Notch and T Cell Function – A Complex Tale

Jyothi Vijayaraghavan and Barbara A. Osborne

Abstract

Notch drives critical decisions in a multitude of developmental decisions in many invertebrate and vertebrate organisms including flies, worms, fish, mice and humans. Therefore, it is not surprising that Notch family members also play a key role in cell fate choices in the vertebrate immune system. This review highlights the critical function of Notch in the development of mature T lymphocytes from hematopoietic precursors and describes the role of Notch in mature T cell activation, proliferation and differentiation.

Keywords

Notch · T cell · $CD4^+$ · $CD8^+$ · Th1 · Th2 · Th17 · Tregs

Abbreviations

CTL	Cytotoxic T lymphocytes.
Dll	Delta-like
GSI, γ	Secretase inhibitor
IFNχ	Interferon gamma
IL17	Interleukin 17
IL2	Interleukin 2
IL4	Interleukin 4
Jag	Jagged
NICD	Notch intracellular domain
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
Tregs	T regulatory cells

1 Introduction

Notch is a protein that is highly conserved throughout evolution and the signaling pathway regulated by Notch performs critical functions in many invertebrates as well as vertebrates. The 300 kilodalton Notch protein is processed in the trans-Golgi by a furin protease resulting in the appearance, on the cell surface, of the Notch heterodimer. This heterodimer consists of an extracellular domain (NECD) that is non-covalently associated with a transmembrane bound peptide referred to as Notch-Tm. Canonical Notch signaling in all species studied to date involves interaction of the NECD with a ligand. In mam-

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mals, four Notch receptors (Notch1, 2, 3 or 4) can interact with five ligands, Delta-like (Dll) 1, 2 or 4 or Jagged (Jag) 1 or 2. Following interaction with ligand, the NECD is forcefully "ripped", from the cell surface of the Notch-bearing cell and endocytosed by the ligand-bearing cell. This exposes a site on Notch-Tm called NRR (negative regulatory region; see also "The Molecular Mechanism of Notch Activation" by Lovendahl/Blacklow/Gordon) making it susceptible to cleavage by an ADAM protease. In most instances, either ADAM 10 or ADAM 17 carry out this cleavage. Following ADAM cleavage, a conformational change occurs in the Notch-Tm, rendering it a substrate for cleavage by the intramembranous protease γ-secretase, resulting in the release of the intracellular domain of Notch (NICD). NICD rapidly translocates to the nucleus, displacing co-repressors bound to the DNA binding protein RBPJ (recombination signal binding protein for immunoglobulin kappa J region), and recruiting co-activators such as Mastermind-like and p300 and initiating a transcriptional program (reviewed in Bray 2016; see also "CSL-Associated Corepressor and Coactivator Complexes" by Oswald/Kovall).

In addition to interaction with RBPJ to initiate canonical signaling, Notch can interact with a variety of other intracellular proteins and participate in non-canonical signaling pathways ("Mechanisms of non-canonical signaling in health and disease: Diversity to take therapy up a Notch?"). In lymphocytes, Notch can interact with such diverse proteins as AKT, mTOR, NF-κB, mitofusin and CARMA1 to name a few (Perumalsamy et al. 2009, 2010; Shin et al. 2006, 2014). In many instances, these "non-canonical" interactions influence Notch function (reviewed in Ayaz and Osborne 2014). For example, Notch interaction with CARMA1 is required for the activation of the IKK complex (Shin et al. 2014) and Notch interaction with mitochondrial proteins such as mitofusion is an important component of Notch mediated survival signals (Perumalsamy et al. 2010).

Notch can also initiate signaling independent of interaction with ligands. The best evidence for ligand independent Notch activation comes from work conducted in *Drosophila melanogaster*, where genetic studies demonstrate that, in some situations, the Notch heterodimer is endocytosed and activated through interaction with Sima, the fly homologue of HIF-1α [Hypoxia inducible factor 1 alpha (Hori et al. 2011; Mukherjee et al. 2011)]. In mammalian cells, ligand-independent activation of Notch may be induced using Ca^{2+} chelators such as EDTA (Rand et al. 2000). Ca^{2+} interaction with the NRR of the Notch-Tm ensures proper folding of the protein and removal of Ca^{2+} disrupts folding and renders Notch-Tm susceptible to cleavage by ADAMs (van Tetering et al. 2009). Whether ligand independent activation of mammalian Notch occurs *in vivo* remains to be determined. However, as discussed below, it is possible that ligand-independent Notch activation may occur in mature T cells.

Notch signaling is important in many cells of the immune system but perhaps the best characterized effects of Notch in the immune system are in early T cell development and mature T cell function (reviewed in Amsen et al. 2015; Shah and Zúñiga-Pflücker 2014; Rothenberg et al. 2016). Indeed, some of the earliest examples of Notch function in mammals comes from studies conducted in the hematopoietic system. Once it was apparent from the report from Ellisen et al. (1991) that activated Notch is aberrantly expressed in T-ALL, many groups focused on the role of Notch signaling in normal T cell development. Before we consider these studies, it is useful to briefly review the important events during T cell development.

2 Notch in T Cell Development

All cells of the immune system are derived from a multi-potent hematopoietic stem cell (HSC). The HSC can differentiate into a common lymphoid progenitor (CLP) that can give rise to either T or B cells depending upon the surrounding environment. In case the CLP migrates to the thymus, this cell progresses through a differentiation program that eventually results in the production of mature $CD4^+$ and $CD8^+$ T cells. In studies pioneered by Zuniga-Pflucker and colleagues, it

is apparent that a key feature of the thymic environment driving this developmental process is Notch/ligand interactions where a Notch-bearing early T cell encounters ligands (likely Dll4) at several points in the developmental process (Schmitt and Zúñiga-Pflücker 2002; Ciofani et al. 2004). The development of a mature T cell from the CLP is a complex process but some of the key steps in this progression are readily recognized by expression of easily identified cell surface markers. During early maturation events, T cell precursors lack both CD4 and CD8 and are called DN (for double negative) T cells. These DN T cells undergo a series of events whereby they acquire various cell surface markers that are detected by flow cytometry. The DN T cell proceeds developmentally through at least four documented stages called DN1, 2, 3 and 4. During the progression through DN1 to DN4, T-cell receptor (TCR) rearrangements occur. Immature thymocytes can mature to express αβ or γδ TCRs however, the majority of mature T cells produced in the thymus express an αβ TCR. In the corticomedullary junction in the thymus, where corticoepithelial cells express Dll4, DN1 cells inevitably are driven to become mature αβ T cells. Thus, the interaction between Notch1 on DN1 cells and Dll4 on cortico-epithelial cells is critical in driving the DN1 cell towards assuming a mature T cell phenotype (Schmitt and Zúñiga-Pflücker 2002; reviewed in Shah and Zúñiga-Pflücker 2014).

In addition to the role of Notch signaling in specifying a cell lineage fate in DN1 cells, Notch is also important in other steps as the DN1 cell begins its progress to become a mature T cell [(reviewed in Shah and Zúñiga-Pflücker 2014; Rothenberg et al. 2016), see Fig. 1]. DN1 cells require Notch signaling to become a DN2 cell. The DN2 cell then begins to rearrange the genes encoding TCR. As mentioned above, TCRs come in two distinct varieties, αβ or γδ. Both TCRs require rearrangement of gene segments to produce a functional TCR and the majority of early T cells become what are termed αβ T cells. A first step in αβ gene rearrangement is the rearrangement of the β chain. The β chain associates in the cytosol of the DN2 cell with an invariant preTCRα chain and is displayed on the cell surface as a dimer with TCRβ associated with preTCRα. The appearance of this heterodimer on the cell surface is a signal to begin the DNA rearrangements necessary to produce a functional mature TCRα chain. Notch signaling is a

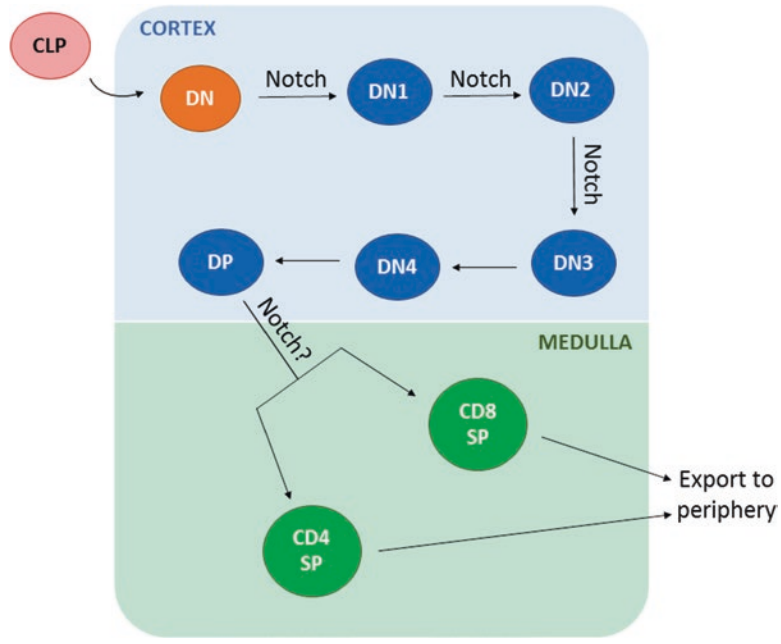

Fig. 1 Notch in early T cell development. CLP - Common Lymphoid Progenitor, DN – Double Negative, DP- Double Positive, SP – Single Positive

key component of this stage of thymic development as it is required for the transition from DN1 to DN2 (Schmitt et al. 2004). At the next stage of development, DN3, a process called β selection occurs whereby preTCR/CD3 signaling drives proliferation as well as inhibits apoptosis allowing progression to the DN4 stage of development and the acquisition of CD4 and CD8. At this point, these immature cells are termed DP (double positive) T cells because they express both CD4 and CD8. The DP cell now must undergo several selective processes. Because TCRs only recognize antigen presented to the cell by self-MHC (major histocompatibility complex), any DP cell that has a TCR that does not recognize self-MHC is deleted or more specifically allowed to die by neglect. The cell receives no stimulatory signals and hence dies. However, in a process called positive selection, if the TCR recognizes self-MHC, this T cell is allowed to survive and mature. Finally, any cell that carries a TCR that strongly recognizes both self-MHC plus self-antigen is negatively selected or instructed to undergo apoptosis. Negative selection ensures that self-reactive T cells, T cells that can cause havoc when mature and functional, are deleted in the thymus.

Notch is critical at the early stages of T cell development up until DN3, failure to encounter DLL4 blocks further thymic development (reviewed in Shah and Zúñiga-Pflücker 2014; Rothenberg et al. 2016). If the early T cell progenitors do not receive Notch signaling, these cells may even turn and become a B cell (Wilson et al. 2001; Koch et al. 2001; Izon et al. 2002). We now understand that Notch signaling not only induces T cell development but also blocks development along the B cell, NK, myeloid and dendritic cell lineages and hence acts a repressor to promote T cell specification. One well-described outcome of Notch signaling is activation of transcription through the canonical Notch signaling pathway. Although several direct targets of Notch including preT-alpha, CD25 and c-myc, have been identified, the mechanism by which Notch drives early T cell development is not fully understood. Therefore, it is likely that Notch assumes many distinct functions during T cell specification and the commitment to a T cell lineage. Indeed, Notch signals in early T cell precursors enhance cell proliferation but are not essential for viability, while at the DN3 stage Notch signaling is essential for survival (Ciofani and Zúñiga-Pflücker 2005). Thus, while we have a detailed understanding of the requirement for Notch signaling in early T cell development, the precise mechanisms that Notch uses to effect T cell specification are unknown.

The influence of Notch on later processes of T cell development are less well-delineated. As described above, DN4 cells acquire the cell surface markers, CD4 and CD8. Early experiments using a truncated version of NICD supported a role for Notch in CD4 versus CD8 lineage decisions with Notch1 overexpression driving DP thymocytes to a CD8 lineage and reducing the number of CD4 single positive (SP) T cells (Robey et al. 1996). However, these findings are controversial because other experiments employing targeted deletion of Notch1 at this point in developing T cells did not observe an effect on CD4 or CD8 lineage decisions (Wolfer et al. 2001). More recently, using thymocytes from mice with targeted deletion of presenilin 1/2, the enzymatic subunit of γ-secretase, a role for Notch1 in CD4 versus CD8 lineages is again supported (Laky et al. 2015). However, because γ-secretase has over 100 identified substrates (Golde et al. 2013) and many of these substrates are expressed in T cells, caution in the interpretation of these experiments is suggested.

3 Notch Activity in Peripheral T Cells

As described above, it is now evident that signaling through Notch plays crucial roles at various stages of T cell development. In more recent years, it has become increasingly evident that Notch is also involved in the activation and differentiation of naïve $CD4^+$ and $CD8^+$ SP T cells into various subsets in the periphery. Here, we describe how this pathway regulates such varied T cell differentiation programs, whether it acts as a molecular switch in peripheral T cell function

and address some of the controversies in this emerging field.

3.1 Notch in T Cell Activation and Proliferation

For a T cell to mount an immune response against an infectious challenge, it needs to be activated. This involves interaction of the TCR with its cognate antigenic peptide presented on the surface of an antigen presenting cell (APC), bound to an MHC Class I (interacts with $CD8^+$ T cells) or II (interacts with $CD4^+$ T cells) molecule. This signal is further augmented by co-stimulatory molecules resulting in full activation of a T cell, subsequent IL2 (Interleukin 2) production, ultimately leading to T cell proliferation. The activation process is complex involving multiple intracellular signaling events and to add to this complexity, recent studies have found that Notch proteins can affect the activation and subsequent proliferation of naïve $CD4^+$ and $CD8^+$ SP T cells.

In 2003, studies by two independent groups revealed previously unrecognized roles for Notch proteins in T cells. Adler and colleagues demonstrated that $CD4^+$ T cell stimulation with anti-CD3 and anti-CD28 not only increases the expression of all four Notch genes but also induces Notch1 activation (Adler et al. 2003). Furthermore, pharmacological blockage of Notch1 activation inhibits T cell proliferation *in vitro,* which is associated with a decrease in CD25 expression and IL2 production. In agreement with the above data, Palaga and colleagues revealed that activation of both $CD4^+$ and $CD8^+$ SP T cells results in Notch activation and subsequent upregulation of the Notch protein (Palaga et al. 2003). They also reported a decrease in T cell activation, proliferation and IFNγ (Interferon gamma) production upon GSI (γ-Secretase Inhibitor) mediated inhibition of Notch in peripheral T cells. A subsequent study by Benson and colleagues demonstrated that Notch1 is upregulated and colocalizes with CD4 on the cell surface following *in vitro* activation of $CD4^+$ T cells (Benson et al. 2005). However, in this study, pharmacological blockage of Notch signaling does not affect proliferation but attenuates cytokine production. This group also showed that upon transfection of a constitutively active form of Notch1, $CD4^+$ T cells fail to proliferate but exhibit enhanced secretion of cytokines on stimulation. In another report, Rutz and colleagues documented that distinct Notch ligands differentially affect early T cell activation and proliferation, where Dll1 and Jag1 inhibit proliferation and the expression of early activation markers – CD69 and CD25, while Dll4 has the opposite effect (Rutz et al. 2005). The binding capacity of the Notch ligands to resting and activated T cells also differ considerably, with Dll4 showing the strongest binding, followed by Dll1 and Jagged1 (Rutz et al. 2005).

Almost a decade later, several studies revealed novel roles for Notch in T cell activation and proliferation. A 2013 study suggested that Notch can directly regulate PD1 (Programmed death 1) expression in activated $CD8^+$ T cells (Mathieu et al. 2013). Following anti-CD3/CD28 stimulation of a co-culture of purified $CD8^+$ T cells and APCs or bulk splenocytes, the authors observed that PD-1 expression was significantly reduced in $CD8^+$ T cells when Notch signaling was blocked using the GSI DAPT. These results are interpreted to suggest that prolonged activation of Notch signaling during chronic infection, due to continued antigen presentation by APCs expressing Notch ligands, may lead to Notch-induced expression of PD-1, thereby regulating the immune response. It is important to note that these experiments, like many of the studies reviewed here, interpret results obtained using GSI as an effect of Notch. γ-secretase substrates number over 100 and the use of GSIs to block Notch activity must be verified by targeted deletion of Notch in the cell in question. Others have used dominant negative forms of mammalian Mastermind (DN-MAML) which is a more direct approach to inhibiting the canonical Notch signaling pathway,

The following year, our laboratory showed that Notch affects activation, proliferation and differentiation of $CD4^+$ T cells in a non-canonical fashion (Dongre et al. 2014). Notch signaling that occurs independent of its canonical partner –

RBPJ, is termed non-canonical Notch signaling. In experiments using conditional Notch1 and RBPJ knockouts, we demonstrated that CD4+ T cell activation and proliferation is impaired in the absence of Notch1 but remains unaffected when RBPJ is deleted. This non-canonical role of Notch in regulating peripheral T cell function is not only novel but may also explain some of the differential effects of Notch. Another group tested the ability of Dll4-bearing APCs to drive CD4+ T cell priming and found that Dll4-deficient APCs less efficiently promote activation, metabolism, proliferation and IL2 secretion of CD4+ T cells (Laky et al. 2015). Furthermore, they documented that APCs can fine tune the antigen sensitivity of CD4+ T cells via Dll4-induced Notch signaling, where Dll4-Notch interaction through PI3K (Phosphoinositide-3-kinase) signaling allows naïve CD4+ T cells to respond to low doses of antigen. This Dll4-induced effect of Notch signaling on T cell activation agrees with the work of Rutz and colleagues (Rutz et al. 2005).

The available data suggest to us that Notch can promote or inhibit T cell activation and proliferation based on environmental cues and the presence or absence of different Notch ligands. Thus, as suggested by others, individual ligands may have differing biological effects and this may be influenced by environmental cues. Lending further credence to this idea are studies showing that distinct Notch ligands can induce differential effects in a particular cell, for instance during human lymphoid differentiation (Jaleco et al. 2001) or T lineage commitment (Lehar et al. 2005). However, because each group uses unique experimental approaches it is difficult to reach an overarching consensus. Differences in the cell populations studied (purified T cells versus T cells in the presence of APCs and other cells), the pharmacological inhibitors used and activation of T cells in the presence or absence of ligands, clearly indicate that more work needs to be done in this direction to obtain a clearer picture of how individual ligands influence Notch in T cell function.

3.2 Notch in CD4+ T Cell Differentiation

CD4+ T cells are multifaceted and therefore an integral part of the immune system. Among other functions, they can orchestrate an immune response against a wide range of pathogens and can also regulate these responses, thereby preventing autoimmune disorders. How does a CD4+ T cell manage to perform such diverse functions? Depending on the cytokine milieu during TCR activation, naïve CD4+ T cells can differentiate into several lineages of T helper (Th) lymphocytes, including Th1, Th2, Th17 and regulatory T (Tregs), that are defined by their function and cytokine production (see Fig. 2). Notch has been found to be important in the differentiation of most Th cells however, whether it acts as a molecular switch or plays a more subtle context-dependent role in Th differentiation remains to be determined.

3.2.1 Notch in Th1 and Th2 Differentiation

Th1 and Th2 cells express T-bet (Th1) and Gata3 (Th2) as the driving differentiation factors and produce IFNγ and IL4 (Interleukin 4) as signature cytokines, respectively. While Th1 cells fight intracellular viruses and bacteria, Th2 cells direct immunity against extracellular helminthic infections and play a role in allergies. IL12 and IL4 are believed to be the major inducers of Th1 and Th2 differentiation, respectively; however, other pathways have been shown to be involved as well (Skokos and Nussenzweig 2007).

The first evidence of a role for Notch in CD4+ T cell differentiation came in 2003, when Maekawa and colleagues showed that the Dll1-Notch3 interaction induces differentiation towards the Th1 lineage (Maekawa et al. 2003). Dll1-Fc stimulation of CD4+ T cells not only substantially increased the number of IFNγ secreting cells over IL4 producing cells, but also induced the expression of T-bet. These results were further strengthened when *in vivo* administration of

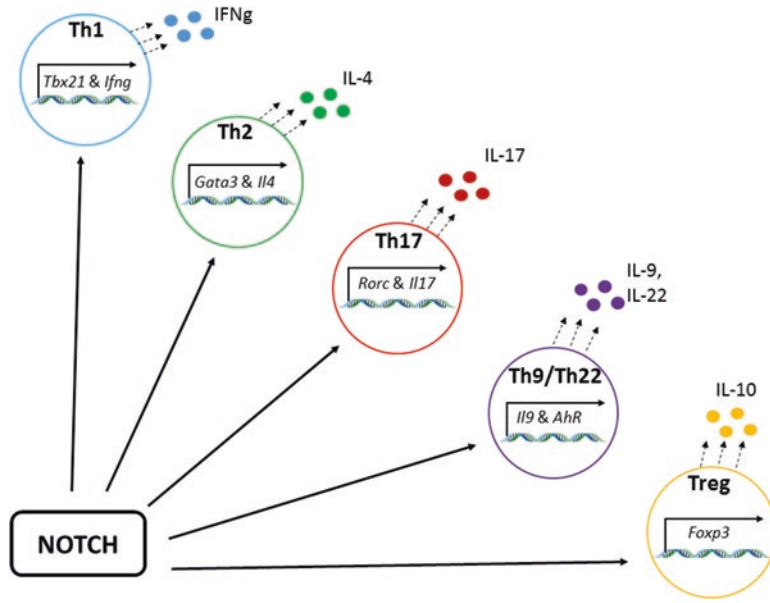

Fig. 2 Notch in CD4⁺ T cell differentiation. Notch can drive CD4⁺ T cell differentiation into most subtypes by regulating the master transcription factors. However, whether Notch does so via its canonical or non-canonical partners requires further study

Dll1-Fc resulted in a Th1 response against *Leishmania major* (*L. major*) infection in normally susceptible BALB/C mice. Moreover, retroviral overexpression of NICD3 in CD4⁺ T cells increased IFNγ secretion while decreasing IL4 production and this skewing towards the Th1 phenotype was found to be dependent on Dll1-Notch3 interaction. In a contrasting report, using Notch1$^{fl/fl}$x CD4-Cre mice where the peripheral T cells are deficient in Notch1, Tacchini-Cottier and colleagues showed that Notch1 is dispensable for Th1 and Th2 differentiation *in vitro* (Tacchini-Cottier et al. 2004). Moreover, *L. major* infection in Notch1$^{-/-}$ CD4-Cre mice resulted in a protective Th1 response characterized by high IFNγ levels and low IL4 levels similar to resistant C57BL/6 mice, indicating that Notch1 is not critical for Th1 differentiation. Challenging these results, Minter and colleagues demonstrated that GSI-mediated inhibition of Notch signaling attenuates polarization towards Th1 by preventing *Tbx21* upregulation, the gene encoding T-bet (Minter et al. 2005). Further, GSI treatment of CD4⁺ T cells reduces the levels of Notch, *Tbx21* and IFNγ on Th1 polarization while IL4 production remains unaffected in polarized Th2 cells. *In vivo*, administration of GSI to mice with experimental autoimmune encephalomyelitis (EAE), a classical Th1 mediated model of multiple sclerosis, significantly reduced the symptoms of EAE. The authors further showed that Notch1 directly regulates *Tbx21* expression by forming a Notch1/RBPJ complex on the *Tbx21* promoter. In contrast to the work of Tacchini-Cottier and colleagues, these results point towards a T cell intrinsic mechanism for Notch1 in Th1 differentiation.

Studies in the subsequent years by both groups resolved some of the controversies regarding the role of Notch in Th1 differentiation. Using mice with T cell specific ablation of both Notch1 and Notch2 on a C57Bl/6 – *L. major*-resistant genetic background, the Tacchini-Cottier laboratory showed that lack of both these receptors renders the mice susceptible to *L. major* infection while mice lacking either Notch1 or Notch2 develop a protective Th1 response (Auderset et al. 2012). Their data point towards a redundant role for Notch1 and Notch2 in driving a Th1 response. Further, in 2013, the Minter laboratory reported that NICD1 is increased in T cells from mice with aplastic anemia, a Th1-mediated disease, and that blocking Notch attenuates the disease (Roderick et al. 2013). In support of their earlier results, they show that NICD1 is bound to the *TBX21* promoter in PBMCs (Peripheral blood mononu-

clear cells) from patients with untreated aplastic anemia. These results highlight a strong role for Notch in regulating Th1-mediated responses.

Several subsequent studies provide a clearer view of Notch in regulating the Th1 differentiation program. A study exploring how dendritic cells induce a Th1 response upon Toll-like receptor (TLR) ligation in the absence of the major inducing cytokine IL12, showed that the Notch ligand Dll4 is involved in this process, implicating Notch signaling in IL12-independent Th1 differentiation (Skokos and Nussenzweig 2007). This suggests that Notch and IL12 are redundant and that this redundancy may explain some of the discrepancies in the contribution of Notch to Th1 differentiation. Another report showed that overexpression of NICD3 in $CD4^+$ T cells during differentiation led to strong IL10 production in Notch-transduced Th1 cells (Rutz et al. 2008). IL10 is an anti-inflammatory cytokine that is involved in controlling immune responses. In this study, Notch signaling was found to be responsible for inducing IL10 production in a STAT4 (Signal transducer and activator of transcription 4) dependent manner converting a pro-inflammatory Th1 response into a regulatory one, thus providing novel opportunities to use this pathway to attenuate Th1-mediated immune disorders. In addition to T cell activation and proliferation (described earlier), our laboratory has also shown that differentiation into the Th1 lineage, although Notch1 dependent, is independent of signaling through its canonical partner, RBPJ (Dongre et al. 2014).

The data described so far suggest that Notch regulates differentiation into Th1 but not Th2 cell fate. However, there is enough evidence to implicate Notch in Th2 differentiation as well. An early study by Amsen and colleagues documented that APCs that express the Notch ligand Dll1 induce a Th1 fate whereas Jagged1 expression potentiates differentiation into Th2 (Amsen et al. 2004). Additionally, the authors report that differentiation into the Th2 lineage requires an intact canonical Notch pathway, which induces Gata3 expression and directly regulates the *Il4* gene but this mechanism is independent of STAT6. They also show that retroviral expression of both NICD1 and NICD2 in $CD4^+$ T cells promotes IL4 production independent of STAT6. In a subsequent study, the same group highlighted that direct regulation of Gata3 by Notch is required to generate optimal Th2 responses (Amsen et al. 2007). These results were confirmed by another group in the same year (Fang et al. 2007). Together, their data reveal that Notch in conjunction with RBPJ binds to the *Gata3* promoter to induce IL4 production, promoting the Th2 phenotype. Furthermore, Amsen and co-authors go on to show that in the absence of Gata3, Notch turns from being an inducer of Th2 to a strong Th1 inducer, indicating that Gata3 acts as a molecular switch in Notch-induced Th differentiation. A separate study demonstrated that signaling through Notch controls the initial IL4 expression by regulating the IL4 enhancer – conserved noncoding sequence-2 (CNS-2) in memory phenotype $CD4^+$ T cells and Natural Killer T (NKT) cells (Tanaka et al. 2006). Their data demonstrate that loss of Th2 development in RBPJ deficient mice is due to the lack of initial IL4 production by CNS-2-regulated T cells, suggesting that Notch/RBPJ-mediated control of initial IL4 production may direct whether naïve $CD4^+$ T cells can adopt a Th2 phenotype. In total, the studies described above clearly demonstrate that Notch, through Gata-3, regulates IL4 expression and this can influence Th2 development. Therefore, it is possible that extrinsic Notch regulation of IL4 production in another cell provides IL4 to a developing Th2 cell. This interpretation is supported by the fact that *in vitro* T helper polarization to Th1 requires Notch while Notch is dispensible for Th2 polarization (Minter et al. 2005; Dongre et al. 2014).

3.2.2 Notch in Th17 Differentiation

Apart from Th1 and Th2, several other subsets of Th cells have been discovered and Notch has been shown to be involved in immune responses through those lineages as well. Th17 cells mount defenses against extracellular fungi and bacteria and are important modulators of several autoimmune disorders. These cells express RORγt (RAR-related orphan receptor gamma t) as their master transcriptional regulator, produce IL17A and IL17F as major cytokines and are induced by TGFβ (Transforming growth factor beta) and

IL6. Through experiments using TCR transgenic cells (DO11.10), a 2009 study revealed that Dll-4 enhances IL17 production in the presence of TGFβ and IL6, while inhibition of Notch signals fail to do so even under skewing conditions (Mukherjee et al. 2009). They further showed that RBPJ, the canonical partner of Notch, directly interacts with the RORɣt and IL17 promoter to regulate IL17 production in response to Dll4. Strengthening these observations, Keerthivasan and colleagues reported that Notch inhibition, using GSI or Notch1 siRNA, reduces IL17 production during mouse and human Th17 polarization (Keerthivasan et al. 2011). Additionally, GSI administration ameliorates EAE symptoms and dampens the Th17-mediated response in this model. This group also found that Notch1 directly binds to both IL17 and RORɣt promoters, implying a direct regulation of Th17 differentiation by Notch1.

3.2.3 Notch in the Differentiation of Other Th Subsets

Th9 cells, another class of Th cells, produce IL9 and are generated under the influence of IL4 and TGFβ. The transcriptional regulation of this subset and whether they act as immune response mediators or sustain inflammation is still not clear. Shedding light on these questions, Elyaman and co-authors showed that Notch1 and Smad3 together bind to the *Il9* promoter and activate IL9 production (Elyaman et al. 2012). Moreover, using an EAE model, they showed that Jag2-induced IL9 production can alleviate or exacerbate EAE symptoms based on whether the mice are pretreated or treated with anti-Jag2 monoclonal antibody at the same time when EAE is induced. This suggests that IL9 producing cells can play dual roles in the immune system, depending on the timing of the co-stimulation and the cytokine microenvironment. IL22 is a cytokine that can be produced by Th1, Th17 cells as well as some other cells. Its production is induced by IL6 and driven by the expression of the aryl hydrocarbon receptor (AhR). Notch was found to be involved in the regulation of IL22 production as well by inducing the production of stimulators of AhR (Alam et al. 2010).

There is no dispute that Notch is important in the differentiation of $CD4^+$ T cells into multiple lineages. Evidence from ligand data suggests that the Delta family of ligands promotes Th1 and Th17 responses, whereas the Jagged family may be important for Th2 and Th9 differentiation. Although limited, there exists adequate evidence pointing towards a role for Notch in regulating differentiation towards Th17 and Th9 lineages as well. Despite the conflicting views on Notch's role in Th1 and Th2, the direct regulation of lineage regulators *Tbx21* and *Gata3* by Notch clearly show that Notch can play crucial roles in both Th1 and Th2 differentiation. Since signaling through the TCR activates Notch, it is possible that Notch acts as a "co-receptor" and cooperates with environmental signals to drive differentiation pathways. Additionally, the interplay between Notch/RBPJ and chromatin is an important feature of Notch signaling (see "CSL-Associated Corepressor and Coactivator Complexes" by Oswald/Kovall; Oswald et al. 2016). In light of ample evidence of epigenetic regulation of T helper lineages (reviewed by Zhu et al. 2010), it is tempting to speculate that the state of the chromatin near Notch target genes likely contributes to T helper lineage decisions. Nevertheless, questions as to which Notch receptors are involved and whether the effects of Notch are cell intrinsic or extrinsic remain unresolved.

3.3 Notch in $CD8^+$ T Cell Differentiation

To add to the already long list of functions for Notch, studies suggest that Notch is also involved in regulating $CD8^+$ T cell responses (see Fig. 3). $CD8^+$ T cells or cytotoxic T lymphocytes (CTLs) are involved in killing tumor cells or virally-infected cells. Data implicating Notch in $CD8^+$ T cell differentiation was provided by the Yasumoto group who showed that retroviral expression of Dll1 on bone marrow-derived dendritic cells (BMDC) enhanced the differentiation of $CD8^+$ cells into CTLs, whereas lack of Notch2 in peripheral $CD8^+$ T cells failed to induce this dif-

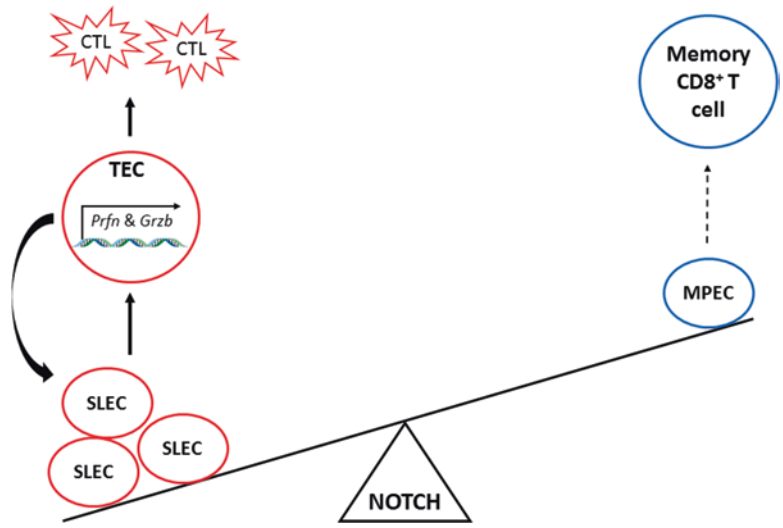

Fig. 3 Notch in CD8⁺ T cell differentiation. CTL – Cytotoxic T Lymphocytes, TEC – Terminal Effector cells, SLEC – Short-lived Effector cells, MPEC – Memory Precursor Effector cells

ferentiation *in vitro* and *in vivo* (Maekawa et al. 2008). Further, Notch2 in a complex with CREB1 was found to directly control the transcription of the gene encoding granzyme B (CTL effector molecule), independent of Eomes – the key regulator of granzyme B and perforin. Using mice that lack Notch2 in CD8⁺ T cells, the same group then went on to show that signaling through Notch2 is essential for antitumor CTL responses *in vivo* (Sugimoto et al. 2010). Consistent with these findings, data from our lab demonstrate that both GSI-mediated inhibition of Notch and genetic reduction of Notch1 decrease the mRNA and protein levels of cytolytic effectors - perforin and granzyme B in CD8⁺ T cells (Cho et al. 2009). This could be the result of direct binding of Notch1 to the promoters of Eomes, perforin and granzyme B, thereby linking Notch signaling to the regulation of these CTLs effector molecules. This effect of Notch held true for human CD8⁺ T cells as well (Kuijk et al. 2013). In another study on the role of Notch in antitumor responses, the authors demonstrated that Notch expression was reduced in T cells from tumors in mice (Sierra et al. 2014). Moreover, transgenic expression of NICD1 in antigen-specific CD8⁺ T cells increased granzyme B levels and resulted in higher cytotoxic effects, suggesting a strong potential for Notch in enhancing the efficacy of T cell based immunotherapies.

Notch was also found to regulate the choice between terminal effector cells (TEC) or memory precursor cell (MPC) fates in CD8⁺ T cells (Backer et al. 2014). Here, Amsen and colleagues describe that signaling through Notch promotes differentiation towards TECs and feeds back into the TEC promoting pathways giving rise to fully protective TECs. Similarly, Mathieu and colleagues document that Notch is crucial for the formation of short lived effector cells (SLECs) but is dispensable for the generation of memory precursor effector cells [MPECs, (Mathieu et al. 2015)]. Their data also suggest a context-dependent role for Notch during CD8⁺ T cell response, where Notch is required for maximal IFNγ production and only selectively required for IL2 and TNFα (Tumor necrosis factor alpha) production after *Listeria monocytogenes* infection and vaccination with dendritic cells. Therefore, the current evidence clearly point towards a crucial role for Notch in immune responses through CD8⁺ T cells, implicating Notch as a strong candidate for immunotherapy in cancer.

3.4 Notch in Regulatory T Cells

Regulatory T cells or Tregs, as the name suggests, are a subset of CD4⁺ T cells that can suppress an immune response. They are defined by

the expression of their master transcriptional factor FoxP3. Tregs that are derived from the thymus are termed naturally occurring Tregs or nTregs. Tregs can also be induced *in vitro* from naïve $CD4^+$ T cells in the presence of TGFβ and these are called induced Tregs or iTregs. Another emerging category of regulatory T cells are $CD8^+$ suppressor T cells. Although, these are less explored than $CD4^+$ Tregs, multiple populations have been described based on the expression of several markers but only a small number of $CD8^+$ Tregs express FoxP3 (Tang et al. 2005; Dinesh et al. 2010).

The first indication of a role for Notch in inhibiting an immune response came with a study reporting that overexpression of Notch ligand Serrate1 (Jag1) on APCs leads to differentiation of antigen-specific $CD4^+$ T cells into regulatory cells (Hoyne et al. 2000). The authors demonstrated that these regulatory cells can inhibit primary and secondary immune responses and can also transfer this antigen-specific tolerance to recipient mice. In the following years, reports from several groups further strengthened the role of Notch signaling in Treg development. In 2003, two studies by the same group revealed that coculture of Epstein-Barr virus lymphoblastoid B cells (EBV-LCL) overexpressing Jag1 with T cells induces the generation of human Tregs that can inhibit proliferative and cytotoxic immune responses towards a specific antigen (Vigouroux et al. 2003) or alloantigen (Yvon et al. 2003). Furthermore, both studies showed that this inhibition of immune response is transferable, since the Notch-induced Tregs could also inhibit immune responses of fresh T cells that have not been exposed to Jag1. Evidence of additional involvement of the Notch receptors in Treg function was provided by the Screpanti laboratory, who showed that the presence of constitutively active NICD3 in the T cells of transgenic mice prevents the development of experimental autoimmune diabetes (Anastasi et al. 2003). Failure to develop disease was associated with an enhanced number of $CD4^+$ $CD25^+$ Tregs and increased expression of the Treg specific cytokine, IL10. Work from our group in collaboration with colleagues concur with the above findings. We have shown that *in vitro* GSI inhibition of signaling through Notch blocks TGFβ-induced expression of FoxP3 and its target genes (Samon et al. 2008). Lending *in vivo* support to this finding, GSI administration to C57BL/6 mice reduced FoxP3 expression resulting in symptoms reminiscent of a disease involving dysregulation of TGFβ and Tregs. Our chromatin immunoprecipitation (ChIP) data further suggest that Notch1 directly regulates FoxP3 expression cooperatively with TGFβ signaling. This result was corroborated by a subsequent study where the authors report that NICD binds to the *Foxp3* promoter in Tregs in a complex with RBPJ (Ou-Yang et al. 2009). On similar lines, the Screpanti group demonstrated that Notch in conjunction with PKC-theta and NFκB controls FoxP3 expression, thereby regulating Tregs generation (Barbarulo et al. 2011). A novel study aimed at generating iTregs *in vitro,* demonstrated that Dll1-mediated Notch signaling efficiently converts human memory $CD4^+$ T cells into iTregs (Mota et al. 2014). Their data further suggest that Notch signaling through Dll1 plays a dual role in promoting iTreg development - by directly regulating FoxP3 expression and interacting with the TGFβ pathway. Therefore, it is evident that Notch channels signals through multiple partners to promote the development of Tregs. Hinting at a role for Notch in $CD8^+$ Tregs, another study showed that pretreatment of alloantigen bearing cells with Dll1 inhibits responses to subsequent exposure of the same antigen, resulting in prolongation of graft survival in a mouse model of cardiac allograft (Wong et al. 2003). Their data further suggest that this inhibition of graft rejection is because Notch ligation on $CD8^+$ T cells enhances their IL10 production, altering their differentiation potential from a T1-type response to an inhibitory one.

The role of Notch in Treg development and function, however, is not without controversy. Evidence opposing the abovementioned findings was provided by Bassil and colleagues, where neutralization of Dll4 using a blocking antibody during the induction phase of EAE alleviated EAE symptoms by drastically increasing the $CD4^+FoxP3^+$ Tregs population in the periphery

and the CNS (Central nervous system) (Bassil et al. 2011). Additionally, the authors reported that Dll4-induced Notch signaling inhibits Tregs development by regulating the phosphorylation of STAT5 (Signal transducer and activator of transcription 5), a key regulator of FoxP3 expression. Adding to an already complex view of Notch in Tregs, a recent study revealed that Tregs-specific deletion of components of Notch signaling augmented Tregs-mediated suppression of Th1 response, whereas NICD1 overexpression reversed this effect (Charbonnier et al. 2015). Their data also suggest roles for both canonical and non-canonical Notch pathways in the dysregulation of Tregs.

The current view on the role of Notch in Tregs is divided. While there is more evidence indicating that Notch signaling promotes Tregs generation than inhibiting development of this population of cells, additional studies are warranted before a consensus can be reached on the matter. Furthermore, numerous studies suggest that Notch ligands are critical with Serrate and Jag inducing Tregs generation, while signaling through Dll ligands appear to have the opposite effect. Therefore, as suggested earlier, it is possible that the opposing evidence on the role of Notch in Tregs function could be the result of signaling through different Notch ligands. Nonetheless, further experiments are needed to test if this idea is indeed true.

4 Notch and Diseases Mediated by T Lymphocytes

Understanding how Notch influences T cell function is important because of the well-defined role it plays in the development of a variety of T cell related diseases. Indeed, the first report of a mammalian Notch homologue was as a translocation in T-ALL (Ellisen et al. 1991) demonstrating a key role for Notch in T cell malignancy. Over the ensuing two decades, Notch has been implicated in many cancers, including those of the immune system (reviewed in Chiang et al. 2016). Perhaps not surprisingly, due to its role in T cell activation, Notch is also known to influence a variety of autoimmune diseases. More than a decade ago, our lab described a role for Notch in mediating EAE, a disease known to require Th1 responses (Minter et al. 2005). Roderick et al. (2013) demonstrated a key contribution of Notch in the development of bone marrow failure, another autoimmune disease mediated by Th1 cells. There also is increasing evidence that Notch may contribute to several other autoimmune conditions (reviewed in Kuksin and Minter 2015). Additionally, data from the Maillard lab (Tran et al. 2013) demonstrate that targeting Notch with blocking antibodies in a mouse model of graft versus host disease (GVHD) may ameliorate GVHD. These data are particularly important since this group used Notch blocking antibodies to abrogate disease, a therapy that may be clinically useful in the near term. Although Notch is implicated in many diseases, including those of the immune system, blockade of Notch in a clinical setting is fraught with potential problems because of the requirement for Notch signaling in a vast array of cells and tissues. Acute blockade using antibodies may possibly alleviate the clinical complications observed with gamma secretase inhibitors.

5 Concluding Remarks

Notwithstanding the contradictory views on how Notch affects T cell activation, differentiation and function, it is beyond dispute that signaling through Notch is critical for T cell function. The available data suggest more and more that Notch plays a highly versatile and context-dependent role in relaying signals downstream and modifying outcomes based on its immediate environmental cues. However, there is still much to be learned to obtain a complete picture and fully understand the implications of Notch-based immunotherapies. Considering the pleiotropic effects of signaling through Notch, the use of consistent experimental approaches and in-depth analysis of their functions are crucial to reach a consensus regarding how this signaling pathway controls so many aspects of T cell-mediated immune responses. However, designing experi-

ments to study the downstream effects of Notch signaling can be very tricky. As mentioned earlier, results from experiments using GSIs need to be interpreted cautiously considering that they have multiple substrates and Notch is only one of them. Knockout experiments are difficult since some Notch receptors (Notch1 and Notch2) are critical during development and therefore blocking signaling through them *in vivo* can cause embryonic lethality. Although, one can get around this issue using conditional knockouts, there is an additional problem of compensation by other Notch receptors when one receptor is knocked out *in vivo*. Further, a Notch loss-of-function phenotype can be mimicked using dominant negative Mastermind-like protein 1 (dnMAML1) that will prevent the binding of wild-type MAML1 to Notch and RBPJ, thus preventing target gene expression downstream of Notch. However, this construct does not account for signaling via non-canonical partners of Notch nor does it take into consideration the effects of MAML1 on other unrelated signaling pathways. These complexities call for careful and detailed design of experiments and cautious analysis and interpretation of results to fully understand T-cell mediated responses regulated by Notch and to develop Notch-based therapies to treat immune disorders.

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