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Review

# The role of Notch receptors and ligands in health and disease

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## ABSTRACT

Notch signalling has an important role in the immune system in directing cell fate decisions in a range of diverse cell types. We are beginning to obtain a better understanding of the roles that the different Notch ligands and receptors play in both cellular differentiation of precursor cells and the regulation of immune responses by mature lymphocytes in the periphery. This review will examine the key findings that have emerged in relation to function of Notch in differentiation of T cells, B cells and dendritic cells and how modulation of Notch signalling seems to have potential for therapeutic applications in immune based diseases.

**KEYWORDS:** T cells, anergy, Notch, Notch ligands, immune suppression, Th1 cells, Th2 cells, Treg cells, dendritic cells

## INTRODUCTION

Notch signalling occurs between cells and is essential for embryonic development through its ability to regulate growth, differentiation and tissue patterning and in adults it is required for tissue homeostasis and stem cell self-renewal. Disruption to Notch signalling has been implicated with a range of human diseases including cancer, neuro-degenerative diseases, and multiorgan congenital syndromes. The use of both gain-of-function and loss-of-function approaches to target specific receptors, ligands and downstream signalling effectors has lead to a better understanding of the diverse roles for Notch signalling in the immune system. These studies have shed light on the role of individual receptor/ligand pairs that direct specific cell fate decisions in the immune system. In recent years there have been some excellent reviews written on the role of Notch signalling in the immune system [1-5] and in this review I would like to focus the discussion on the role of Notch receptors and ligands and their role in immune function concentrating more specifically on T cells, B cells and dendritic cells.

Notch was first described in *Drosophila melanogaster* as a Type 1 transmembrane receptor protein that interacts with Type 1 transmembrane ligands: Delta and Serrate [6]. In mammals, four Notch receptors (Notch 1, 2, 3 and 4) can interact with five canonical Notch ligands of the Delta-Serrate-Lag2 (DSL) family. Mammals express two Serrate-like ligands named Jagged1 and Jagged2 and they differ structurally from the three Delta-like ligands (Dll-1, -3 and -4). The Jagged ligands have more EGF-like repeats on their extracellular domain compared to the Dll ligands, and they also contain an additional cysteine -rich like region on the extracellular domain that is absent on the Delta-like ligands [7, 8]. Ligand binding to Notch receptors requires the cysteine-rich DSL domain together with the N-terminal domain (NTD) and the first two EGF-like repeats that physically interact with EGF repeats 11 and 12 on the Notch receptor [9]. The intracellular domain of the ligands do not display sequence homology but three of the five ligands (Jagged 1, Dll1 and Dll4) possess a PDZ (PSD-95/Dlg/ZO-1) domain on the C-terminus that is thought to facilitate interactions

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with the cytoskeleton PDZ ligand. The PDZ ligand of Jagged1 can mediate cellular transformation [10].

A number of non-canonical Notch ligands have been described in the literature and they represent a structurally diverse group of proteins that include integral membrane proteins (e.g. Dlk-1), GPI-linked proteins (e.g. contactin 1 and 6) and secreted ligands (CCN3, MAGP-1 and MAGP2). None of these proteins have yet been described to have a function in the immune system and so are beyond the scope of this review. However, readers are referred to a recent review on this area by D'Souza *et al.* for more detail [11].

**Mechanism of Notch signalling**

Binding of a Notch ligand via the DSL domain to its receptor leads to a metalloproteinase-dependent cleavage at S2 which lies in the extracellular portion of the N-terminal portion of transmembrane domain (NTM), creating a short lived membrane-bound form of NTM [12]. The final cleavage at site S3 catalysed by γ-secretase protease complex, releases the intracellular domain of the NTM (NICD) which translocates to the nucleus to form a short lived transcription complex by associating with a DNA binding transcription factor known as RBP-Jκ (Recombination signal Binding Protein or CSL (Core Binding Factor-1, Suppressor of Hairless, Lag1)) and transcriptional co-activators of the Mastermind-like family (MAML) [9]. This converts RBP-Jκ from a transcriptional repressor to an activator. NICD binds firstly to RBP-Jκ with high affinity through its RAM domain, which stabilises binding of ankyrin to RBP-Jκ. Ankyrin is absolutely required in all Notch functions because MAML does not bind free RBP-Jκ or free NICD but binds with high affinity to RBP-Jκ/ankyrin binary complex [9]. Three MAML proteins have been identified [13, 14]. The C-terminal domain of MAML binds to complexes containing RNA polymerase [15] and other histone acetyl transferase activity, including p300 and pCAF [14-16]. Nuclear NICD is short-lived and degradation is promoted by phosphorylation of residues on the C-terminal end of the PEST domain [15].

In turn, this RBP-Jκ -NICD-MAML complex alters the structure of chromatin into a form that is transcriptionally active. This results in transcription of Notch target genes such as *NF-κB* [17], members of the *hairy enhancer of split* (*Hes*) family such as *Hes1* and *Hes5* (beta helix-loop-helix transcriptional repressors), *Hes-related repressor protein* 1-3 (*HERP*) [18, 19], *Deltex1* [20], *c-myc* [2], *pTα* and *Meltrin*β [21], and *PTEN* [22]. Expression of Notch transcriptional targets in early T cell development such as *Hes1* and *Deltex1* mirror *Notch1* expression, suggesting that Notch signalling accompanies Notch1 surface expression [23]. In addition to the classical RBP-Jκ -dependent Notch pathway, there is also evidence for RBP-Jκ -independent Notch signalling [11]. The RBP-Jκ-independent pathway has been mainly characterized in *Drosophila* and exactly how it functions has not been clarified.

**Regulators of Notch signalling**

Notch signalling is also regulated by positive and negative regulators such as Deltex, Fringe, Numb, MAML, Presenilin1, Notch-regulated ankyrin-repeat protein (Nrarp) and Msx interacting protein 2 (Mint). A dominant-negative version of MAML1 (DNMAML1) retains the ability to interact with NICD but does not potentiate transcriptional activation of Notch target genes [24]. Instead, DNMAML1 constructs can inhibit signalling by all four Notch1-4 receptors [25] by interrupting the recruitment of critical co-activators to the NICD/ RBP-Jκ complex, to inhibit transcriptional activation [26]. This is supported by an *in vivo* study revealing that expression of DN-MAML1 in the murine haematopoietic stem cells prevents T cell development and leads to B-cell development in the thymus [25]. Another negative regulator of Notch is Numb protein which antagonises the Notch signalling pathway by promoting the ubiquitination of the membrane-bound Notch1 receptor and degradation of NICD domain following activation [27]. This in turn, blocks the nuclear translocation of Notch and downstream activation of Notch1 target genes [27].

***Cis* and *trans* signalling by Notch ligands**

Notch signalling is transmitted through interaction between Notch ligands on one cell (i.e. signal

sending cell) and Notch receptors expressed on a neighbouring cell (i.e. a signal receiving cell). This interaction in *trans* elicits a series of specific proteolytic cleavage events that culminate in the release of the intracellular domain of NICD [28] and signal transduction to the nucleus as described above. In mammals, both classes of Notch ligands (i.e. Jagged and Delta-like) can inhibit Notch signalling when expressed in the same cell as Notch; this phenomenon is referred to as *cis*- or cell autonomous inhibition of Notch signalling [8]. There is clear evidence from studies in *Drosophila* that *cis*-inhibition by DSL ligands controls a subset of Notch dependent developmental fates [29-32]. However, the physiological relevance of *cis*-signalling via Notch in mammals remains poorly understood.

Truncated ligands that lack the intracellular domain of the ligand can act as effective inhibitors of Notch signalling that can disrupt tissue patterning during embryonic development *in vivo* [33-36]. Unlike the other canonical Notch ligands, the Dll3 protein lacks the conserved DSL domain as well as the key lysine residues in its C-terminal domain which are important for ubiquitination and association with PDZ domain proteins [37-39]. Several studies have shown that Dll3 is unable to activate Notch in *trans* but can function in *cis*-inhibition of Notch signalling [38-40]. Thus Dll3 appears to have evolved a divergent function compared to other canonical Notch ligands and therefore may assume a more regulatory role in controlling Notch signalling. The function of Dll3 in the immune system will be discussed further below.

**Regulation of Notch ligand binding by modification of glycosylation**

Notch receptors and ligands have conserved sequences in specific EGF-like repeats that can be modified with O- and N-linked glycans, in particular the O-fucose and O-glucose modifications mainly influence Notch signaling [41]. In *Drosophila* there are two enzymes located in the endoplasmic reticulum, O-fucosyltransferase-1 (OFUT-1) and Rumi a glycosyl transferase, that both modify Notch with O-fucose and O-glucose respectively. OFUT-1 has both enzymatic and chaperone activities that promote folding and trafficking of the Notch protein, while Rumi adds O-glucose to Notch and affects folding, stability or conformation in a temperature dependent manner [41-43]. In mammals POFUT1 is not required for surface expression of Notch receptors but it does appear to be important for ligand binding and efficient Notch signalling, but as yet there is no evidence for O-glucosyl modification of Notch receptors in mammals [44]. The addition of O-fucose to Notch EGF-like repeats is a prerequisite for the modification of Notch by the β1,3-N-acetylglucosaminyltransferase enzyme Fringe that adds N-acetylglucosamine to O-fucose residues [43, 45-48]. This additional posttranslational modification of the Notch receptor influences ligand binding by the receptor. Fringe modification of Notch promotes binding of the Delta ligands to the receptor and this may help facilitate Notch signalling when ligand concentrations are low. On the contrary Serrate/Jagged ligands are unable to bind to Fringe modified Notch receptors [49-51]. Mammals express 3 different Fringe proteins Lunatic, Manic and Radical. The three enzymes are required during embryonic development and play important roles in tissue patterning [41]. In the immune system Lunatic Fringe is the enzyme that influences the Notch dependent process of T cell lineage specification in the thymus [52], while Lunatic and Manic Fringe are required to regulate Notch signalling in relation to the Marginal Zone B cell fate decision in the spleen [53] and these functions will be described in more later.

**Ligand endocytosis and signalling**

In *Drosophila* there is a requirement for both ligand and receptor endocytosis for efficient Notch signalling to occur and DSL ligands can follow two different rates after endocytosis [54-56]. Following ligand binding to the Notch receptor, the ligand needs to be internalized by the ligand expressing cell and it has been proposed that this interaction may provide a pulling force that leads to a conformational change in the Notch receptor exposing the S2 ADAM cleavage site. This events leads to the proteolytic release of the extracellular domain of Notch (NECD) and this can be shed or endocytosed by the DSL ligand expressing cells. Alternatively, endocytosis of

DSL ligands is thought to direct ligands into an intracellular compartment where they can undergo posttranslational modification before being recycled to the cell surface where they can engage in receptor binding. Studies in *Drosophila*, *Xenopus* and Zebra fish have shown that endocytosis of the DSL ligand is directed by monoubiquitination of the cytoplasmic tail that requires the E3 ubiquitin ligases Neuralized and Mind bomb and these are critical for Notch signalling [57-61]. In mammals there are two genes encoding both classes of ubiquitin ligases Neuralized 1, 2 and Mind bomb 1 , 2 and it appears that Mind bomb is the major E3 ligase required for ligand internalization [57, 58, 62-65]. For a more thorough description of the role of endocytosis of Notch ligands and receptors the readers are directed to some comprehensive reviews written on this subject [7, 8].

**Role of Notch in T cell differentiation in thymus**

Notch signalling is important in haematopoeisis. It maintains a pool of self-renewing haematopoietic stem cells (HSC), uncommitted pool of lymphoid, myeloid and erythroid precursors in the bone marrow and is essential for the generation of definitive hematopoietic stem cells in early mouse embryos [66]. Notch signalling has a diverse role in the immune system as it can regulate the development and differentiation of T cells, B cells, monocytes, macrophages, dendritic cells, osteoclasts and natural killer cells [67]. The Notch signalling pathway is most studied and best understood in the earliest steps of T cell development. Common lymphoid progenitors (CLPs) travel form the bone marrow through the blood to seed the thymus. Notch1 is known to commit the common lymphoid progenitors into the T/NK cell lineage at the expense of the B cell, conventional and plasmacytoid dendritic cell lineages allowing efficient T ell specification [68]. Notch signalling is undetectable prior to the early T cell progenitor (ETP) stage and increases as the cells differentiate toward DN3 stage. At this stage, the cells must pass the ß-selection checkpoint, which requires two signals generated by the pre-TCR (pTα) complex and Notch1. Cells that receive these signals proliferate rapidly and following the success of ß-selection, the Notch signal is abruptly downregulated to slow down cell division [4].

The conditional deletion of Notch1 or RBP-Jκ within mouse lymphoid progenitor cells results in a marked decrease in thymus size with an early arrest of T cell development and a striking accumulation of intrathymic B cells (Radtke *et al*. 1999). The failure of other Notch receptors to compensate for the loss of Notch1 suggests that Notch1 has a nonredundant role in T cell development in the thymus by directing lineage commitment of CLPs along the T cell rather than a B cell lineage [69, 70]. Notch2 and 4 are not obligatory for T cell commitment [71, 72], while Notch3 is required during the DN-DP transition of T cell development especially around the time of β-selection [71, 73]. The progression of thymocytes beyond the β-selection checkpoint requires the combined signalling of Notch and pTα and in particular Notch signalling enhances survival by modulating glucose metabolism [74, 75].

**Role of DSL ligands in T cell development**

The thymus expresses both the Delta-like and Jagged ligands and these are largely restricted to stromal and epithelial cells [76, 77]. Using the OP9 stromal cell culture system it was thought that Dll1 and Dll4 were redundant in the thymus as ectopic expression of either Dll1 or Dll4 on OP9 cells was sufficient to support T cell development *in vitro* [78-80]. Animal studies have determined unequivocally that Dll4 is the major ligand directing T cell differentiation in the thymus. Conditional deletion of Dll4 but not Dll1 in stromal cells leads to an early arrest of T cell differentiation and an accumulation of thymic B cells [81, 82]. The role of Jagged ligands in T cell development is less clear. OP9 stromal cells expressing Jag1 or Dll-1 inhibited the differentiation of DN1 thymocytes into B-cell lineage [80]. Unlike OP9- Dll1 cells which promote αβT cell maturation, Jagged1 failed to promote αβT cell maturation but instead favoured γδ̄T cell development [80]. Likewise, the loss of Jagged2 does not affect T cell lineage specification and differentiation but *Jagged2* knockout mice displayed decreased numbers of TCRγδ+ cells in the thymus [83]. However the precise role of Jagged2 in this step of TCRγδ cell differentiation has yet to be resolved.

Fringe is an important modulator Notch signalling through posttranslational modification of Notch receptors. Fringe modified Notch receptors favours the binding of the Delta ligands at the expense of Jagged/Serrate ligands [49]. Overexpression of Lunatic Fringe provides thymocytes with a competitive advantage *in vivo* promoting T cell lineage commitment compared to Notch1$^{+/-}$ cells. This finding can now be reconciled with the known role for Dll4 ligands in directing T cell differentiation, as Fringe modified Notch receptors on thymocytes should bind more avidly to Dll4 ligands expressed on stromal cells to allow these cells to enter cellular niches where they can contact the ligands and cytokines for their survival and differentiation [52]. Endocytosis of Notch ligands is important for T cell development as the E3 ubiquitin ligase Mind bomb 1 is essential in cortical epithelial cells to induce Notch signalling in response to ligand binding by the Notch1 receptor expressed by thymocytes [84].

While most studies have focused on the role of Notch ligands expressed on stromal cells, a recent study revealed that Dll3 is expressed by thymocytes and is important in regulating Notch signalling in thymocytes. Dll3 is not required for T cell lineage specification but it does appear to help attenuate Notch signalling in DP cells [40]. In the absence of Dll3 there is an increase in mature T cells that are exported from the thymus and these cells enter the peripheral circulation. The loss of Dll3 does not affect negative selection of DP cells but it does lead to enhanced positive selection of DP thymocytes leading to production of mature CD4$^+$ and CD8$^+$ T cells [40]. This was the first reported study of Dll3 function in the immune system and showed that Dll3 acts cell autonomously in thymocytes to regulate Notch signalling. Since Dll3 does not activate Notch in *trans* it suggests that Dll3 may act in *cis*-inhibition but the exact mechanism of how this occurs has yet to be determined [40].

## Role of Notch in regulation of peripheral immune responses

The induction of immune responses to foreign antigens is dependent on the coordinated responses of both the innate and adaptive immune systems. The differentiation of CD4$^+$ T helper (Th) cells is guided by specialised antigen presenting cells that are able to translate environmental signals to help stimulate an appropriate response to the antigen. CD4$^+$ T cells were originally shown to differentiate along the Th1 and Th2 pathways that were defined by the specific patterns of cytokine secretion [85, 86]. However this paradigm has shifted to incorporate the additional Th cell lineages that have been defined including Th17 cells, T follicular helper (Tfh) cells and adaptive or inducible regulatory T (Treg) cells [87]. The differentiation of CD4$^+$ Th cells along the different lineages *in vivo* and *in vitro* is guided by the nature of the cytokines present within the immediate environment where T cell priming occurs and the expression of lineage specific transcription factors [87].

## Notch and DC differentiation

Antigen presenting cells such as dendritic cells (DCs) are crucial for T cell priming and there are two major subsets of DCs namely (i) conventional DCs (cDCs) and (ii) plasmacytoid DCs (pDCs) [88-90]. There are two types of cDCs which are referred to as resident cDCs and migratory cDCs. The resident CD8$^+$ cDCs are found predominantly in T cell areas of lymphoid tissues, whereas the migratory CD8$^-$ cDCs reside in tissues and migrate to the regional lymph node following activation. The pDCs are characterized by their ability to secrete high levels of type 1 interferon in response to viral infection and precursors are found in the peripheral blood and upon activation can migrate to nonlymphoid tissues at sites of chronic inflammation [88, 91]. There have been conflicting reports for the effect of Notch signalling on DC differentiation. Culturing bone marrow progenitors with GM-CSF and Dll1 can promote the differentiation of DCs [92]. Similarly culturing human blood monocyte derived precursors in the presence of the Dll1 ligand and the cytokines GM-CSF and IL-4 can promote their differentiation into DCs and Langerhans cells [92]. DC differentiation is impaired in Notch1 antisense mice and conditional deletion of RBP-Jκ in bone marrow cells results in the loss of the CD8$^-$ migratory DCs that reside in the marginal zone of the spleen but there was a corresponding increase in pDCs in these animals

[93]. On the other hand conditional deletion of Notch1 was shown not to have any effect on thymic DCs or on cDCs or Langerhans cells or pDCs [94, 95].

ES cells that lack Notch1 have an impaired ability to differentiate as DCs [96]. To investigate the relationship between Notch and DC differentiation further, Zhou *et al*. [97] showed that Dll1-mediated Notch signalling in HPCs can direct the differentiation of cDCs via activation of the Wnt signalling pathway. Culturing Notch1 deficient ES cells with Wnt ligands can restore cDC differentiation indicating that Wnt must be a downstream target of Notch signalling. Dll1 facilitates Wnt signalling in HPCs by inducing expression of the Frizzled proteins which are the receptors of Wnt ligands [97].

**Notch ligands expressed by DCs and immune regulation**

During their circulation in nonlymphoid tissues and blood, DCs display an immature phenotype but following recognition of microbial antigens through pattern recognition receptors such as the Toll-like receptors (TLRs), NOD-like receptors or C-type lectins, DCs become activated and migrate to draining lymph nodes where they present peptide/MHC complexes to stimulate antigen-specific naïve T cells. Naive T cells need to make stable contacts with DCs in the lymph node to achieve activation and the nature of cell surface ligands expressed by DCs at the time of antigen priming as well as the cytokines that are present will be determine the outcome of the immune response and will influence T effector cell differentiation [90, 98, 99]. Therefore DCs are well placed to directly impact the process of $CD4^+$ and $CD8^+$ T cell differentiation *in vivo*.

TLR ligands are an efficient means of directing maturation of DCs but these innate signals can also influence Notch ligand expression on APCs. The response of bone marrow derived DCs to LPS has been shown to be rely on RBP-Jκ-dependent signalling through Notch [100]. Inhibiting Notch signalling in BM DCs prevented the up regulation of MHC II expression, reduced their mobility and their antigen presenting capacity to T cells [100]. The $CD8^-$ DCs upregulate Dll4 ligand in response to LPS stimulation and can promote Th1 cell differentiation, whereas in response to LPS $CD8^+$ DCs, which reside in the T cell areas of the lymphoid tissues, upregulate IL-12 in a MYD88 dependent fashion but do not express Dll4 [101, 102]. Murine DCs treated with multiple TLR agonists specific for TLR2/4 (LPS), TLR5 (Flagellin) and TLR9 (CpG) could induce expression of Dll4 in DCs whereas Dll1 and Jagged 1 was not affected by the same treatment [103]. Murine pDCs have been shown to constitutively express high levels of Dll4 and when they present antigen to Th1 cells can induce IL-10 secretion during T cell priming both *in vitro* and *in vivo* [104]. Human DCs appear to respond in a similar way to TLR signals to that observed by murine DCs. Immature human DCs express Jagged 1 but do not express Dll4. However following stimulation with TLR agonists to TLR3 and TLR8 this lead to robust induction of Dll4 expression but had minimal effect on Jagged 1 expression. These TLR stimulated DCs expressing Dll4 could also induce Th1 responses [105].

Ectopic expression of Dll1 on allogeneic APCs could induce long lasting immune tolerance to cardiac allografts in mice [106]. Regulation in this model was mediated by $CD8^+$ T cells that when activated *in vitro* could induce IFN-γ. and IL-10 secretion [106]. A similar pattern of co-expression of IFN-γ and IL-10 has also been observed with $CD4^+$ Th1 cells recognizing antigen in the presence of Dll4 ligand on DCs [103]. Recent studies have confirmed the immunoregulatory role of Notch signalling in alloreactive T cells and that intervening in this pathway may be beneficial in inducing tolerance to alloantigens *in vivo* [106-108]. It has been proposed that antigen recognition by Th1 cells in the context of Dll4-signalling on DCs can produce IL-10 secreting cells and this may allow the diversion of the immune response from an inflammatory to regulatory activity [103].

**Th1 and Th2 responses**

Dissecting the role of Notch signalling in the regulation of peripheral immune responses is made difficult due the large number of Notch receptors and ligands that can be expressed by peripheral T cells as well as the diversity of

experimental systems that have been employed to address this issue. Antigen presenting cells expressing Jagged 1 or Dll1 ligands give rise to distinct responses following priming. Antigen recognition in the context of the Dll1 ligand can induce Th1 responses whereas recognition in the presence of the Jagged1 ligand on APCs induces Th2 responses [109-111]. The role for Notch signalling in Th1 cell differentiation is less compelling compared to that of Th2 cell differentiation. Genetic inactivation of Notch1 alone, or the deletion of both Notch1 and Notch2, RBP-Jκ or the transgenic expression of the DNMAML1 in $CD4^+$ T cells did not affect Th1 cell differentiation [109, 112-114]. T cells in each of these mouse strains were able to maintain robust IFN-γ secretion a hallmark of Th1 immunity. However several studies have described that Notch signalling can favour Th1 cell differentiation. Antigen presented by Dll1 expressing APCs could direct Th1 cell differentiation; ectopic expression of Notch1 ICD or Notch3 ICD in $CD4^+$ T cells could promote Th1 cell differentiation; or treatment of $CD4^+$ T cells with the γ-secretase inhibitor GSI could inhibit Th1 differentiation *in vitro* [115, 116]. However if Notch does have a role in Th1 differentiation it would appear from the gene knockout studies that this would have to be independent of the canonical Notch signalling pathway involving RBP-Jκ [109, 112, 113]. There is evidence in *Drosophila* that RBP-Jκ independent signalling can occur downstream of Notch, but this pathway remains poorly defined.

In contrast abrogation of Notch signalling in $CD4^+$ T cells prevented Th2 differentiation in mice primed with various Th2 polarizing antigens such as nematode infection or protein antigens immunized in alum adjuvant. The loss of Notch signalling lead to decreased secretion of IL-4 and a failure to upregulate GATA-3 the master regulator of Th2 cell differentiation, whereas IFN-γ secretion was unaffected [109]. Supporting the role for Notch signalling in Th2 cell differentiation APCs expressing Jagged ligands can favour Th2 responses *in vivo* [109, 111, 117]. Molecular studies have identified that both *Il4* and *Gata3* are direct Notch target genes in $CD4^+$ T cells [109, 118]. Notch and RBP-Jκ were immunoprecipitated from the HS5 *Il4* enhancer and Notch/RBP-Jκ complexes were also detected bound to the upstream GATA-3 promoter in $CD4^+$ Th cells [118]. Amsen and colleagues identified that culturing RBP-Jκ deficient and Notch1/Notch2 deficient $CD4^+$ T cells could still give rise to IL-4 secretion *in vitro*, but when allowed to differentiate *in vivo* following antigen priming, Th2 cell differentiation was completely blocked [112]. This discrepancy highlights important differences between *in vitro* and *in vivo* systems and that the *in vitro* polarizing culture conditions used by many laboratories around the world appear to override the Notch signal that would normally regulate Th2 differentiation.

Previous studies have shown that stimulation of Th1 cells with IL-12 or IL-27 can induce IL-10 production without affecting IFN-γ secretion [119-121]. The Dll4 mediated IL-10 secretion by of Th1 cells requires the presence of either IL-12 or IL-27 and is mediated by STAT4 signalling and IL-10 induction could be blocked by the treatment of the γ-secretase inhibitor indicating that induction of IL-10 secretion is mediated by the canonical Notch signalling pathway[103]. pDCs that constitutively express Dll4 ligand can promote IL-10 secretion by Th1 cells *in vitro* [104]. Therefore this may be an important mechanism for immune regulation *in vivo* mediated by Notch signalling. Ectopic expression of Jagged ligands on DCs can promote Th2 immunity but there is also evidence that Jagged ligands can also directly influence the development of regulatory T cells *in vivo*.

The requirement of Notch signalling in human $CD4^+$ Th cell differentiation has not been clearly resolved. Using an RNAi knockdown approach Stallwood *et al*. examined the role different ligands in both human monocyte derived DCs and $CD4^+$ T cells [122]. They found that the knockdown of specific ligands in DCs enhanced IFN-γ production by alloreactive $CD4^+$ T cells. Knockdown of the Dll1 ligand in human $CD4^+$ T cells enhanced IFN-γ, IL-2 and IL-5 production following stimulation with anti-CD3/anti-CD28 antibodies while knockdown of either Jagged 1 or Jagged 2 ligands had no effect on cytokine production by human $CD4^+$ T cells [122]. Treatment of $CD4^+$ T cells in this model system

with a γ-secretase inhibitor did not influence cytokine production indicating that the Dll1 modulation of cytokine secretion in human T cells occurs by a CSL independent pathway [122]. These studies would suggest that there are potential implications for modulation of Notch signalling in ligand expressing cells through expression of the ligands which may be reminiscent of *cis*-inhibition. The canonical Notch ligands Delta and Serrate can both function in *cis*-inhibition of Notch when expressed in the same cell.

**Regulatory T cells**

Autoimmunity arises following a failure in either central or peripheral tolerance mechanisms and is mediated by Th1 and Th17 responses that can give rise to either organ specific or systemic autoimmune diseases. In the periphery the immune system has a range of mechanisms available to control the fate of autoreactive T cells, including immune privilege, immune ignorance, activation induced cell death, clonal anergy and immune suppression mediated by regulatory T (Treg) cells. Regulatory $CD4^+$ T cells play a central role in maintaining immune homeostasis to limit aberrant responses to antigen by effector T cells to both self and foreign antigens but the control of immune responses to these different antigens is mediated by distinct group of Treg cells [87, 123-125]. Natural Treg cells arise in the thymus during T cell differentiation they express the winged helix transcription factor Foxp3 and differentiate to become mature cells with suppressive function. Once they enter the peripheral circulation they can circulate through lymphoid tissues and act to suppress immune responses to self antigens through both cell contact and through the secretion of inhibitory cytokines such as IL-10 and TGF̄β. Inducible Treg (iTreg) cells differentiate from naïve $CD4^+$ $CD25^-$ cells when activated through their TCR in the presence of TGF-β and IL-10. These cells can also express *Foxp3* and differentiate as an iTreg cell that can suppress the response of naïve $CD4^+$ effector T cells through a mechanism that relies on the secretion of inhibitory cytokines such as IL-10, TGF-β or IL-35 [123].

The immunoregulatory cytokine TGF-β plays an important role in dampening T cell proliferative responses. In more recent years it has become apparent that the function of TGF-β in the immune system is more pleiotropic and its influence on $CD4^+$ T cell differentiation depends on the types of cytokines that are present at the time of T cell priming. Naïve T cells activated in the presence of TGF-β and IL-6 can switch on the transcription factor ROR-γτ and differentiate to become Th17 cells which are involved in bacterial immunity at mucosal surfaces. When T cells encounter TGF̄β alone, this can induce *Foxp3* expression and direct iTreg development [126-129]. In contrast, the presence of IL-6, antagonizes Foxp3 expression by a direct affect on serine threonine kinase AKT [130]. TGF-β signalling by the TGF-βIIR leads to the phosphorylation of Smad2/3 which forms a complex with Smad4 and this complex is translocated to the nucleus to regulate transcription of target genes. One of the targets is Smad7 which is an inhibitory Smad used to attenuate TGF-β signalling by competing with Smad 2/3 competes for binding to the receptor [131, 132].

**Notch ligand expression on APCs and iTreg development**

It is more than 10 years since the first report that Notch ligands expressed on APCs could influence the outcome of a peripheral immune response. Hoyne *et al*. showed that ectopic expression of Jagged 1 on spleen DCs presenting allergen-derived peptides could inhibit immune responses to the house dust mite allergen Der p 1 [133]. The suppression was long lasting and was transferable to naïve recipient animals via $CD4^+$ iTreg cells in an antigen-specific manner [133]. These findings have been corroborated by several studies suggesting that Jagged ligands expressed by APCs favour iTreg cell differentiation in $CD4^+$ T cells in both mouse and human culture systems [134-137]. Injection of Jagged2 expressing haematopoietic cells into nonobese diabetic (NOD) mice lead to the expansion of iTreg cells *in vivo* and these cells could decrease the incidence of spontaneous type1 diabetes in NOD mice recipients. The induction of iTregs cells in this system could be inhibited by the addition of neutralizing antibodies to either Jagged2 or Notch3 dependent manner and suppression by Treg cells required cell-cell contact that was independent of TGF̄β [135].

Mouse and human T cells appear to have different preferences for the Notch ligands required to induce Treg differentiation, as a recent report highlighted that the Dll1 ligand can induce differentiation of human $CD4^+$ $Foxp3^+$ Treg cells *in vitro* from $CD34^+$ cord blood haematopoietic progenitor cells grown on OP9-Dll1 stromal cells [138]. It is important to note that the effect of Notch signalling on Treg differentiation appears restricted to the iTreg population and does not affect nTreg differentiation. Blockade of Notch signalling by conditional deletion of Notch receptors, RBP-Jκ signalling, or the expression of the DNMAML1 transgene in $CD4^+$ T cells, does not affect the development or differentiation of the nTreg cells in the thymus and these animals show no evidence of spontaneous autoimmunity suggesting nTreg development and homeostasis must be normal despite the absence of Notch signalling [70, 71, 112, 114, 118].

**Notch and TGF–β signalling in iTreg development**

The induction of the master regulator gene *Foxp3* is critical for $CD4^+$ Treg development and it appears that *Foxp3* is a target gene of Notch signalling in $CD4^+$ T cells [139]. Expression of the constitutively active NICD gene can induce expression of a *Foxp3* promoter and the NICD-RBP-Jκ complex has been immunoprecipitated from the *Foxp3* promoter in T cells [140]. Samon *et al*. showed that treatment of $CD4^+$ T cells with the γ-secretase inhibitor could block the induction of TGF-β-induced *Foxp3* expression in $CD4^+$ T cells and the differentiation of Treg cells [141]. Likewise, blockade of Notch1 signalling in $CD4^+$ T cells could prevent TGF-β̃ mediated induction of iTreg differentiation *in vitro*. Collectively these studies showed that *Foxp3* is a direct target of Notch signalling and this was further supported by animal studies which showed that blockade of Notch1 *in vivo* reversed the immunosuppressive effect of $CD4^+$ $Foxp3^+$TGF-$\beta^+$ cells in allergic airway inflammation in mice. The iTregs cells in this model expressed membrane bound TGF-β and this was crucial to induce Notch signalling in target cells [142]. Soluble TGF-β is unable to induce Notch signalling even though it can induce STAT3 signalling. The relationship between Notch and TGF-β iTreg effector function was further explored by Asano *et al*. who showed that Tregs can express Notch ligands, Jagged1 and Dll4, and that blockade of Notch signalling can inhibit the suppressive function of Tregs[143]. The NICD can interact with phospho-SMAD3 and promote its translocation to the nucleus where it can modulate the expression of TGF-β target genes. Furthermore culturing $CD4^+$ T cells in the presence of TGF-β and Dll4 could inhibit iTreg differentiation *in vitro* by inhibiting Jak3 induced Stat5 phosphorylation which is required for *Foxp3* expression [134, 144]. At present there is no definitive knowledge about the specific receptor-ligand pairs that are required to mediate Treg differentiation *in vivo*. Jagged ligands appear to favour iTreg differentiation but the precise Notch receptor involved in this process has not been resolved and may be either Notch1 or Notch3 [143, 145, 146]. Further studies using conditional knockouts of these genes are required to resolve this issue.

The current view is that the Delta-like ligands are strong inducers of Th1 immunity and to demonstrate the functional relevance of Notch signalling on immune regulation *in vivo* different experimental models have been used to study the effect blockade of Notch signalling on the outcome of disease pathogenesis. Experimental allergic encephalomyelitis (EAE) is an autoimmune inflammatory disease of the central nervous system in mice mediated by Th1/Th17 cells. Blockade of Dll4 on APCs during the induction phase of EAE reduced the clinical severity of the disease and was associated with an expansion of $CD4^+$ Treg cells in the periphery and the CNS [134, 144, 147]. Modulation of Notch signalling has been attempted in a variety of Th2 animal models. Firstly, aerosol delivery of allergens can induce allergic sensitization and a Th2 mediated disease in the airways. Administration of an anti-Dll4 antibody during a period of allergic sensitization in mice exacerbated the development of allergic airway disease. Mice treated with an anti-Dll4 antibody showed evidence of increased airway hyper-reactivity and mucus production [148]. Pharmacological blockade of Notch signalling using a γ-secretase inhibitor *in vivo* during allergic sensitization was capable of reducing Th2 mediated

airway hyperreactivity [149]. In addition treatment of mice with an anti-Dll4 antibody could block the development of allergic conjunctivitis in mice [150]. Finally treatment of mice with an antibody to Dll4 could also alleviate symptoms of respiratory syncytial virus infection a Th1 mediated disease. The virus specific effector $CD4^+$ T cells that emerged following anti-Dll4 treatment displayed a Th2 phenotype and an increase in activated $CD8^+$ T cells in the lung [151].

**T cell anergy**

T cell activation is dependent on the delivery of two separate signals. Signal one is mediated through the TCR and the second signal is through the costimulatory receptor CD28 in response to binding of its ligands CD80/CD86 which leads phosphorylation of a number of intracellular signalling pathways including phospholipase γ-1, protein kinase-θ, MAPK, JNK, PI3K and Iκ-B kinase (IKK) that leads to the recruitment of transcription factors (e.g. NFAT, AP-1 and NF-κB) critical to the transcription of the *Il2* gene [152]. Ligation of TCR on T cells in the absence of CD28 costimulation leads to the development of clonal anergy which is characterized by the failure to activate the MAPK, PI3K/AKT and the IKK pathways, and results in reduced activity of the nuclear factors AP-1 and NF-κB and deficient IL-2 gene transcription, but there is elevated NFAT signalling in anergic cells [153]. Clonal anergy is an active process that requires new protein synthesis and is associated with an anergic gene expression profile that is characterized by increased expression of a number of E3 ubiquitin ligases including Cbl-b, Itch, Grail [154]. Recent studies have also identified that the induction of anergy is associated with induction of numerous negative regulators of TCR signalling including growth arrest and DNA-damage-inducible 45 β (Gadd45β), *diacyglycerol kinase*, *caspase3*, *Traf6*, *Ikaros*, *Egr2*, *Egr3* and *CREM* (cyclic AMP response element modulator) [154-160]. Egr2 and Egr3 can regulate the expression of Cbl-b [159]. Deltex is induced during $CD4^+$ T cell anergy mediated by treatment of cells with the calcium ionophore, ionomycin or treatment with CTLA-4Ig which blocks delivery of costimulatory signals to T cells [161] and human anergic T cells [162]. Genetic deletion of *Dtx1* does not affect T cell development which is surprising given that it is a downstream target of Notch signalling [20, 161, 163]. Loss *Dtx1* prevents induction of Cbl-b expression during T cell anergy and *Dtx1* deficient mice display a mild splenomegaly and T cells are hyperproliferative *in vitro* compared to wild type T cells when stimulated with anti-CD3 and anti-CD28. The mice also produce spontaneous auto-antibody formation and exhibit pulmonary inflammation suggesting a defect in immune regulation that leads to spontaneous autoimmunity [161]. The data implies that Dtx1 plays an important role in dampening TCR signalling and is another E3 ligase that plays an important role in the induction of T cell anergy to control the fate of effector T cells [164-166].

**Marginal zone B cells**

B cell development occurs in the bone marrow from CLPs that progress through a well defined differentiation process characterized by the expression of different cell surface and intracellular proteins [167, 168]. Three populations of mature B cells migrate exist in the periphery that reside in distinct anatomical locations and display distinct physiological roles. B1 B cells are found primarily in pleural and peritoneal cavities and are thought to provide an important response to bacteria and provide humoral immunity against invading gut pathogens. B2 follicular B cells circulate through the blood and spleen and are the predominant type of B cell found in lymph nodes [167-169]. They localize adjacent to the T-cell enriched areas of lymphoid tissues and respond to T-dependent antigens where they form germinal centres to help generate high affinity antibodies an undergo isotype switching. B2 Marginal Zone (MZ) B cells take up residence in the marginal sinus in the marginal zone of the spleen. MZ B cells are also thought to contribute to immunity to bacterial pathogens as they respond to T-independent antigens [167-169].

A further step of B cell maturation occurs in the spleen and progresses through two transitional stages defined as Type 1 (T1) and Type 2 (T2) and these help to give rise to the MZ B cells and follicular B cells in the spleen. T1B cells represent

the recent BM cell migrants and have a surface phenotype ($IgM^{hi}IgD^{-}CD21^{-}$ $CD23^{-}$) and they can develop into ($IgM^{+}IgD^{+}CD21^{+}$ $CD23^{+}$) T2 B cells. The T2 cells can further differentiate to become recirculating B cells and it was proposed that this population of cells may contain the MZ B precursors [169]. The signals that direct the MZ versus follicular B cell fate had been largely unknown but recent studies have provided convincing evidence that Notch signalling plays a crucial role in this decision process.

Conditional deletion of either Notch2 or the downstream signalling molecule RBP-Jκ leads to the selective loss of MZ B cells in the spleen [71]. Conversely, the conditional deletion of Mint a negative regulator of Notch signalling leads to the preferential production of MZ B cells at the expense of B2 follicular cells [170]. Collectively these studies reveal that specification of the MZ B cell fate requires a canonical Notch signal that is mediated by Notch2 and RBP-Jκ and that this pathway can be inhibited by Mint. Jagged ligands do not play a role in the MZ B cell fate decision but rather Dll1 is the major ligand required for the MZ B cell fate decision [81]. It was recently shown that Dll1 is expressed on endothelial cells in the red pulp and marginal zone in the spleen and conditional deletion of Dll1 in B cells leads to the selective depletion of MZ B cells [53]. Lunatic and Manic Fringe appear to cooperate to modify Notch receptors on B cells to enhance the initial weak interaction between Notch2 and Dll1 [53]. Fringe modified receptors on B cells enhances precursor competition for Dll1 in defined niches and this directs the formation of marginal zone precursors and these can differentiate to become mature MZ B cells.

Given the important role that Notch signalling has in the regulation of Th1/Th2 responses *in vivo*, it is interesting that relatively little is known about the role of Notch signalling in controlling humoral immune responses. A study by Santos showed that Dll1-Notch signalling in B cells could enhance the production of antibody secreting cells by naturally activated MZ B cells and B1 B cells [171]. Conversely suppression of Notch signalling by either conditional deletion of Notch1 or expression of the DNMAML1 could block enhancement of antibody secretion in LPS activated B cells [171]. There is a lot more to learn about the role of Notch in regulating B cell responses to antigen. T follicular helper (Tfh) cells play a critical role in initiating the germinal centre response in the spleen by directly regulating the activation of follicular B cells. At present there is nothing known about the role of Notch in Tfh cells and this will be an important area to explore with the use of conditional knockouts with receptors and/or ligands.

## CONCLUSIONS AND FUTURE PROSPECTS

Notch is an evolutionary conserved signalling pathway and over the last 15 years there has been a considerable advance in our knowledge about how Notch influences cell fate decisions and differentiation in a wide range of cell types in the immune system. The preeminent cell fate decisions regulated by Notch include the T/B cell fate decision in the thymus and the differentiation of marginal zone B cells in the periphery where the precise Notch receptor - ligand pairs have been unequivocally determined. The Notch ligands appear to have exclusive roles like that observed in other developmental systems, this is best observed in mature T cells in regulating the Th1/Th2 cell fate decision where Delta and Jagged ligands induce distinct responses but this response may depend on integration of signals from more than one Notch receptor. The general consensus of the studies suggests that Notch-Notch ligand signals mediate an inductive signal to regulate cell fate specification. There does not appear to be any evidence for lateral inhibition having a role in regulating cell fate decisions in the thymus or in any other tissues in the immune system.

The role of Notch signalling in the development of iTreg cells continues to receive some focus and this has important implications for therapeutic modulation of immune based diseases such as allergy, autoimmunity and transplantation tolerance. There is strong evidence for the role of Jagged ligands inducing iTreg differentiation in both mouse and human T cells, but exactly how these ligands are able to induce iTreg cells requires further dissection. Some studies indicate that ectopic expression of Notch ligands on APCs can direct iTreg differentiation while others suggest

that it is the expression of Notch ligands by Tregs themselves that help mediate the suppressive function. Further studies on this line will be helpful and maybe both types of signals are required during the response *in vivo* as inductive Notch signals delivered by APCs could help direct iTreg differentiation and then through the release of cytokines such as IL-10 and Jagged expression by the emerging iTreg cells they could reinforce Notch signalling between other iTreg cells and this could help stabilize their differentiation program.

During the last decade there have been new insights provided into the plasticity of $CD4^+$ Th cell differentiation and this plays an important role in shaping the effector response to a pathogen. Pivotal to this new development is the role that TGF-β plays, where it can influence the development of two distinct Th lineages one associated with inflammatory diseases (Th17) and the other for immune regulation (iTregs). In addition IL-10/IFN-γ secreting Th1 cells have been observed in mouse and human Th cells, and the Dll4-Notch signal together with IL-12 or IL-27 can induce strong induction of IL-10 in established Th1 cells. This may represent another mechanism by which the immune system tries to dampen immune responses once an infection has been controlled. Different DC subsets display Notch ligand expression and in particular Dll4 expression appears to be upregulated in response to TLR signals in both mouse and human DCs.

The therapeutic potential of Treg cells in clinical medicine has been mooted for a while but the key is to be able to grow out these cells stably in culture and in some cases to derive antigen-specific iTreg cells. Culturing T cells with Jagged DCs can induce a population of iTreg cells *in vitro* which have suppressive effects on naïve T cells. Perhaps improved culture systems for expanding iTreg cells *in vitro* could be facilitated by eliminating Fringe expression by T cells. Jagged ligands cannot bind to Fringe modified Notch receptors and so this could be a useful way together with cytokines IL-10, TGF-β and IL-2 to bias iTreg differentiation and expansion *in vitro*. These cells could then be used in a variety of settings to help modulate disease [91, 107, 108, 144, 149].

Although Notch signalling appears to influence immune regulation it is interesting to note that many of the conditional knockouts of receptors, ligands or downstream signalling components do not develop significant autoimmune diseases that lead to perinatal death such as that observed with the loss of *Foxp3* in Scurfy mice and in X-Linked Autoimmunity-Allergic Dysregulation (XLAAD)/ Immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) patients. This indicates that Notch signals are not required for the differentiation of the nTreg population and even though Notch signalling can influence immune regulation there must be sufficient checkpoints in place in the periphery to control cellular immune responses. It is going to be fascinating to see how the field develops in the next 10 years and perhaps Notch signalling could be targeted in therapeutic interventions in a range of immune mediated diseases.

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