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REVIEW

Notch signaling and its emerging role in autoimmunity

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Abstract **Studies of notch signaling in immune cells have uncovered critical roles for this pathway both during the differentiation and effector function phases of immune responses. Cells of the myeloid lineage, including macrophages and dendritic cells, function as key components of innate immune defense against infection and, by acting as antigen presenting cells, can instruct cells of the adaptive immune response, specifically CD4 and CD8 T cells. Tight regulation of this functional interaction is needed to ensure a well-balanced immune response and its dysregulation may indirectly or directly cause the tissue damage characteristic of autoimmune diseases. In this review, the focus will be placed on those recent findings which support a role for notch signaling in inflammatory responses mediated by macrophages and other myeloid lineage cells, as well as peripheral T cells, and their relevance to inflammatory and autoimmne diseases.**

Keywords notch signaling, inflammation, autoimmunity

Introduction

In mammals, the notch family of transmembran receptors and ligands consists of four receptors, notch14 and five ligands, Jagged (Jag) 1, 2 and Delta-like (Dll) 1,3 and 4. Mature notch receptors are fucosylated in the endoplasmic reticulum, glycosylated in the trans-Golgi and, after furin-mediated processing at their “S1” site, are assembled as non-covalently bound heterodimers and inserted into the cell membrane. Canonical notch signaling is initiated upon ligand-receptor engagement which is thought to induce a conformational change in the receptor’s structure, exposing an ADAM protease-specific cleavage site just proximal to the exterior of the cell membrane. Following cleavage at this “S2” site the ligand and extracellular domain of the notch receptor are endocytosed into the ligand-bearing cell. Subsequently, the remaining portion of the notch receptor is internalized in endocytic vesicles of the receptor-expressing cell, where it is further processed by the enzymatic actions of gamma-secretase to liberate the signaling-competent intracellular domain (NICD). Translocation of NICD to the nucleus positions it to interact with its DNA binding partner, RBP-jκ/CSL. This association converts the transcriptional complex in which RBP-jκ/CSL is found from a repressor to an activating complex. Blocking notch signaling in transgenic mice has been achieved genetically by i) conditionally-deleting RBP-jκ/CSL, notch1 or notch2 or both; ii) generating transgenic mice deficient for notch3 or notch4; iii) preventing signaling though another important nuclear binding partner, mastermind-like (MAML), by expressing a dominant negative (DN) form; iv) conditionally deleting notch ligands; or v) deleting *pofut1* which plays an essential role in fucosylating notch receptors (Radtke et al., 1999; Krebs et al., 2000; Han et al., 2002; Krebs et al., 2003; Shi and Stanley, 2003; Hozumi et al., 2004; Mancini et al., 2005; Tu et al., 2005; Brooker et al., 2006; Kiernan et al., 2006; McCright et al., 2006; Hozumi et al., 2008). Pharmacological methods have also been developed to block notch signaling by inhibiting the final, activating cleavage mediated by gamma-secretase. Similar to deletion of RBP-jκ/CSL or expression of DN-MAML, administering gamma-secretase inhibitors (GSI) affects signaling through all notch receptors. It remains unclear, however, whether each of these means of interrupting notch signaling affects the downstream biological outcomes equivalently (Fig. 1).

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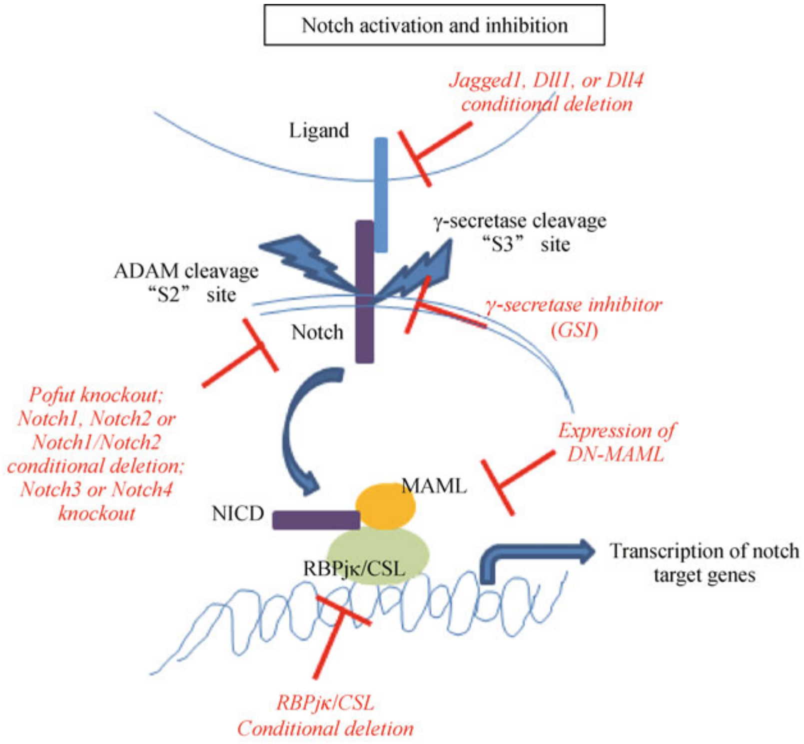

Figure 1 Activation and inhibition of notch signaling. Following ligand binding notch receptors are sequentially cleaved to release their intracellular, transcriptionally active components. This includes cleavage at the S2 site by an ADAM protease followed by cleavage at the S3 site by a gamma-secretase containing complex. Cleavage by gamma-secretase liberates the signaling-competent intracellular domain of notch (NICD), which translocates to the nucleus and interacts with its canonical nuclear binding partners, MAML and RBPjκ/CSL to transcribe notch target genes. Notch signaling can be inhibited genetically or pharmacologically, as indicated in red above.

The involvement of the notch pathway in lineage commitment of hematopoietic cells and thymocyte development has been extensively reviewed elsewhere, as has its signaling in peripheral T cell activation and differentiation (Radtke et al., 2002; Osborne and Minter, 2007; Amsen et al., 2009; Radtke et al., 2010; Billiard et al., 2011), thus, the focus here will be on how notch signaling regulates pro-inflammatory responses mediated by cells of the innate and adaptive immune systems and how it may contribute to chronic inflammation and autoimmunity.

Expression of notch receptors and ligands in cells of the immune system

Various notch receptors and ligands are expressed on most cells of the immune system. Some discrepancies in their expression patterns exist between murine and human cells and they are summarized in Table 1.

Myeloid lineage cells

Transcripts of *notch1* and *notch4* are detected at high levels in mature resting murine macrophages while that of *notch3* can be detected at all stages of myeloid lineage development using single-cell RT-PCR or precursor slot blot assay (Singh et al., 2000). Expression of notch1 and notch2 proteins in mature macrophages can be readily detected by Western blots and at a single cell level by flow cytometry (Monsalve et al., 2006; Palaga et al., 2008; Zhang et al., 2010; Zhang et al., 2012). While all notch receptors are expressed in human macrophages, only notch3 is upregulated during differentiation from monocytes (Fung et al., 2007). Notch ligands can also be detected in resting and activated murine and human macrophages, with Jag1, Dll1 and Dll4 being the major ligands expressed (Yamaguchi et al., 2002; Fung et al., 2007).

The expression patterns and levels of notch receptors and ligands found on murine and human macrophages change following activation of these cells by various stimuli such as agonists for the pattern recognition receptors (PRR), infection with intracellular pathogens, growth factors or cytokines (Hu et al., 2008; Kim et al., 2008; Narayana and Balaji, 2008; Palaga et al., 2008). Upon activation by PRR agonists, human and murine macrophages upregulate Jag1 (Foldi et al., 2010) and pro-inflammatory stimuli induce expression of Dll4 in human macrophages (Fung et al., 2007). Expression of notch receptors and ligands on granulocytes has also been reported whereby notch1, notch2, Jag1 and Jag2 were detected (Singh et al., 2000; Jönsson et al., 2001). Mast cells also

Table 1 Summarized expression of notch receptors and ligands in immune cells

Cell type	Notch receptors	Notch ligands	References
Myeloid lineage			
(1) Monocyte/macrophage			
Mice	Notch1, 2, 3, 4 (RT-PCR, Western blot, Flow cytometry)	Jag1, Dll1, Dll4 (RT-PCR)	Jönsson et al., 2001; Monsalve et al., 2006; Palaga et al., 2008; Zhang et al., 2010
Human	Notch1, 2, 3, 4 (RT-PCR, Western blot)	Jag1, Dll1, Dll4 (RT-PCR, Western blot)	Fung et al., 2007; Foldi et al., 2010
(2) Granulocytes			
Mice	Notch1, 2 (RT-PCR)	Jag1, Jag2 (RT-PCR)	Singh et al., 2000; Jönsson et al., 2001; Yamaguchi et al., 2002
Human eosinophils	Notch1, 2 (RT-PCR, Flow cytometry)	Jag1, Jag2 (Western blot, Flow cytometry)	Radke et al., 2009
(3) Mast cells			
Mice	Notch1, 2 (Flow cytometry)	Jag1 (RT-PCR)	Singh et al., 2000; Nakano et al., 2011; Sakata-Yanagimoto et al., 2011
(4) Dendritic cells			
Mice	Notch1, 2 (RT-PCR, Western blot)	Jag1, Jag2, Dll1, Dll3, Dll4 (RT-PCR, Western blot)	Cheng et al., 2010; Hoyne et al., 2000; Weijzen et al., 2002; Yamaguchi et al., 2002
Human	Notch1, 2 (Flow cytometry, immunofluorescence)	Jag1, Jag2, Dll1, Dll4 (Flow cytometry, immunofluorescence)	Pérez-Cabezas et al., 2011
Lymphoid lineage			
(5) CD4 T cells			
Mice	Notch1i(4), 2i(24), 3i(24), 4 (Flow cytometry; RT-PCR; Western blot)	Jag1, Jag2i, w(48) (qRT-PCR, Flow cytometry)	Adler et al., 2003;Palaga et al., 2003; Amsen et al., 2004; Benson et al., 2005; Minter et al., 2005; Jurynczyk et al., 2008; Moriyama et al., 2008; Fiorini et al., 2009; Koyanagi et al., 2012
Human	Notch1i(24) (Flow cytometry, Western blot)		Keerthivasan et al., 2011; Minter, unpublished results
(6) CD8 T cells			
Mice	Notch1i(4), 2i(24), 3i(24) (Flow cytometry)	Jag2i, w(48) (Flow cytometry)	Palaga et al., 2003; Fiorini et al., 2009; Koyanagi et al., 2012
Human	Notch1i(12) (Flow cytometry)		Minter, unpublished results
(7) Th1 cells			
**Mice	Notch1^{+++}, 2^{+++} , 3i(24) (Flow cytometry)	None detected (Flow cytometry)	Maekawa et al., 2003; Minter et al., 2005; Koyanagi et al., 2012
Human	Notch1^{+++} (Flow cytometry)		Minter, unpublished results
(8) Th2 cells			
**Mice	Notch1^{++} , 2^{+}, 3w, i (24) (Flow cytometry)	None detected (Flow cytometry)	Minter et al., 2005; Koyanagi et al., 2012
(9) Th17 cells			
**Mice	Notch1^{+}, 2w, 3i(24) (Flow cytometry, Western blot)	None detected (Flow cytometry)	Keerthivasan et al., 2011; Koyanagi et al., 2012
Human	Notch1 (Western blot)		Keerthivasan et al., 2011
(10) T regulatory cells			
Mice	Notch1i(24), 2w, i(24), 3vw, i(24) (Flow cytometry)	Jag1, Jag2vw, Dll1, Dll4 (Flow cytometry)	Kared et al., 2006; Ostroukhova et al., 2006; Koyanagi et al., 2012
(11) CD19^{+} B cells			
Mice	Notch1, 2 (RT-PCR, Flow cytometry)		Moriyama et al., 2008; Bertrand et al., 2000

(*Continued*)

Cell type	Notch receptors	Notch ligands	References
Human	Notch1 (RT-PCR)		Bertrand et al., 2000

*: The techniques used to detect expression are shown in parentheses; **: T helper cells were polarized *in vitro* for 10 days before receptor and ligand expression were determined by flow cytometry in fully-polarized and restimulated (i) cells; $^{+++}$: Strong expression; $^{++}$: Moderate expression; $^{+}$: Low level expression; w: Weak expression; vw: Very weak expression; i(hr): Induced upon stimulation for length of time designated in parentheses.

constitutively express notch1 and notch2 (Nakano et al., 2009; Nakano et al., 2011).

Multiple studies have focused on the expression of notch ligands on dendritic cells (DCs), which are likely to play an essential role in priming T cells during activation. All notch ligands are detected on human and murine DCs which originate from various tissues such as thymus, spleen and bone marrow (Hoyne et al., 2000; Yamaguchi et al., 2002). Among these, Jag1 and Jag2 are the most abundant ligands, and are found on most DCs. Dll1 and Dll3 are found on splenic DCs and bone marrow-derived DCs, respectively (Cheng et al., 2010). Human DCs express notch1and notch2, but not notch3, similar to the expression patterns observed on murine bone marrow-derived DCs (Pérez-Cabezas et al., 2011).

Other components of the notch signaling pathway can also be detected in myeloid cells, including RBP-jκ/CSL, Fringe, and ADAMs (Fung et al., 2007), as well as the negative regulators, Numb and Itch (Kuenjinda and Palaga, unpublished data). Taken together, these observations suggest that, beyond their developmental stages, notch signaling may function to regulate the effector phase of innate immune cells.

Interactions between toll-like receptors and notch signaling in macrophages

The innate immune system utilizes genome-encoded pattern recognition receptors (PRR) to recognize the molecular patterns associated with danger signals derived from pathogens and foreign molecules. One of the most extensively studied families of PRRs is the Toll-like receptors (TLR) which recognize various components foreign to hosts, such as lipopolysaccharide (LPS), lipopeptides, unmethylated CpG DNA and dsRNA (Takeuchi and Akira, 2010). Other PRRs include the NOD-like receptors and RIG-I-like receptors. Recognition of specific agonists by these PRRs leads to the activation of diverse intracellular signaling cascades and results in the production of pro-inflammatory cytokines and release of cytotoxic granules. Furthermore, engagement of PRRs by their agonists also leads to increased expression of cell surface molecules necessary for T cell activation, including co-stimulatory molecules CD80 and CD86 and secreted cytokines which instruct T helper cell differentiation and further govern the outcome of an immune response.

In macrophages, stimulation by various TLR agonists, such as TLR2, 3, 4 and 9, results in activation of the notch signaling pathway. This conclusion is supported by the presence of the cleaved form of the notch receptors and/or increased expression of one or more notch target genes, including Hairy/Enhancer of Split (Hes), bHLH proteins and related genes (*Hes/Hey*) and *Deltex* (Hu et al., 2008; Palaga et al., 2008). Notch activation by LPS and lipopeptide via TLR4 and TLR2, respectively, is mediated by MyD88, the adaptor molecule of TLR signaling, because MyD88$^{-/-}$ macrophages fail to upregulate notch expression upon stimulation (Palaga et al., 2008). Just how, then, is notch signaling initiated when a TLR is engaged by its ligand? Detailed analyses of the expression of notch ligands following stimulation with TLR agonists identify Jag1 in mice and Dll4 in humans to be the probable ligands responsible for activating notch signaling in macrophages (Fung et al., 2007; Foldi et al., 2010). The differences in responses observed between humans and mice are likely due to distinct notch receptors expressed on the cell surface of macrophages; whereas human macrophages upregulate notch3, murine macrophages express notch1 and notch2.

Activation of TLR signaling induces Jag1 expression through NF-κB- and JNK-dependent mechanisms (Foldi et al., 2010; Tsao et al., 2011). Activating TLR in the presence of IFNγ enhances Jag1 expression and the expression of Jag1 auto-amplifies notch signaling (Foldi et al., 2010). Furthermore, treating macrophages with LPS in combination with IFNγ induces more robust levels of cleaved N1ICD than treatment with either one alone (Palaga et al., 2008). In contrast, it has also been reported that IFNγ treatment inhibits TLR-mediated activation of notch signaling in macrophages by suppressing activation of notch2 (Hu et al., 2008). Therefore, the detailed mechanism of how IFNγ influences notch signaling in macrophages through the TLR pathway awaits further investigation (Fig. 2).

Notch signaling in regulating inflammatory responses in macrophages

The functional significance of notch signaling in macrophages following stimulation has been characterized using both inhibitory and activating approaches. Notch signaling can be abrogated by the use of gamma-secretase inhibitors (GSI) or by utilizing transgenic animals with targeted deletions of components of the notch signaling pathway such as RBP-jκ/CSL. Blocking notch signaling decreased the

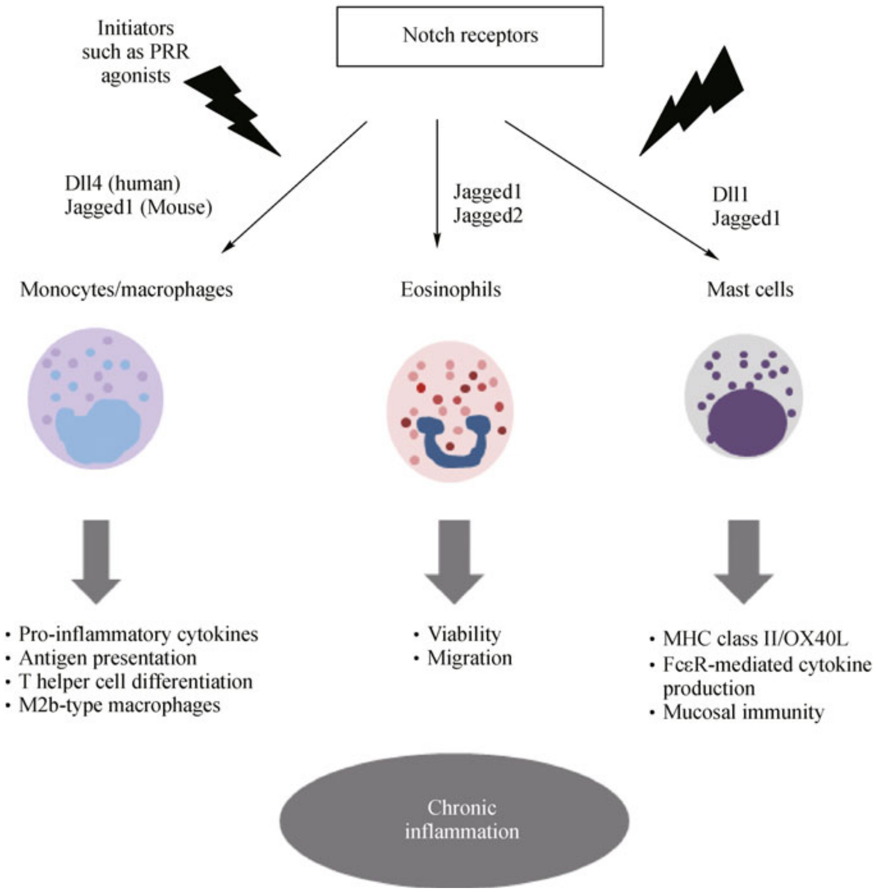

Figure 2 Notch signaling regulates effector functions of myeloid lineage cells. PRR-recognition of their specific ligands initiates the activation of notch signaling. Notch receptors are engaged by different ligands which are induced upon activation of the myeloid lineage cells. The activation of notch signaling functions cooperatively with other signaling pathways to produce a wide array of outcomes. It promotes pro-inflammatory responses in macrophages and regulates migration and effector functions of other myeloid lineage cells.

production of various pro-inflammatory cytokines, including TNFα, IL-1β, IL-6 and IL-12 (Hu et al., 2008; Palaga et al., 2008; Tsao et al., 2011). Furthermore, decreased expression of *iNOS* and the production of nitrite were also observed (Palaga et al., 2008). Targeted deletion of RBP-jκ/CSL yielded results in agreement with those obtained from the use of GSI, and suggests that signaling through this nuclear binding partner is critical to mediating downstream effects (Hu et al., 2008). Activating approaches included the use of soluble notch ligands and overexpression of truncated, constitutively-active notch receptors and produced nearly opposite results, suggesting that activating notch signaling is essential for inflammatory responses in macrophages.

Interestingly, however, inhibiting notch signaling does not entirely abrogate responses to TLR stimulation and some residual pro-inflammatory cytokines are still detectable. Therefore, it is likely that notch signaling is necessary for optimal responses of TLRs, but may not be sufficient to induce a full-scale pro-inflammatory response in-and-of itself.

How does activating notch signaling regulate TLR-responsive genes? Evidence suggests that it can directly and/or indirectly modify TLR responses. Notch signaling has been shown to directly associate with the promoters of target genes of the TLR pathway such as *il-6* (Wongchana and Palaga, 2011). In this case, activating notch receptor alone only minimally induced expression of *il-6* transcript, but in the presence of LPS, a synergistic effect on *il-6* mRNA level was observed. Interestingly, within the *il-6* promoter, the consensus binding site for RBP-jκ/CSL overlaps with that of NF-κB, suggesting that they may cooperatively regulate IL-6 expression. Furthermore, activated notch signaling in macrophages is associated with increased activation of the NF-κB pathway, which also plays a critical role in regulating pro-inflammatory responses (Palaga et al., 2008; Monsalve et al., 2009). Notch activation promotes nuclear localization of some members of the NF-κB family, including p65 and p50, partly by promoting phosphorylation of the IκB kinase complex (Palaga et al., 2008; Monsalve et al., 2009). Thus, the cross-talk between notch and TLR pathways may converge at the level of NF-κB signaling, although additional studies will be needed to confirm this.

Notch signaling in other myeloid lineage cells

Multiple notch receptors and ligands are expressed in myeloid cells other than monocytes and macrophages; however, the involvement of notch signaling in the effector phase of other myeloid lineages has not yet been fully investigated.

Mast cells play a critical role in allergic reactions and IgE-mediated immune responses. Furthermore, they can regulate T helper cell differentiation via secreted cytokines. Notch1 and notch2, but not notch3 or notch4, are expressed on the cell surface of murine bone marrow-derived mast cells (Nakano et al., 2009). Activating notch signaling in mast cells by co-culturing with ligand-expressing cells identified Dll1 and Jag1 as the functional ligands and this activation induced MHC class II and OX40L expression. Furthermore, interaction of notch with Dll1 enhanced FcεRI-mediated cytokine production in mast cells, including TNFα, IL-4 and IL-13 (Nakano et al., 2009). Detailed analysis revealed that notch signaling induces MHC class II expression by increasing binding of the transcription factor PU.1 to the promoter of MHC class II (Nakano et al., 2011). Dll1 on mast cells also functions as an adhesion molecule, allowing the accumulation of mast cells at inflammatory sites (Murata et al., 2010). Notch2 in mast cells regulates localization of intestinal mast cells, since notch2$^{-/-}$ mast cells failed to localize to the epithelial layers of the intestine (Sakata-Yanagimoto et al., 2011). Taken together, these data suggest a critical role for notch signaling in regulating mucosal immunity and Th2-type immune responses via regulating the effector functions of mast cells.

Eosinophils are phagocytic granulocytes involved in innate immune defense against parasitic infection. Human eosinophils express notch1, notch2, Jag1 and Jag2 on their cell surface (Radke et al., 2009). GM-CSF treatment increased notch ligand expression on eosinophils. Inhibiting notch signaling with GSI enhanced their viability although, at the same time, it reduced their capacity to migrate in the presence of GM-CSF (Radke et al., 2009).

DCs play an important role during T cell priming which dictates the outcome of immune responses. Notch signaling in DCs was shown to be necessary but not sufficient to induce DC differentiation (Cheng et al., 2003). Most studies have focused primarily on the notch ligands expressed on DCs and their effects on T helper cell polarization. However, the intrinsic role notch signaling plays in DC effector functions has also been recently addressed. Targeted deletion of *notch2* in DC subsets decreases CD11b^{+} DCs in the spleen and intestines, which in turn decreases the number of Th17 cells in the intestine (Lewis et al., 2011). Furthermore, genetic deletion of RBP-jκ/CSL, specifically in CD8^{-} splenic DCs, had a profound effect on their homeostasis, suggesting that RBP-jκ/CSL-dependent notch signaling is essential for maintaining homeostasis of this DC subset (Caton et al., 2007). Recently, it was reported that TLR stimulation results in a pattern of notch receptor expression that differs between human conventional and plasmacytoid DCs (Pérez-Cabezas et al., 2011). Ligation of notch receptors in DCs increased IL-10 and CCL9 expression, while gamma-secretase inhibitor treatment reversed this trend (Pérez-Cabezas et al., 2011). Furthermore, in plasmacytoid DCs, notch activation induced TNFα production (Pérez-Cabezas et al., 2011).

Altogether, these data suggest that notch signaling may regulate a broad array of functions in numerous cell types to mediate innate immune responses.

Notch signaling during T cell activation, proliferation and survival

In naïve peripheral T cells, notch1 is expressed at low levels on CD4 and CD8 T cells whereas notch2 is undetectable in these cells. Stimulation through the T cell receptor (TCR) activates the notch pathway. Notch1 is rapidly upregulated and remains high in CD4 and CD8 T cells for up to 48 h (Adler et al., 2003; Palaga et al., 2003; Fiorini et al., 2009). In contrast, notch2 displays a delayed upregulation, its expression peaking at 24 h after stimulation and decreasing by 48 h (Fiorini et al., 2009). This temporal expression of notch receptors can be observed in T cells activated with antibodies directed against CD3 and CD28, as well as under more physiologically relevant conditions such as following infection with LCMV, indicating the critical importance of notch activation downstream of signaling through the TCR (Fiorini et al., 2009).

Notch has been shown to co-localize with CD4 at the site of the immunological synapse (Benson et al., 2005; Luty et al., 2007) and can physically interact with p56^{lck} following T cell activation (Sade et al., 2004). We have found, additionally, that notch may serve as a scaffold to aid in the formation of the supramolecular aggregate known as the Carma1-Bcl10-MALT1 (CBM) complex through its physical interactions with PKC-θ and Carma1 (Shin et al., unpublished data). The CBM complex links signals from the TCR to the liberation of NF-κB (Schulze-Luehrmann and Ghosh, 2006), sustaining T cell activation and providing potential intersection between the notch and NF-κB pathways.

Blocking notch signaling with pharmacological inhibitors that prevent its cleavage by the gamma-secretase complex dramatically reduced proliferation, NF-κB nuclear localization and secretion of IFNγ by peripheral T cells (Palaga et al., 2003). In contrast, ectopic expression of constitutively-active N1ICD increased expression of CD25, the high-affinity alpha chain of the IL-2 receptor, as well as production of IL-2 by CD4 T cells (Adler et al., 2003). Thus, activating notch during T cell signaling increases proliferation by positively regulating the IL-2/IL-2R axis, and this may further be mediated through NF-κB (Palaga et al., 2003, Shin et al., 2006).

In addition to promoting cell proliferation in stimulated T cells, notch signaling can potentially initiate multiple mechanisms to actively suppress cell death. When N1ICD was overexpressed in the DO11.10 thymoma cell line, it bound to the orphan nuclear receptor, Nur77, preventing Nur77-mediated transcription of downstream pro-apoptotic target genes (Jehn et al., 1999). Additionally, p56^{lck}-notch association was required to promote phosphatidylinositol-3-kinase (PI3K) -mediated phosphorylation of Akt/PKB and upregulation of the inhibitors of apoptosis (IAP) protein family, as well as Flice-like inhibitor protein (FLIP), both of which act to antagonize caspase-induced apoptosis (Sade et al., 2004).

In CD8 T cells, a cell-intrinsic role for notch signaling in the development of cytolytic T lymphocytes (CTL) has been described. N1ICD was detected, together with NF-κB, bound to the promoters of *perforin* and *granzymeB*, two effector molecules upregulated in CTLs (Cho et al., 2009). Furthermore, preventing notch cleavage using GSI abrogated the recruitment of N1ICD, as well as NF-κB, suggesting these two transcriptional elements may coordinately regulate CTL effector functions (Cho et al., 2009). Notch2 signaling is also implicated in the development of CTLs. Transgenic mice in which *notch2* was conditionally deleted in CD8 T cells showed reduced CTL differentiation, *in vitro* and *in vivo*, in response to antigen-specific stimulation. Further analyses suggested that notch2 was acting to integrate RBP-jκ/CSL and CREB1 complexes on the *granzymeB* promoter to facilitate its transcription (Maekawa et al., 2008).

Thus, notch signaling in activated CD4 T cells may promote cell growth and survival through coordinated events that synergize with proliferative determinants while suppressing mediators of cell death. In CD8 T cells, notch signaling contributes both to activation and to acquisition of effector functions.

Notch signaling during T helper cell differentiation: the importance of ligands

Cell-intrinsic roles for notch receptor signaling have been described for the differentiation of naïve CD4 T cells into T helper-type1 (Th1), -type2 (Th2) and -type17 (Th17) cells. This includes evidence that notch regulates expression of the “master” transcription factors for each cell type: T-bet in Th1 cells, GATA3 in Th2 cells and RORγ in Th17 cells (Maekawa et al., 2003; Amsen et al., 2004; Minter et al., 2005; Amsen et al., 2007; Fang et al., 2007; Keerthivasan et al., 2011). Although much attention to date has focused on signals initiated after engagement of notch receptors in T cells, the importance of specific ligands and their unique abilities to impart instructive signals to notch receptors is the subject of growing interest. While polarizing cytokines present during T cell differentiation may provide the context for delivering these signals (Ong et al., 2008), it is becoming increasing clear that the ligands themselves may prove to be the linchpins, integrating extra-cellular cues with cell-intrinsic responses.

In general, a pattern influencing differentiation is emerging whereby signaling through the family of Delta-like ligands promotes Th1 and/or Th17 differentiation and, reciprocally, Jagged ligands promote Th2 cell differentiation. These specificities were suggested by studies showing notch3-Dll1 interactions upregulated T-bet expression and production of Th1 cytokines (Maekawa et al., 2003), and were expanded upon to show distinct regulation of T cell fate depended upon engagement of notch receptors with delta-like *versus* jagged ligands (Amsen et al., 2004). Since then, additional studies have demonstrated the strong Th1-polarizing effects of Dll1 and Dll4 expressed on dendritic cells (Skokos and Nussenzweig, 2007; Sun et al., 2008). Furthermore, although both of these reports implicate MyD88 in the upregulation of Delta-like ligands, the latter suggested that signaling through Delta-like ligands blocked Th2 differentiation and redirected T cells toward a Th1 phenotype by default, rather than through instructive signals. This observation was further supported by studies in which naïve T cells were cultured in the presence of Dll4-expressing antigen presenting cells together with Th17-polarizing cytokines, IL-6 plus TGF-β. Here, a block in Th2 cytokines was also noted, along with increased expression of *rorc* and IL-17, both of which were shown to be directly regulated by notch signaling (Mukherjee et al., 2009).

Reciprocal studies have identified a role for Jag1 in promoting Th2 polarization, which is augmented by the appropriate cytokine milieu. Human, naïve peripheral T cells upregulated their GATA3/T-bet ratio and Th2 polarization when they were cocultured with myeloid dendritic cells expressing high levels of Jagged1 (Liotta et al., 2008). This phenomenon required the actions of IL-4 since adding an IL-4-neutralizing antibody abrogated the increased GATA3/T-bet ratio. Similarly, maturing the dendritic cells in the presence of TLR ligation downregulated Jag1 expression, increased the surface expression of Dll4, and inhibited Th2 polarization.

Apart from influencing T helper cell differentiation, notch ligands may function to regulate T cell activation. Silencing ligand expression in dendritic cells using ligand-specific siRNA effectively increased IFNγ production by CD4 T cells in a mixed lymphocyte reaction, and this effect was not altered by the addition of GSI (Stallwood et al., 2006). Additionally, there are also reports that Jag1- and, to a lesser extent Dll1-engagement inhibits T cell activation, compared to Dll4 signaling (Rutz et al., 2005), and blocking Jag1- or Dll1-mediated signaling by dendritic cells *in vitro* or *in vivo* enhanced T cell proliferation (Elyaman et al., 2007). In a complementary system, incubating naïve CD4 T cells with immobilized Dll4-Fc, Dll1-Fc or Jag1-Fc fusion peptides together with anti-CD3 and anti-CD28 decreased expression of two markers of activation, CD25 and CD69, compared to stimulating cells in the presence of control Ig. This occurred in a hierarchical fashion, with Dll4-Fc inducing the least inhibition and Jag1-Fc the most, and correlated positively with the strength which with individual ligands bound notch receptors (Rutz et al., 2005). These effects required concomitant stimulation through the TCR and were also independent of gamma-secretase-mediated notch receptor cleavage. The exact mechanism responsible for this response has yet to be defined, although activation of NF-κB, AP-1 and NF-AT, all essential for full T cell activation following TCR ligation, were completely abrogated when T cells were stimulated in the presence of Jag1-Fc. In contrast, no difference in proliferation was observed when T cells were cocultured with antigen-pulsed CHO cells engineered to express Dll1 or Jag1 together with MHC class II and B7 (Ong et al., 2008).

Although not definitive, it appears that notch ligands vary with respect to their binding affinities. This in turn may affect the strength of T cell activation and, as a result, influence Th cell differentiation (Tao et al., 1997; Badou et al., 2001). These observations are consistent with the notion that T helper cells receiving a strong signal, perhaps conveyed in conjunction with Dll4 or Dll1, may promote Th1 responses. On the other hand, those receiving weak signals through the TCR, or signals potentially dampened by concomitant association of notch receptors with Jagged ligands, may be more inclined to adopt a Th2 cell fate.

There exists further evidence that signals delivered by Jag1 are strongly inhibitory and can result in the induction of anergic or regulatory T cells rather than Th2 cells. In one study, mice were immunized with Jag1-expressing antigen presenting cells (APC) that had been pulsed with the house dust mite protein, Der p 1, then challenged directly by immunization with Der p 1 antigen. When lymph node (LN) cells were removed and stimulated *ex vivo* with Der p 1 peptide, their proliferative response was greatly diminished compared to control cells. This suppression required antigen stimulation, since transferring Jag1-expressing APCs that were not pulsed with Der p 1 did not instill the same unresponsiveness in LN cells restimulated *ex vivo*. Furthermore, the lack of response to Der p 1 also conferred tolerance to subdominant peptides effectively inducing a linked suppression (Hoyne et al., 1999). Similar results were noted when human naïve T cells were stimulated with an antigen-presenting human B cell line engineered to overexpress Jag1 (Vigouroux et al., 2003; Yvon et al., 2003). CD 8 T cells were less effective at killing specific target cells if they had been primed initially in the presence of CD4 T cells and B cells expressing Jag1. Responses to autologous or third party antigens remained unchanged, reinforcing the notion that Jag1-mediated suppression is antigen-specific. In a slightly different system, human peripheral T cells incubated with immobilized Jag1-Fc fusion peptides, together with immobilized antibodies against CD3 and CD28, proliferated far less efficiently than control cells and produced decreased levels of Th1 and Th2 cytokines (Kostianovsky et al., 2007). Consistent with previous studies (Rutz et al., 2005), adding GSI to the cultures did not reverse the suppressive effects provided by Jag1. Interestingly, the gene related to anergy in lymphocytes (GRAIL) was upregulated following T cell stimulation with co-ligation of Jag1-Fc. Unlike anergic cells however, T cells initially stimulated in the presence of Jag1-Fc proliferated similarly to control cells upon restimulation, although the level of pro-inflammatory cytokines they produced were significantly lower.

Multiple notch receptors and ligands have been observed to be upregulated on regulatory T cells (Ostroukhova et al., 2006; Asano et al., 2008; Koyanagi et al., 2012). Furthermore, strong evidence has also been put forth supporting a role for specific notch ligands in expanding or maintaining inducible regulatory T cell (iTreg) populations. In an interesting and well-controlled study, when bone marrow-resident hematopoietic stem cells (HSC) were mobilized into the periphery they adopted the slightly more mature phenotype of hematopoietic progenitor cells (HPC) and concomitantly upregulated expression of Jag2 (Kared et al., 2006). Subsequently, transferring these Jag2-expressing HPCs into NOD mice could prevent progression to spontaneous autoimmune diabetes through their ability to significantly expand a host-derived population of iTreg cells. Similar expansion of iTregs did not occur after transferring Jag2^{-} HPCs or Jag2^{lo} bone marrow-derived HSCs, indicating expression of Jag2 on the mobilized HPCs was sufficient and necessary for the *in vivo* expansion of the iTreg subset. Blocking antibodies specific for Jag2 or for notch3, which was increased on the iTregs, abrogated the suppressive effects mediated by the HPC transfer, and indicated that Jag2-notch3 ligation was responsible for driving the expansion of the inducible Treg population in this model. These data are in agreement with earlier work which showed that expressing a constitutively active notch3 receptor in CD4 T cells expanded the Treg population, increased expression of IL-4 and IL-10, and could protect mice from streptozotocin-induced autoimmune diabetes (Anastasi et al., 2003). Together, these data argue strongly in favor of a notch3-Jag2 signaling axis that functions to promote (i)Treg expansion *in vivo*, and which may prove to be therapeutically beneficial.

Utilizing a mouse model of allergic airway inflammation, several groups have shown that therapeutic transfer of Tregs can mitigate disease severity, and this action was shown to proceed through a mechanism involving membrane-bound transforming growth factor-β (TGF-β^{m+}) (Ostroukhova et al. 2006; Asano et al., 2008). CD4^{+}CD25^{+}TGF-β^{m+} Tregs express high levels of Jag1, Dll1 and Dll4 compared to CD4^{+}CD25^{+}TGF-β^{m-} controls. The suppressive activity of the TGF-β^{m+}-expressing Tregs was dependent upon interaction with notch1 in the target cell population, since incubating cocultures of Tregs and effector cells in the presence of a notch1-neutralizing antibody inhibited the suppressive capabilities of the TGF-β^{m+}-expressing Tregs. Addition of neutralizing antibodies specific for Jag1 or Dll4 (Huang et al., 2009) could similarly abrogate Treg-mediated suppression. These observations are consistent with an earlier study which used gamma-secretase inhibition and notch1 antisense mice to demonstrate that intact notch signaling was necessary to maintain Foxp3 expression in peripheral Tregs (Samon et al., 2008).

Ligand specificity, therefore, seems to play an important role in influencing the activation and differentiation of naïve T cells, and signals initiated in response to unique ligands are not equivalent. Additionally, the contribution of cytokines within the microenvironment and engagement of the TCR seem to be required for ligand-mediated modulation of T cell responses. Although some inconsistencies can be noted, fundamentally the studies to date support a role for Dll family ligands in facilitating Th1 and/or Th17 responses, while Jagged ligands promote a Th2 response in the presence of IL-4 or may induce a suppressive phenotype in its absence. The exact means by which individual ligands impart their unique cellular outcomes await further elucidation.

Notch signaling in acute and chronic inflammatory responses

An immune response is a double-edged sword; it can protect the host from pathogens or damage host tissue with a sometimes-fatal outcome. Both innate and adaptive immune processes are involved in initiating inflammatory responses as well as maintaining chronic inflammation.

Following infection with respiratory syncytial virus, Dll4 is upregulated on bone marrow-derived macrophages. Blocking the actions of Dll4 in this mouse model, exacerbated airway hyper-responsiveness by increasing infiltration of Th2 cytokine-producing CD4 T cells, and reducing the influx of those secreting IFNγ (Schaller et al., 2007). Thus, notch signaling may contribute positively to anti-viral immunity, while altered ligand-receptor interactions may precipitate pathogenic conditions.

Sepsis is a systemic inflammatory response characterized by a massive pro-inflammatory cytokine storm which can rapidly lead to multiple organ failure and death. Two studies examined the effect of inhibiting notch signaling on the outcome of experimental sepsis. When *rbpjκ/csl* was specifically deleted in the myeloid cells of transgenic mice, endotoxin-induced lethality was partially rescued (Hu et al., 2008). Furthermore, in a cecal ligation and puncture model of endotoxemia, GSI treatment partially reduced mortality (Tsao et al., 2011). During wound healing, wherein macrophages participate in resolving tissue injury, notch1 deficiency dampened inflammatory responses, decreasing recruitment of macrophages to wounds and reducing the secretion of pro-inflammatory cytokines such as IL-6, IL-12 and TNFα (Outtz et al., 2010). Collectively, these studies strongly suggest that blocking notch signaling during sepsis and inflammation helps alleviate the severity of the responses.

Atherosclerosis results from chronic inflammation in the arterial walls. Lipid-laden macrophages found within the lesions play a significant role in sustaining inflammatory responses and in promoting the instability of these atherosclerotic plaques (Libby and Aikawa, 2002). In human atherosclerotic plaques, notch3 and Dll4 expression were found to co-localize with CD68$^+$ macrophages (Fung et al., 2007). More importantly, GSI treatment reduced atherosclerosis in an ApoE$^{-/-}$ murine model, with documented reduction in plaque area and plaque burden. Furthermore, a significant reduction in macrophage accumulation in plaques was also seen following GSI treatment (Aoyama et al., 2009). Thus, sustained notch signaling may promote conditions of chronic inflammation and may prove to be a novel target for therapeutic intervention.

Aberrant notch signaling and autoimmunity

Autoimmune diseases were originally thought to be mediated primarily by an aggressive adaptive immune response against self-antigens resulting in damage to various tissues. The involvement of the innate immune system, however, has been found to be equally important in some instances of autoimmunity. Studies in *motheaten* mice showed that T and B cells are not required for an inflammatory autoimmune phenotype (Yu et al., 1996). Furthermore, mice deficient in the enzyme α-mannosidase II have a systemic lupus erythematosus (SLE)-like phenotype even in the absence of an adaptive immune system, suggesting that inflammatory autoimmune phenotypes can be mediated solely by innate immune cells (Green et al., 2007; Paulson, 2007).

Within the context of the adaptive immune system, a genetic role for notch signaling in T cell-mediated autoimmunity was initially described in *itchy* mice. Mice deficient for *itch*, a HECT E3 ubiquitin ligase, developed a progressive autoimmune-like disease characterized by lymphoproliferation in the thymus and secondary lymphoid organs and this phenotype was exacerbated and accelerated when *itch*$^{-/-}$ mice were crossed with mice overexpressing a notch1 transgene in CD4 T cells (Matesic et al., 2006). An additional study examined notch signaling in a mouse model of autoimmune and lymphoproliferative disease. Here, mice treated with GSI showed a marked amelioration of symptoms compared to control animals (Teachey et al., 2008).

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoantibodies and immune-complex deposition in tissues which affects multiple organs. In the murine lupus model, an increased percentage of renal macrophages were found to have a unique cytokine profile of high IL-10/TNFα/IL-1β/IL-6/MCP-1 and low IL-12 (Zhang et al., 2010). These types of macrophages, referred to as M2b macrophages, expressed activated, cleaved notch1 and high levels of *Hes1/Hey1* (Palaga et al., unpublished data; Zhang et al., 2010). Using both inhibitory and overexpression approaches, it was found that notch signaling contributes to some key M2b phenotypes. As a result, GSI treatment prevented accumulation of M2b macrophages in the kidney and ameliorated aspects of a lupus-associated phenotype including lowering anti-dsDNA titers, decreasing kidney score and reducing IgG deposition (Zhang et al., 2010). These results may appear contradictory to the pro-inflammatory role of notch signaling in macrophages described above. However, recent studies showed that overexpressing activated notch1 and notch2 promoted anti-inflammatory IL-10 production and notch signaling seemed to function as a regulatory feedback mechanism to control TLR-mediated inflammation (Zhang et al., 2012). Therefore, it is likely that notch signaling itself is not the determining factor as to whether the response of macrophages is anti- or pro-inflammatory. Rather, it seems that notch signaling serves to re-enforce and optimize the responses of macrophages.

Notch expression in peripheral T cells from patients with active SLE was also recently examined. In contrast to its upregulation in murine M2b macrophages, notch1 was downregulated in T cells from patients with active SLE, but its expression returned to normal in patients with inactive SLE (Sodsai et al., 2008). Decreased notch1 expression was accompanied by decreased proliferation in response to stimulation with phytohemaglutinin and decreased expression of CD25 and *Foxp3*. Since the murine study focused on macrophages and the human study on peripheral T cells, it is unclear whether T cells in the mouse model also downregulate notch1 during active disease and, similar to mice, if M2b macrophages exist in humans and exhibit increased levels of notch1. Nonetheless, the complexity of dysregulated notch signaling in SLE does highlight the fact that, since treatment with GSI affects all aspects of the immune system, a more refined way to address the direct involvement of notch activation in lupus may be warranted.

Rheumatoid arthritis (RA), a condition characterized by painful swelling of affected joints, is another autoimmune disease in which the role of notch signaling is being more closely examined. RA can be simulated in mouse models by immunizing susceptible strains with collagen II. One inquiry using this model found that delivering a plasmid encoding soluble Jag1 reduced the severity of disease, even when treatment was begun after disease induction (Kijima et al., 2009). Although Jag1 did not directly inhibit the actions of CD8 T cells in this model, both Jag1 and CD8 T cells were required to attenuate disease. The exact manner by which Jag1 acts to prevent CD8 T cell-mediated destruction under these experimental conditions awaits further investigation. In a reciprocal study, a blocking antibody specific for notch3 attenuated collagen-specific T cell proliferation and pro-inflammatory cytokine production, while culturing T cells with Dll1 promoted these phenomena (Jiao et al., 2011). In follow-up experiments, synoviocytes were isolated from patients with RA then cultured *ex vivo* in the presence of TNFα, a pro-inflammatory cytokine frequently elevated in inflamed joints. Exposure to TNFα increased expression of *notch2* and its downstream target, *Hes1*, as well as *Dll1* and *Dll3* (Jiao et al., 2012). IL-6 secretion was also increased following culture with TNFα but was abrogated after GSI was added to the culture, suggesting that notch signaling mediated IL-6 production. Consistent with earlier observations, coculture with a Dll1-Fc fusion peptide promoted cytokine secretion. The complex involvement of notch signaling in RA is highlighted by the fact that multiple cell types are capable of responding to a pro-inflammatory milieu by upregulating notch ligands and receptors and perpetuating the ongoing inflammatory state.

In recent studies using mice with experimental autoimmune encephalomyelitis (EAE), an autoimmune model of multiple sclerosis, macrophages which were activated by LPS in the presence of an immune complex (similar to M2b) were shown to alleviate disease severity (Tierney et al., 2009). Compared to LPS-stimulated macrophages, this type of macrophage produced more IL-10 and less IL-12 when FcγR was engaged with an immune complex (Sutterwala et al., 1998). Interestingly, we found that GSI-treatment of LPS-stimulated macrophages decreased expression of *IL-12p40* and *IL-23p19*, subunits of IL-12/IL-23 and IL-23, respectively (Boonyatecha et al., unpublished data). The pathology of EAE is mediated largely by Th1 and Th17 responses and IL-12 and IL-23 are two important cytokines that drive differentiation of both types of T helper cells (Domingues et al., 2010). In light of this finding, inhibiting notch signaling in macrophages may alleviate the severity of EAE, in part, by decreasing IL-12 and IL-23 production.

Consistent with its attenuating effects on macrophages in EAE, GSI treatment also inhibited notch signaling in T cells and effectively reduced symptoms of disease by inhibiting Th1 and Th17 responses. In an early report, we showed that blocking notch signaling in T cells with GSI reduced expression of T-bet, the master transcriptional regulator of Th1 differentiation, and overexpressing an active form of notch1 restored its expression (Minter et al., 2005). Subsequently, we demonstrated that inhibiting notch signaling using GSI also prevented upregulation of *rorγt*, the transcriptional regulator of Th17 differentiation *in vitro*, and reduced disease symptoms *in vivo* (Keerthivasan et al., 2011). Inhibiting notch1 in human peripheral T cells using RNAi silencing techniques reduced IL-17A and IL-17F secretion, while expressing active, intracellular notch1 increased the production of both. These data contradict an earlier study which showed that abrogating notch3, but not notch1 signaling with neutralizing antibodies, reduced proliferation and Th1 and Th17 responses through its negative regulation of PKC-θ (Jurynczyk et al., 2008). Whether mouse and human T cells differ in their control of Th1/Th17 responses as mediated through individual notch receptors remains to be determined.

Manipulating signals mediated by notch ligands can also modulate disease severity in models of EAE. In one experimental set up, inhibiting Jag1 with neutralizing antibodies increased IFNγ production and exacerbated disease, while treating mice with antibodies neutralizing

Dll1 reduced IFNγ and ameliorated disease symptoms (Elyaman et al., 2007). Administering Jag1-Fc or Dll1-Fc fusion peptides modulated Th1 and Th2 responses *in vivo*, as well, with Jag1-Fc inducing more IL-10-producing cells and fewer IFNγ-producing cells in the central nervous system and Dll1-Fc having the opposite effect. Interestingly, a recent analysis of samples of cerebrospinal fluid from patients with primary progressive multiple sclerosis (MS) found that expression of Jag1 was reduced by more than threefold compared to patients diagnosed with relapsing remitting MS (Stoop et al., 2010). Further investigations of notch ligand expression in patient samples may provide additional clues as to their role in mediating autoimmune destruction, or serve as biomarkers that correlate with disease status.

Additional studies, focused on Dll4, showed it strongly induced Th1 and Th17 responses. One report examined the effects of inhibiting Dll4 signaling in Theiler's murine encephalomyelitis, a virally-induced mouse model of MS. Here, blocking Dll4-mediated signaling did not alter relative percentages of Th1 and Th2 cells in the spleens of diseased animals, but the absolute numbers of T cells infiltrating the spinal cords were markedly reduced, as were transcripts of *ifng* and *il17* (Takeichi et al., 2010). In another report, preventing Dll4-notch signaling induced a Th2/Treg immune response that lessened disease severity by counteracting Th1/Th17-mediated pathology. Mechanistically, it was shown that signaling through Dll4 blocked TGF-β-induction of *Foxp3* and the development of regulatory T cells (Bassil et al., 2011). A complementary report assessed the impact of blocking Dll4 on innate immune cells present in central nervous system lesions after induction of EAE. Administering a Dll4-blocking antibody *in vivo* altered the expression of chemokine receptors CCR2 and CCR6 and effectively decreased CNS-infiltration of antigen-specific T cells (Reynolds et al., 2011). Collectively, these findings suggest that notch ligand-receptor interactions may control multiple aspects of autoimmune pathology in EAE, not only the regulation of cytokine production.

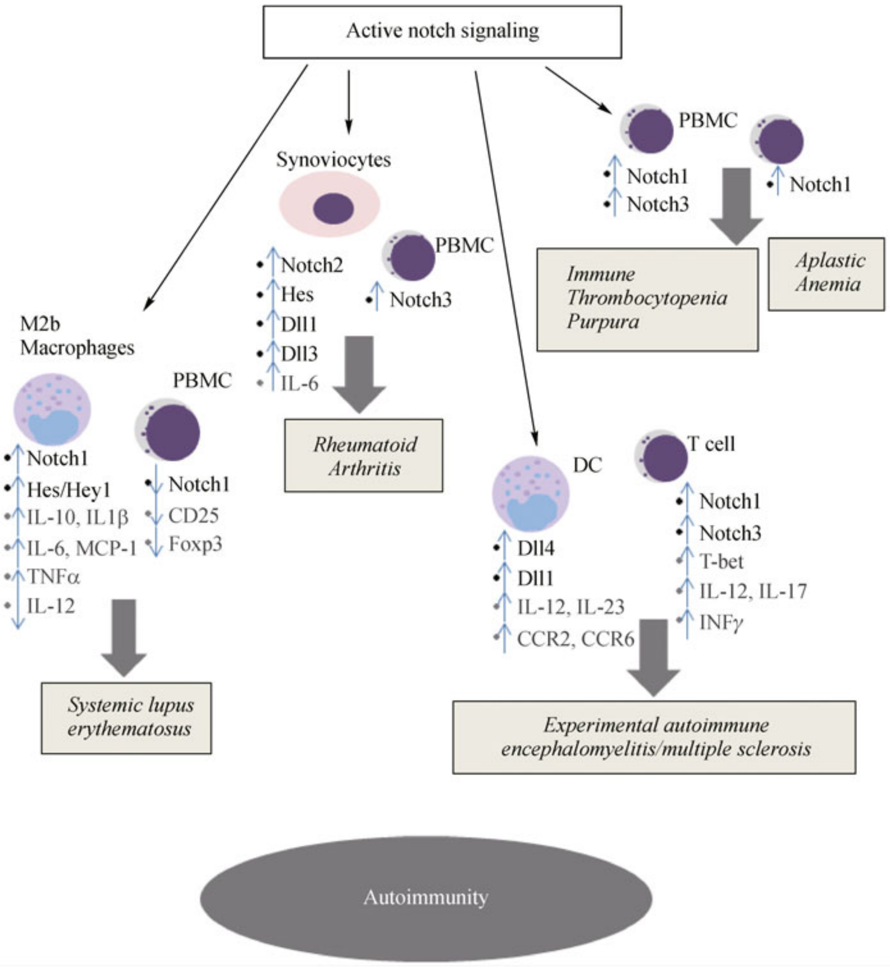

Figure 3 Notch signaling in autoimmune diseases. Notch signaling has been implicated in various autoimmune conditions including systemic lupus erythematosus, rheumatoid arthritis, experimental autoimmune encephalomyelitis/multiple sclerosis, immune thrombocytopenia purpura, and aplastic anemia. Increased or decreased expression of individual notch receptors and/or ligands may be disease specific and, as such, may provide useful biomarkers of disease or targets for therapeutic intervention. See text for discussion of individual autoimmune conditions and notch receptor/ligand expression.

Finally, a role for notch signaling in mediating some autoimmune cytopenias may also be emerging. Aplastic anemia is a Th1-mediated autoimmune bone marrow failure syndrome characterized by immune-meditated destruction of hematopoietic stem and progenitor cells, as well as their supportive stromal compartment (Chen et al., 2004). Data from our laboratory revealed elevated N1ICD in peripheral T cells of patients with aplastic anemia who had not received previous immunotherapy (Roderick et al., unpublished data). Echoing these findings, a recent study of patients with immune-mediated thrombocytopenia purpura, an autoimmune condition which destroys circulating platelets, demonstrated increased levels of notch1 and notch3 in patient samples compared to controls (Ma et al., 2010). Precisely how notch signaling contributes to the pathology of these and, perhaps, other immune-mediated cytopenias will require further investigation (Fig. 3).

Summary and therapeutic implications

Activating notch signaling in macrophages and other myeloid lineage cells favors, overall, a strong pro-inflammatory response. This is fine-tuned by the adaptive immune response as T cells differentiate into appropriate T helper cell subsets in the context of unique notch receptor-ligand interactions and instructive cytokines. Given our increasing understanding of notch signaling during immune responses and its complex and tightly managed regulation, it is not surprising that aberrant notch signaling is now coming to light as a critical contributing factor in a number of autoimmune conditions. Furthermore, it is becoming more evident that cells both of the innate and adaptive immune systems may facilitate this dysregulation.

Based on evidence presented here, inhibiting notch signaling by GSI treatment or by other, more specific methods holds great promise for reducing the severity of autoimmune diseases. Several reports have implemented novel dosing regimens or administered GSI together with adjuvant therapies with effective and well-tolerated outcomes (Teachey et al., 2008; Real et al., 2009). Substrates in the immune system, in addition to notch receptors and ligands are likely to be targets of the inhibitory actions of GSI treatment (Haapasalo and Kovacs, 2011) and further investigation will tease out just what these are and on which cell types they are expressed. The more important take-home message here, however, is that targeting notch signaling promises to be a novel, alternative or adjunct approach to treating chronic inflammatory and autoimmune diseases.

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