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Notch and Inflammatory T Cell Response: New Developments and Challenges

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Abstract

Inflammatory T cell response is important for protecting the host against infections and tumors. However, dysregulated generation of effector T cells that produce high levels of inflammatory cytokines and cytotoxic molecules may cause inflammatory disorders, such as chronic infections, autoimmune diseases and graft-versus-host disease after allogeneic bone marrow transplantation. Thus, tight regulation of effector T cells is essential. Accumulating evidence indicates that Notch plays critical roles in regulating the differentiation of antigen-activated T cells into distinct lineages of effector T cells. Furthermore, aberrant regulation of Notch signaling has been shown to implicate in several inflammatory disorders and tumorigenesis. On the other hand, significant progresses have been made in the development of new methods to modulate Notch for the disease treatment. In this review, we will discuss recent findings that help to gain insight into the impact of Notch in regulation of effector T cells and highlight the beneficial effects of modulating Notch on inflammatory diseases.

Keywords

Notch effector T cell differentiation; inflammatory disorder; modulation of Notch signaling

INTRODUCTION

Inflammatory T cell response is a part of complex immune response against antigens, such as pathogens, autoantigens, alloantigens and tumor antigens^{1–4}. Upon antigenic stimulation, naïve T cells are activated to become different lineages of effector T cells that produce high levels of inflammatory cytokines (e.g., TNF- α , IFN- γ , IL-4 and IL-17, etc.) and cytotoxic molecules (e.g., granzyme B, perforin and Fas ligand, etc.). It is well known that this differentiation of naïve T cells to different effector subsets is regulated, first by T cell receptor (TCR) and costimulatory signals, and then by cytokines^{1–4}. For example, IL-12 and IL-4 induce T helper 1 (Th1) and Th2 differentiation, respectively^{2,5}. Accumulating evidence indicate that Notch signaling plays important roles in regulating effector T cell differentiation via a mechanism independent of IL-12 and IL-4^{6–9}. In the setting of allogeneic bone marrow transplantation (BMT), inactivation of Notch signaling leads to reduced production of a broad spectrum of inflammatory cytokines in alloantigen-activated

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T cells¹⁰. Thus, Notch signaling may represent a critical pathway regulating inflammatory T cell responses.

In mammals, there are five Notch ligands (i.e., Delta-like 1 (Dll1), Dll3, Dll4, Jagged1 (J1) and J2) and four Notch receptors (Notch 1, 2, 3 and 4)^{9,11,12}. Notch receptor composed of an extracellular ligand-binding domain, a single-pass transmembrane domain and an intracellular domain. In the canonical signaling pathway, binding of a Notch ligand to its receptor results in the cleavage of the receptor by γ -secretase complex and the subsequent release of its Notch intracellular domain (NICD)^{9,11–14}. NICD associates with the DNA-binding factor RBP/J and co-activator Mastermind-like 1 (MAML1) in the nucleus, where it recruits additional co-activators to activate the transcription of Notch target genes. This review will focus on discussing the impact of Notch ligands and their interactions with Notch receptors in effector T cell differentiation (Fig. 1) and the beneficial effects of modulating Notch on inflammatory diseases.

A. DELTA-LIKE NOTCH LIGANDS AND EFFECTOR T CELL DIFFERENTIATION

Dll1 and Dll4 induce Th1 cell differentiation

Th1 cells produce IFN- γ to promote the clearance of intracellular pathogens and inflammatory antigens^{2,5,6}. Several studies have demonstrated that Dll1 may promote Th1 cell differentiation^{6,15–18}. *In vitro* stimulation of activated T cells with functional Dll1 induced Th1 cells^{15,16}. *In vivo* administration of active Dll1 increased IFN- γ secretion in CD4⁺ T cells in mice infected with *Leishmania major* (*L. major*)¹⁵ and in mice with experimental autoimmune encephalomyelitis (EAE)¹⁸, where as blocking Dll1 by its neutralizing antibody (Ab) decreased the frequency of IFN- γ -producing CD4⁺T cells in these EAE mice¹⁸.

Dll4 was also reported to promote Th1 cell differentiation^{19–22}. Gain-of-function assays showed that activation of CD4⁺ T cells with Dll4-expressing I-E^k fibroblast L cells enhanced the generation of Th1 cells in cultures¹⁹. In contrast, soluble Dll4 fused to mutant Fc fragment of immunoglobulin G (sDll4-mFc), which can effectively compete with endogenous Dll4 on binding to Notch receptors, decreased IFN- γ production in activated CD4⁺ T cells without impairing their proliferation and activation¹⁹. Most importantly, Dll4 is found to be critical to the development of IL-12-independent Th1 cell differentiation¹⁹.

Dll1 and Dll4 suppress Th2 cell differentiation

Th2 cells produce IL-4, IL-5 and IL-13 to promote the control of parasite infection^{2,6,16,23,24} and are thought to be important for mediating allergic inflammation such as airway hyper-responsiveness and inflammation^{18,22,25–28}. Several studies suggest that both Dll1 and Dll4 may modulate Th2 cell differentiation^{18,22,25–28}. DCs over-expressing Dll1 suppressed IL-4 production in OT-II CD4⁺ T cells *in vitro* under a Th2-skewed culture condition²². In a mouse model of airway hyper-responsiveness and inflammation, administration of active Dll1-Fc led to increased levels of IFN- γ in bronchoalveolar lavage fluid but decreased Th2 responses in the lung²⁶.

Dll4 is also found to negatively influence Th2 cell differentiation^{20,24}. Anti-Dll4 Ab treatment caused an increase in airway hyper-responsiveness in respiratory syncytial virus (RSV)-infected animals as compared with control Ab treatment²⁴. CD4⁺ T cells from the lung of these anti-Dll4 Ab-treated mice demonstrated a significant increase in Th2-type cytokines and a decrease in IFN- γ production than those derived from control mice²⁴. *In vitro* culture assays showed that addition of recombinant Dll4 reduced the production of both IL-4 and IL-13 in CD4⁺ T cells recovered from RSV-infected mice²⁴. Interestingly, this suppressive effect of Dll4 on Th2 cytokine production was observed not only in wild type but in MyD88 deficient lymphocytes, which suggested this suppressive effect was independent of MyD88²⁴, a pathway used by all toll-like receptors (TLRs)^{29,30}. A recent study also revealed the negative role of Dll4 in Th2 cell development²⁷. In this study, *in vivo* anti-Dll4 Ab treatment during induction phase significantly augmented Th2 cell-mediated experimental allergic conjunctivitis in WT mice but not in IFN- γ -deficient mice²⁷. This finding implies that the suppressive effect of Dll4 on Th2 differentiation might be associated with IFN- γ . However, a separate, *in vitro* study suggested that Dll4-mediated inhibition of Th2 cell development could occur independently of IFN- γ or T-bet²². Thus, an alternative explanation could be that Dll4-mediated Th2 inhibition might result in a block in IL-4-initiated commitment to the Th2 lineage²², which has yet to be formally examined.

Dll4 induces Th17 cell differentiation

Th17 cells are recently characterized Th cells that produce IL-17 and recruit neutrophils to the site of inflammation^{2,31–33}. Several experimental studies reveal an important role of Dll4 in regulating IL-17 production^{21,28,34}. *In vivo* administration of neutralizing Dll4 Ab reduced the production of IL-17 and IL-17-related cytokines IL-6 and IL-21 in the lung of mice immunized with mycobacteria³⁴. A separate study showed that *in vitro* treatment with anti-Dll4 Ab markedly reduced the mRNA levels of IL-17 and ROR γ t in mice with Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease (TMEV-IDD)²¹. *In vitro* assays further demonstrate that blocking Dll4 inhibited IL-17 production by TCR transgenic DO10.11 CD4⁺ T cells, whereas stimulating these CD4⁺ T cells with active Dll4 promoted their generation of Th17 cells²⁸. However, how Dll4 integrates other signals to promote T cell production of IL-17 and whether Dll4 is required for the generation of Th17 cells or maintenance of differentiated Th17 cells have not been reported.

Dll1 plays differential roles in the generation of CD8⁺ cytotoxic T lymphocytes (CTL)

The acquisition of cytotoxic effector function by antigen-specific CD8⁺ T cells is crucial for the control of intracellular infection and tumor invasion³. Several studies have demonstrated that Dll1 may play important roles in CD8⁺ CTL differentiation. DCs over-expressing Dll1 induced higher levels of granzyme B in alloantigen-activated CD8⁺ T cells with than control DCs³⁵. CTL induced by Dll1-expressing DCs had enhanced capability to kill target leukemic cells³⁵. Silencing Dll1 in DCs significantly reduced their ability to induce the generation of granzyme B-producing T cells³⁵. Further studies showed that Dll1-expressing DCs augment CD8⁺CTL-mediated anti-tumor immunity³⁶. These data suggest that Dll1 promotes CTL differentiation.

Controversially, one early study reports that constitutive expression of Dll1 on alloantigen-bearing non-professional I-E^k fibroblast L cells rendered them nonimmunogenic and able to induce specific unresponsiveness to a challenge with the same alloantigen, even in the form of a cardiac allograft³⁷. These effects could be reversed by depletion of CD8⁺ T cells at the time of transplantation³⁷. Furthermore, ligation of Dll1 on CD8⁺ T cells resulted in a dramatic decrease in IFN- γ with a concomitant enhancement of IL-10 production. These data suggest that Dll1 can alter the differentiation potential of CD8⁺ T cells³⁷. The mechanisms by which Dll1 expressing I-E^k fibroblast L cells induce CD8⁺ T cell-dependent specific unresponsiveness is unclear³⁷. Since DCs have different features in many aspects as compared to non-professional APCs such as fibroblasts, it will be intriguing to investigate whether the impact of Notch ligands such as Dll1 on T cell responses may depend on the presence of other signals delivered by APCs. (On the other hand, the effect of Dll4 on the differentiation of CD8⁺ cytotoxic T lymphocytes is largely unknown.)

B. JAGGED NOTCH LIGANDS AND EFFECTOR T CELL DIFFERENTIATION

The impact of Jagged Notch ligands in Th1 and Th17 has not been well characterized. A recent study reports that curdlan, one of the β -glucans derived from *Candida albicans*, was able to upregulate the expression of J1 in human monocyte-derived DCs that stimulated Th17 differentiation³⁹. Addition of polyclonal anti-J1 Ab inhibited DC-mediated Th17 differentiation in the culture. Further studies are needed to assess the impact of J1 in regulating Th1 and Th17 differentiation *in vivo*.

Jagged Notch ligands induces Th2 cell differentiation

Jagged ligands have been demonstrated to be important for the induction of Th2 responses^{16,18,40}. Initial studies demonstrate that BM-derived DCs expressing high levels of J1 and I-E^k-fibroblast cells that were transduced with J1 stimulated Th2 cell differentiation *in vitro*¹⁶. Later studies suggested that *in vivo* administration of active J1-Fc enhanced the generation of CD4⁺ T cells producing IL-4 and IL-10 in EAE mice, whereas treatment of mice with anti-J1 Ab resulted in a decrease of IL-4- and IL-10-producing CD4⁺ T cells¹⁸. It has also been shown that *in vivo* treatment of J1-Fc enhanced Th2 cell response-mediated airway hyper-responsiveness and inflammation in mice⁴⁰. These data support the influence of J1 on Th2 cell differentiation.

However, available data indicates that although J2 is found to be induced in response to several stimuli, including *Schistosoma* egg antigen (SEA), dust-mite extracts, prostaglandin E₂ (PGE₂) and cholera toxin^{16,41,42}, inactivation of J2 in APCs showed no effect on either Th1 or Th2 cell differentiation *in vivo*⁴². In addition, suppression of J2 expression using retrovirally delivered small interfering RNA failed to affect Th2 cell responses⁴².

C. NOTCH SIGNALING IN EFFECTOR T CELL DIFFERENTIATION

Notch signaling in Th1 cells

Notch ligands regulate Th1 differentiation through signaling mediated by Notch receptors. Treatment of T cells with γ -secretase inhibitors (GSI), which can block signaling of all four Notch receptors, decreased IFN- γ and IL-2 production in TCR-activated T

cells^(Adler et al, Palaga et al). In addition, blockade of Notch signaling by dominant-negative MAML (DNMAML), which is a pan Notch inhibitor, inhibited alloantigen-activated donor CD4⁺ T cells to produce IFN- γ in the setting of allogeneic BMT¹⁰. These studies provide strong evidence that Notch signaling is critical to Th1 cell differentiation.

Several studies tried to dissect the role of individual Notch receptors in Th1 cell differentiation. Some *in vitro* studies suggested that inhibition of Notch 1 by its antisense or neutralizing Ab reduced production of IFN- γ in activated CD4⁺ T cells^{17, Palaga,43}. However, Notch 1-deficient CD4⁺ T cells develop Th1 immune responses in mice infected by *L. major*⁴⁴. Furthermore, Notch 1 and Notch 2 double-deficient CD4⁺ T cells that were sensitized by parasite antigens showed no consistent effect on reduced production of IFN- γ ⁴⁵. In contrast, some other studies suggested that silencing Notch 3 resulted in a decrease in production of IFN- γ by TCR-activated T cells, whereas ectopic expression of NICD3 increased IFN- γ secretion by CD4⁺ T cells¹⁵. Most recently, it has been shown that *in vivo* treatment with anti-Notch 3 Ab but not anti-Notch 1 Ab significantly inhibited IFN- γ production of lymphocytes from mice with EAE⁴⁶. It appears that Notch 3 may play a dominant role in regulating the production of Th1 type cytokine during EAE. These apparent discrepancies might be the consequence of different experimental systems.

Molecular cues directing Th1 differentiation include expression of the Th1-specific transcription factor T-bet, encoded by Tbx-21. It has been found that activation of Notch by Dll1 induces T-bet expression in activated CD4⁺ T cells early after stimulation^{22,15}. This finding supports an earlier study showing that inhibition of Notch signaling by GSI extinguished expression of T-bet in activated CD4⁺ T cells, whereas ectopic expression of activated Notch 1 restored Tbx21 transcription¹⁷. Activated Notch binds to the RBP/J(CSL) complex, displacing the SMART-SKIP corepressors, and recruits the coactivator MAML1 to convert RBP/J(CSL) to a transcriptional activator. Chromatin immunoprecipitation assay (ChIP) revealed that both active Notch 1 and RBP/J bound to the promoter region of Tbx21, suggesting the direct effect of Notch on regulating Tbx21 transcription¹⁷. This may explain that Dll1 or Dll4 activation of Notch signaling induce T-bet expression independent of IL-12^{22,15}. However, controversial results were reported about the role of IFN- γ in Delta like ligand mediated Th1 cell development. Early study showed the neutralizing IFN- γ reduced T-bet expression in Dll1 or Dll4 inducing CD4⁺ T cells and the subsequent generation of Th1 cells²², while recent study demonstrated the independence of anti-IFN- γ regulating T-bet expression.

Other studies also show that Notch may promote the generation of Th1 cells by interacting with the NF- κ B pathway. Complexes of NICD1 and NF- κ B1 (p50) or NICD1 and RelA (p65) could be immunoprecipitated on the Ifng promoter, in regions lacking RBP/J(CSL) binding sites^{47–49}. This raises the possibility that Notch 1 can contribute to Th1 cell differentiation in a RBP/J(CSL)-independent manner^{47–49}. Interestingly, activated Notch may also involve in molecular pathway(s) associated with TCR signaling⁴⁶. Inhibition of Notch in T cells is correlated with the down-regulated expression of protein kinase C (PKC) θ , which is an important regulator of the mature T cell activation and polarization⁴⁶.

Notch signaling in Th2 cells

Notch signaling is an important regulator of type 2 immunity. Initial studies suggest that inactivation of RBP/J in mature T cells resulted in the defect in production of IgG1 and IgE, an indication of Th2-related immune responses⁵⁰. Inactivation of RBP/J in mature T cells also reduced their capability to generate Th2 cells producing IL-4 upon *in vitro* stimulation with LPS-pretreated MyD88-deficient DC and *in vivo* in mice immunized with SEA¹⁶. Later studies demonstrate that mature T cells expressing the pan Notch inhibitor DNMA1 failed to develop protective Th2 responses against the gastro intestinal helminth *Trichuris muris*⁵¹, suggesting the critical role of Notch in regulating Th2 cell differentiation. However, mice lacking individual Notch genes have no defect in T helper differentiation⁴⁵. In contrast, Notch 1-and Notch 2-double deficient CD4⁺ T cells failed to make IL-4⁴⁵. These experiments reveal that either Notch 1 or Notch 2 are required for Th2 cell differentiation.

The transcription factor GATA3 is critically required for promoting IL-4 production and Th2 cell differentiation^{2,5,6,45,51,52}. Several studies indicate that GATA3 is a direct target of Notch signaling. Ectopic expression of active NICD greatly increases the expression of GATA3, whereas inactivation of Notch signaling results in markedly diminished GATA3 expression^{45,52}. Furthermore, mature T cells lacking RBP/J have significantly reduced expression of GATA3 as compared to WT T cells and loss of RBP-J may cause the failure of efficient induction of GATA3 expression, leading to impaired Th2 differentiation⁵⁰. ChIP assays confirm that both active Notch and RBP/J directly bind to the promoter region of *Gata3* to regulating the gene transcription^{45,52}. Interestingly, several RBP/J(CSL) binding sites are found in enhancer region downstream of the *Il4* coding region, and one site in the *Il4* promoter^{16,52}. Therefore, *Il4* gene is also a direct target of Notch effector¹⁶. This may explain that naïve T cells lacking STAT6, which is required for IL-4 mediated Th2 cell differentiation, retained their capability to differentiate into Th2 cells. Furthermore, ectopic expression of either NICD1 or NICD2 in STAT6-deficient T cells promotes IL-4 production in effector T cells^{16,52}. Thus, Notch signaling acts on both *Il4* and GATA3 genes to activate IL-4 expression⁵².

Notch signaling in Th17 cells

Recent studies suggest that Notch signaling may play an important role in Th17 cells. Inhibition of Notch signaling by the pan Notch inhibitor DNMA1 drastically reduced the number of alloreactive CD4⁺ T cells producing IL-17 in the setting of allogeneic BMT¹⁰. Treatment of EAE mice induced by proteolipid protein (PLP) immunization with anti-Notch 3 Ab, but not anti-Notch 1 Ab, impaired Th17 responses, leading to amelioration of EAE in animals⁴⁶. Thus, it appears that Notch 3 may play an important role in Th17 cells.

Several studies show that Dll4 activation of Notch signaling results in increased production of IL-17 in activated CD4 T cells^{21,28,34}. *Rorc*, which is a transcription factor required for Th17 differentiation^{2,31,32}, was significantly increased in CD4⁺ T cells cultured under Th17 skewing condition, and further enhanced when cells were incubated with Dll4 in a time dependent fashion²⁸. A second required factor for Th17 differentiation is STAT3 protein activation (pSTAT3)^{2,31,32}. While Th17 skewing condition increased pSTAT3, addition of Dll4 did not alter pSTAT3 in these cells. Thus, increased Th17 cell differentiation by Dll4

appears to be associated with increased expression of *Rorc*, but not altered STAT3 activation²⁸.

Notch signaling in CD8⁺effector T cells

Notch signaling directly regulates the transcription of effector molecules in CTLs. Naïve CD8⁺ T cells express higher levels of Notch 2 on their surface than Notch 1 as assessed by flow cytometry, whereas activated CD8⁺ T cells expressed similarly high levels of Notch 1 and Notch 2³⁵. In contrast, both naïve and activated CD8⁺ T cells expressed extremely low levels of Notch 3 and Notch 4³⁵. Conditional inactivation of Notch 2 but not Notch 1 in mature CD8⁺ T cells led to impaired generation of functional CTLs^{35,36}. Mechanistic analysis revealed that active NICD2 interacted with a phosphorylated form of the transcription factor CREB1, and subsequently bound to the transcription coactivator p300 to form a complex on the promoter of the gene encoding granzyme B³⁵. Thus, Notch signaling may control CTL differentiation through phosphorylated CREB1 and p300 coactivator³⁵.

Transcription factors T-bet and Eomes control CD8⁺ CTL differentiation^{1,3}. Previous reports have suggested that Notch could control the expression of these genes^{17,35,36,49}. Notch 1 signaling was also found to regulate the expression of perforin and granzyme B through direct binding to the promoters of these crucial effector molecules⁵⁴. However, Notch 2-mediated CTL differentiation appears to be independent of Eomes³⁵. Interestingly, a recent study demonstrates that inhibition of pan Notch signaling by DNMAML reduced the expression of granzyme B and perforin in alloantigen-activated CD4⁺ and CD8⁺ T cells without altering the expression of T-bet and Eomes¹⁰. Furthermore, *Tbx21* mRNA was preserved in alloreactive DNMAML T cells, while *Eomes* transcripts were significantly elevated¹⁰. These observations suggest that Notch signaling-mediated CD8⁺ CTL differentiation may involve multiple molecular pathways.

D. REGULATION OF NOTCH LIGAND EXPRESSION

Notch ligand in APCs

APCs are crucial in directing the differentiation of distinct subsets of effector T cells^{6,55}. We now appreciate that Notch ligands presented by APCs are also important for promoting the generation of different lineages of effector T cells⁶. Many studies have demonstrated that different inflammatory stimuli may stimulate APCs to express different type of Notch ligands at different levels^{6,15,16,18–20,24,28,35,56}. As summarized in Table 1, TLR ligands or TLR agonists stimulate APC expression of both Delta ligands and Jagged ligands, whereas other inflammatory stimuli such as helminthes, allergens, toxins and PGE2 may cause the selective increase in the expression of Jagged ligands^{6,15,16,18–20,24,28,35,56}. These observations could explain, in part, how different pro-inflammatory stimuli induce the development of different type of immune responses.

APC expression of Notch ligands under steady condition

Flow cytometry analysis of Notch ligand revealed that under steady condition different APC subsets in lymphoid organs expressed distinct patterns of Notch ligands (Table 1). Myeloid CD11b⁺CD11c⁺ cells expressed low to moderate level of Dll1, J1 and J2 but not Dll4,

whereas lymphoid CD11b⁻ CD11c⁺ cells expressed low to moderate levels of Dll1 and J2 but not Dll4 and J1⁵⁷. A similar pattern of Notch ligand expression in CD8⁺CD11c⁺ DCs and CD8⁻ CD11c⁺ DCs has been reported⁵⁸. Macrophages expressed low to moderate levels of Dll1 and Jagged 2 but not Dll4 and J1⁵⁹. Interestingly, plasmacytoid DCs (pDCs) expressed high levels of Dll1 and Dll4 but lacked J1 and J2⁵⁷. These pDCs had greater ability than either myeloid or lymphoid DCs to induce the generation of IL-10-producing Th1 cells. This effect of pDCs could be reduced by GSI or by inactivating the Notch effector RBP/J(CSL)⁵⁷. Whether these Notch ligands impact mature T cells under steady condition and what kind of Notch ligands is expressed in APCs resided in non-lymphoid tissues have yet to be carefully examined.

TLR ligand induction of Notch ligands in APCs

TLRs are potent stimuli for the induction of Notch ligands in DCs^{16,19,24,28,60–63}. These included TLR-specific signals, poly(I:C) (TLR3), LPS (TLR4), and CpG (TLR9), as well as RSV, which activates by both RIG-I and TLR-mediated pathways^{16,25,28,61–63}. LPS could rapidly induce Dll4 expression in DCs in mice^{61–63}. Similar results were observed by using human monocyte-derived DCs stimulated by LPS⁶³. Combined stimulation of human DCs with LPS with TLR7 agonist R848 further increased the expression of Dll4 and down-regulated J1⁶³. These data suggest that activation of TLRs may lead to opposing effects on the expression of Notch ligands in DCs. Notably, while LPS induction of Dll4 in BM-derived DCs was found to be dependent on MyD88¹⁶, cytokines including IL-1 β and IL-18, which are two IL-1R family MyD88-dependent signals, did not induce the expression of any Notch ligands²⁸. This suggests that activation of MyD88 is not sufficient to the induction of Dll4 in DCs. Further studies are necessary to determine the molecular mechanisms by which LPS induces Dll4 expression in APCs.

An elegant study recently demonstrates that high but not low concentration of LPS induced a marked upregulation of both Dll4 and J1 mRNA in DCs in mice.⁶² In contrast Dll4 mRNA expression was only weakly upregulated by low concentration of LPS, while it was sufficient to lead to the upregulation of J1⁶². This coincided with the finding that high concentration of LPS induces Th1 and low concentration of LPS resulted in Th2 responses in an allergic lung inflammatory disease mouse model⁶². Furthermore, LPS has different effects on regulating Notch ligand expression in distinct APC subsets. Skokos et al. report that LPS stimulation rapidly induced Dll4 expression in CD8⁻ DCs but not in CD8⁺ DCs¹⁹. In contrast, Dll1, Dll3, J1 and J2 were not up-regulated in these DCs upon LPS stimulation¹⁹. It appears that different mechanisms may be involved in regulating the expression of distinct Notch ligands in APCs.

Non-TLR ligand regulation of Notch ligands in APCs

Several studies have demonstrated that different antigens may possess the ability to regulate the expression of distinct Notch ligands in APCs. SEA stimulation moderately enhanced the expression of J1 and J2, whereas *P. acnes* infection resulted in notable reduction in expression of J2 in DCs⁴¹. Other studies showed the stimulation of DCs with *P. acnes* induces the expression of both Dll1 and Dll4, with Dll4 the most extent⁴². In an EAE mouse model, immunization of mice with antigen MOGe multified in CFA induced the expression

of Dll1 and J1 mRNA in DCs in the spleen and lymph node, but did not affect the expression of Notch ligands in macrophages¹⁸. Interestingly, viruses such as RSV and TMEV also increase the expression of Dll4 in DCs^{21,24,25}. Thus, a complex mechanism is involved in regulating the expression of Notch ligands in APCs during immune response. Some studies suggested that proinflammatory cytokines were able to regulate Notch ligand expression during inflammatory response. *In vitro* assays showed that IFN- γ and TNF- α down-regulated the expression of J1 on astrocytes. In contrast, a significant increase in J1 mRNA levels was detected in cells exposed to TGF- β 1¹⁸. Cholera toxin and PGE-2 are found to upregulate the expression of J2¹⁶. However, none of these inflammatory stimuli shows the impact on APC expression of Dll4^{16,18}.

Non APC expressing Notch ligands

In slot blot analysis, Dll1 was detected in all immature lineages as well as mature hematopoietic cells. In the same analysis, expression of J1 transcripts was restricted mainly to terminally maturing cells, most strikingly to mast cells and megakaryocytes, and at lower levels in B cells and macrophages (Singh, JExp Hem 2000). In a separate study, bone marrow stroma cell (BMS) were shown to express a substantially higher level of J1 than D1, while spleen stroma (SS) had just the opposite expression pattern. Interestingly, those two stroma cells have different roles in DC development. BMS stimulated accumulation of DC precursors but prevent their transition to terminally differentiated DCs, while SS promoted generation of fully differentiated DCs (Cheng, Blood 2006). Another study showed that CD4⁺ and CD8⁺ T cells also express mRNA of J1 more abundant in DC (Hoyone, IntImm 2000). In Airway hyperresponsiveness (AHR) mouse model, increased expression of Dll1 in effector CD8⁺ T cells were shown which skewed the T cell response from a Th1 to Th2 phenotype (26). Although non APC expressing Notch ligands are intriguingly investigated, it is still controversial that the precise roles of those Notch ligands in effector T cell differentiation.

E. MODULATION OF NOTCH SIGNALING TO CONTROL INFLAMMATORY DISEASES

Approaches to modulate Notch signaling

Significant progresses have been made in understanding the impact of each individual Notch ligands and receptors in various inflammatory conditions⁶. These studies clearly demonstrate that Notch signaling may play critical and multiple roles in inflammatory T cell-mediated diseases⁶. Both genetic and pharmacologic approaches have been used for modulating Notch to control inflammatory diseases and improve tumor immunity. These include the pharmacologic Notch inhibitor GSI, neutralizing Abs specific for Notch receptors and ligands, ectopic expression of Notch ligands and Notch receptors and transgenic expression of pan Notch inhibitor-DNMAML.

GSI has been in the clinical development for the treatment of advanced solid tumor, acute T lymphoblastic leukemia and Alzheimer's disease^{64,72}. Preclinical experimental studies suggest that mice treated with a GSI resulted in significantly delayed onset of EAE, reduced clinical symptoms and decreased inflammatory lesions in the spinal cord¹⁷. This inhibitory

effect of GSI was associated with significantly decreased proliferation and reduction of Th1-associated with cytokines¹⁷. Treatment of systemic lupus erythematosus (SLE) mice with GSI resulted in a reduction of autoreactive T cells and a decrease in production of inflammatory cytokines (e.g., IFN- γ , TNF- α , IL-6, and IL-2) and improved nephritis⁶⁵. Despite the potential benefit in cancer and immune mediated disease, GSI treatment causes significant accumulation of goblet cells in the small intestine⁶⁶. This results in severe diarrhea and has hindered the clinical application^{67,68}. However, progress has recently been made in reducing the adverse effects of GSI on GI tract, while preserving its beneficial effects⁶⁷.

Abs that specifically antagonize Notch 1 and Notch 2 have been developed to elucidate the discrete functions of Notch 1 and Notch 2 and reduce intestine toxicity⁶⁹. These Abs specifically reacted to each receptor paralogue and cross-reacted with the human and mouse sequences, enabling the discrimination of Notch 1 versus Notch 2 function in human patients and rodent models⁶⁹. Selectively blocking of Notch 1 inhibited cancer cell growth and induced deregulation of angiogenesis. Importantly, both antibodies inhibited signaling induced through the ligands J1, J2, Dll1 and Dll4, and thus inhibition occurs irrespective of the ligand⁶⁹. Furthermore, whereas inhibition of Notch 1 plus Notch 2 caused severe intestinal toxicity, inhibition of either receptor alone reduced or avoided this effect, demonstrating a clear advantage over pan-Notch inhibitors⁶⁹.

Alternatively, targeting individual Notch ligands may have significant implications in modulating Notch signaling. Many Abs specific to Notch ligands are available now^{21,27,35,53,59,70}. Different Notch ligands may differentially regulate the outcome of inflammatory responses via interacting with distinct Notch receptor. It is possible that blocking one Notch ligand may allow the other Notch ligands to trigger the corresponding Notch receptor, leading to the preservation of some Notch functions in the target cells and possible reduction of toxicity resulted from inhibition of pan Notch signaling¹⁰.

Notch and multiple sclerosis (MS)

Tight regulation of Th1 and Th2 responses is critical, as aberrant Th1 conditions perpetuate destructive inflammation characteristic of autoimmune diseases such as MS^{71–76}. MS is a chronic demyelinating disease of the human central nervous system (CNS). Although its etiology remains unclear, MS is generally considered to be an autoimmune Th1/Th17-mediated disease^{71–76}. EAE represents a useful animal model for studying human MS, in which antigen-specific CD4⁺ Th1 and Th17 cells mediate inflammatory damage in CNS, with consequent demyelination and progressive paralysis^{71–76}. Data from recent studies indicate that selective inhibition of Notch 3 by its neutralizing Ab ameliorated EAE^{46,77}. Furthermore, treatment of EAE mice with anti-Dll1 Ab or active J1-Fc from the induction phase led to reduced CD4⁺ T cells secreting IFN- γ , increased frequency of CD4⁺ T cells producing IL-4 and IL-10, and the subsequent amelioration of the disease¹⁸.

A separate study demonstrates that administration of anti-Dll4 mAb ameliorated demyelinating disease induced by TMEV²¹. Treatment with mAb to Dll4, especially during the effector phase, resulted in significantly decreased expression of transcripts of Th1- and Th17-derived cytokines, reduced number of infiltrating mononuclear inflammatory cells in

the spinal cords and suppression of the disease development²¹. Thus, aberrant activation of Notch signaling by Dll4 may be also important to the development of Th1 cell-mediated demyelinating disease, in particularly during effector phase. Most recently, in EAE mouse model, anti-Dll4 treatment was shown to reduce T cell surface expression of the CCR2 and CCR6, and decreased clinical severity with diminished T cell accumulation in CNS. This result suggests a role of Dll4 in EAE pathogenesis through modulation of T cell chemokine receptor expression and migration to the CNS (Reynolds JI 2011). All these observations suggest that Notch ligands or Notch receptors may be new and attractive targets in treating MS.

Notch and airway hyper-responsiveness and inflammation

A dysregulated Th2 milieu can promote airway hyper-responsiveness and inflammation^{20,23,24,26,40}. Allergic asthma is characterized by persistent airway hyperresponsiveness and inflammation due to cellular and molecular responses induced by allergen in sensitized hosts^{20,23,24,26,40}. Antigen-specific memory T cells, especially CD4⁺ T cells, are believed to play an integral role in orchestrating the disease process through the secretion of a variety of Th2 cytokines, including IL-4, IL-5 and IL-13^{20,23,24,26,40}. Treatment of allergen-sensitized mice with active Dll1-Fc resulted in decreased airway hyper-responsiveness and inflammation in response to secondary allergen rechallenge²⁶. This effect was associated with a significant increase in number of CD4⁺ T cells producing IFN- γ and the reduction of eosinophils in the lung tissues²⁶.

Emerging evidence indicate that Dll4 also plays important roles in the pathogenesis of airway hyper-responsiveness. *In vivo* administration of anti-Dll4Ab substantially increased airway hyper-responsiveness in mice infected by RSV²⁶. Examination of lymphocytic lung infiltrate revealed a significant increase in total CD4⁺ T cells and activated CD8⁺ T cells in RSV-infected mice following anti-Dll4 Ab treatment. These lung CD4⁺ T cells produced high levels of type 2 inflammatory cytokines²⁵. All these observations suggest that both Dll1 and Dll4 could be therapeutic targets for modulating airway hyper-responsiveness and inflammation.

Notch and graft-versus-host disease (GVHD)

GVHD is caused by donor T cells that recognize and react to alloantigens of the recipient. Upon APC activation, donor T cells become alloreactive effector T cells producing high levels of inflammatory cytokines (e.g., TNF- α , IFN- γ , IL-4 and IL-17) and cytotoxic molecules, leading to host tissue injury and the subsequent life-threatening complication^{78–85}. However, blockade of individual effector molecules has limited efficacy in controlling GVHD^{79,85}. We recently demonstrate that Notch signaling is a potent regulator of T cell activation, differentiation and function during acute GVHD¹⁰. Inhibition of canonical Notch signaling by expressing pan Notch inhibitor DNMAML in donor T cells markedly reduced GVHD severity and mortality in mouse models of allogeneic HSCT. Whereas Notch-deprived T cells proliferated and expanded in response to alloantigens *in vivo*, they were incapable of producing several inflammatory cytokines, including TNF- α , IFN- γ , IL-4 and IL-17¹⁰. Furthermore, inactivation of Notch resulted in a decrease in expression of cytolytic effector molecules in both CD4⁺ and CD8⁺ T cells activated by

alloantigens¹⁰. Most importantly, Notch-deprived alloreactive CD4⁺ T cells retained significant cytotoxic potential and anti-leukemia activity, leading to improved overall survival of the recipients. These results identify Notch as a novel essential regulator of pathogenic CD4⁺ T cell responses during acute GVHD and suggest that Notch signaling in T cells should be investigated as a therapeutic target following allogeneic HSCT¹⁰.

This work presents the first example of a disease model in which Notch inhibition prevents otherwise lethality in recipients after allogeneic BMT¹⁰. Since systemic Notch inhibition can cause adverse reactions, in particular gastrointestinal toxicity, strategies are needed to overcome this important limitation. To this end, further experiments are needed to identify the individual Notch ligand(s) and Notch receptor(s) that are critical for the induction of effector differentiation of alloantigen-activated T cells.

Notch and cancer immunotherapy

CD8⁺ T cells play a central role in cancer immune surveillance, and the efficient induction of CTLs against tumor antigens is required for successful immunotherapy for cancer patients^{86,87}. Several studies suggest that Notch signaling is critical to effector differentiation of antigen-activated CD8⁺ T cells^{17,35}. For instance, Dll1 activation of Notch 2 signaling directly regulates granzyme B expression and promotes CTL differentiation.³⁵ Consistent with their higher expression of granzyme B, CD8⁺ T cells stimulated with Dll1 upregulated DC lysed allogeneic EL-4 (mouse thymoma) target cells more efficiently than those stimulated with control DC. Most recently, Sugimoto et al. report that Notch 2 signaling is required for potent anti-tumor immunity *in vivo*³⁶. Mice with Notch 2-deficient-but not Notch 1-deficient-CD8⁺ T cells die earlier than control mice after inoculation with OVA-expressing EG7 thymoma cells³⁶. These data suggest that new strategies capable of enhancing Notch 2 signaling in CD8⁺ T cells may increase the anti-tumor activity *in vivo*. Indeed, administration of anti-Notch 2 agonist Ab or Dll1-overexpressing DCs augment tumor immunity³⁶.

F. FUTURE PERSPECTIVES

Significant progresses have been made during the past few years in understanding the important roles of Notch in inflammatory T cell responses. However, many critical issues have yet to be determined. For example, why do distinct Notch ligands have differential roles in regulating development of different lineages of effector T cells? It has been shown that while Dll1 and Dll4 promote the development of Th1 response, they may repress the production of Th2-type immune responses under some circumstances^{20,24}. In sharp contrast, J1 can instruct Th2 cell differentiation but has little effect on the generation of Th1 cells^{6,16,18,40,45}. One possible explanation is that different Notch ligands may activate distinct Notch receptors for the subsequent development of Th1 and Th2 cells. Indeed, whereas Notch 3 is critical to Th1 differentiation^{15,17,46}, both Notch 1 and Notch 2 play a redundant role in regulating the development of Th2 cells⁴⁵. However, there is no evidence indicating that J1 is incapable of binding to Notch 3^{6,15,16,45,70}. Furthermore, both Dll1 and Dll4 were also shown to be able to bind to both Notch 1 and Notch 2^{69,70}. It would be interesting to identify whether additional mechanisms such as co-stimulatory/co-inhibitory

signals, or Notch ligands on other cell populations such as stroma cells may determine the consequence of Notch regulation of effector differentiation from antigen-activated T cells.

Another important question is what the role of Notch ligands is in regulating APC development and function. Accumulating evidence indicates that Notch receptors are highly expressed in APCs and Notch signaling may be critical to the development and functional differentiation of APCs^{58,88–90}. Inactivation of the Notch effector RBP/J(CSL) led to impaired differentiation and survival of DCs⁸⁸. Blocking of Notch ligands drastically reduced the number of DCs⁵⁸. Thus, a better understanding of the role of Notch in APC generation and function will be important for the development of novel strategies to modulate inflammatory responses.

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Executive summary

Introduction

Delta-like Notch ligands and effector T cell differentiation

- Both Dll1 and Dll4 promote Th1 cell differentiation.
- Dll4 is related the development of IL-12 independent Th1 cell differentiation.
- Both Dll1 and Dll4 suppress Th2 cell differentiation.
- Dll4 is related the promotion of Th17 cell differentiation.
- Dll1 may promote CD8⁺ cytotoxic T lymphocyte differentiation.

Jagged Notch ligands and effector T cell differentiation

- J1 induces Th2 cell differentiation.
- The role of J2 in effector T cell differentiation is unclear.

Notch signalling in effector T cell differentiation

- Notch signaling is critical to Th1 cell differentiation.
- Notch signalling acts GATA3 directly, and promotes Th2 cell differentiation.
- Notch signalling acts ROR γ t directly, and may play a role in Th17 cell differentiation.
- Notch signalling directly regulates the transcription factor CREB1 and EOMES in CD8⁺ effector T cells.

Regulation of Notch ligand expression

- Under steady condition, different APC subset expressed distinct pattern of Notch ligands (?).
- TLRs stimulate APC expression of both Delta and Jagged ligands (Table ?).
- Non-TLR ligands have the ability to regulate the expression of distinct Notch ligands in APC (Table ?).
- The precise role of non-APC expressing Notch ligands is yet to be determined.

Modulation of Notch signalling to control inflammatory diseases

- Both genetic and pharmacologic approaches have been used for modulating Notch to control inflammatory diseases and improve tumor immunity.
- These include the pharmacologic Notch inhibitor GSI, neutralizing Abs specific for Notch receptors and ligands, ectopic expression of Notch ligands and Notch receptors.
- **GSI,**
- **Abs to Notch R,**
- **Abs to Notch Ligands....**

- **Engineering APCs**
- **Engineering stromal cells/fibroblast cells**
- Notch 3, Dll1, Dll4, and J1 may be new targets in treating MS.
- Both Dll1 and Dll4 could be therapeutic targets for modulating airway hyper-responsiveness.
- Notch signalling is an essential regulator of allogeneic CD4⁺ T cell response during acute GVHD, and should be investigated as a therapeutic target following HSCT.
- Enhancing Notch 2 signalling in CD8⁺ T cells may increase the anti-tumor activity in vivo.

A. Delta family **B. Jagged family** **Fig. 1. Notch ligand regulation of effector differentiation of antigen-activated T cells**

(A), Delta-like ligand regulation of effector T cell differentiation. Activation of dendritic cells (DCs) with inflammatory stimuli upregulates the expression of Dll1 and Dll4. Dll1 can induce Th1 differentiation of antigen-activated CD4⁺ T cells and the generation of CD8⁺ cytotoxic T lymphocytes (CTL). Notch 3 and Notch 2 are found to be important for the development Th1 cells and CTLs, respectively. Whether Dll1 may affect Th17 cell differentiation remains unknown. Dll4 can promote the development of both Th1 and Th17 cells, whereas its impact on CD8⁺ CTL differentiation is poorly understood. Both Dll1 and Dll4 are found to repress Th2 differentiation via an unknown mechanism. (B), Jagged ligand regulation of effector T cell differentiation. J1 instructs the differentiation of antigen-specific Th2 cells. However, the impact of J1 in the generation of Th1 cells, Th17 cells and CD8⁺ CTL has yet to be determined. Unlike J1, J2 has been shown to be incapable of stimulating Th2 cell differentiation. In addition, whether J2 is engaged in regulation of other lineages of effector T cells has not been reported.

Table I

Regulation of Notch ligand expression.

A. TLR stimulation							
Cells	Stimulation	mRNA				J2	Reference
		Dll1	Dll4	J1	J2		
Splenic DC: CD8(-)/CD11c(+)	LPS	No change	Increase	No change	No change	No change	19
Mediastinal LN DC: CD11c(+)	OVA + LPS(hi)		Increase	Increase			62
	OVA + LPS(lo)		No change	Increase			62
BM DC	LPS	Increase	Increase	No change	No change	No change	28
	LPS		Increase	Increase		No change	16
	LPS			Increase		Decrease	41
Monocyte derived DC	LPS + R848		Increase	Decrease			63

B. Non-TLR stimulation							
Cells	Stimulation	mRNA				Reference	
		Dll1	Dll4	J1	J2		
Astrocyte	TGF-β			Increase			18
	IFN-γ, TNF-α			Decrease			18
BM DC	CT		No change		Increase		16
	CT			Increase	Increase		41
BM DC	SEA	No change	No change	No change	No change		42
	SEA			Increase	Increase		41
BM DC	PGE2		No change		Increase		16
	PGE2			Increase	Increase		41
BM DC	RSV	Increase	Increase				28
	RSV		Increase				24
BM DC	TMEV		Increase				21
BM DC	P. acnes	No change	Increase	No change	Decrease		42
	P. acnes			Increase	Decrease		41