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## Notch Governing Mature T Cell Differentiation

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## BRIEF REVIEWS

## Notch Governing Mature T Cell Differentiation

Shin-ichi Tsukumo and Koji Yasutomo<sup>1</sup>

*The differentiation of naive T cells to effector/memory T cells is regulated by a variety of factors. The recent advance of the contribution of Notch signaling in this differentiation step has provided a new path to better understand the acquisition or persistence of effector function of mature T cells. In this review, we summarize emerging and, in some points, conflicting evidence for Notch signaling on mature T cell activation and differentiation. The Journal of Immunology, 2004, 173: 7109–7113.*

The immune system has evolved to combat infectious organisms (1). During the evolution of immune systems, T cells with Ag-specific receptors have emerged and an acquired immune system was established to help or collaborate with the innate immune system (2). T cells that strongly recognize self-peptide MHC complexes undergo apoptosis, whereas cells that receive an “appropriate” weak signal differentiate further in the thymus, which prevents self-reactivity of mature T cells (3, 4). Furthermore, the immature T cells undergoing positive selection commit to the CD4 vs CD8 lineages based on the duration/strength of intracellular signals arising from the interaction between the TCR and self-MHC molecules (5–7). After completion of T cell maturation in the thymus, naive CD4<sup>+</sup> or CD8<sup>+</sup> T cells migrate into peripheral lymphoid organs and further differentiate into a variety of effector T cells to eliminate pathogens by recognizing pathogen-derived Ags presented by their own MHC (8–10).

When naive CD4<sup>+</sup> T cells recognize peptides on MHC, they proliferate and differentiate into Th1 or Th2 effector cells (10). Th1 cells secrete IFN- $\gamma$  and play major roles in resistance to intracellular pathogens, including *Leishmania major*, *Toxoplasma gondii*, and *Mycobacterium tuberculosis*. Th2 cells secrete IL-4 and enhance humoral immunity that takes part in protection from extracellular pathogens. The balance of CD4<sup>+</sup> T cell differentiation toward Th1 and Th2 is a critical determinant in some infections or autoimmune diseases (9, 10). In addition to Th1 and Th2 differentiation, naive CD4<sup>+</sup> T cells can differentiate into regulatory T cells (Treg)<sup>2</sup> by Ag recognition (11, 12). Treg have roles in preventing autoimmunity and inflammation by excessive immune responses (13). Regarding CD8<sup>+</sup> T cells, naive CD8<sup>+</sup> T cells differentiate into effector T cells with cytotoxic functions and/or IFN- $\gamma$  secretion after Ag stimulation.

A recent article has revealed that a transcriptional factor, Eomesodermin, is one of the factors that regulates CD8<sup>+</sup> T cell effector functions (14), but the direct target genes of this transcriptional factor remain unclear.

Although there are many articles addressing the molecules that regulate effector functions or activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (10, 15, 16), the entire cellular and molecular mechanisms governing the effector functions in mature T cells have not been revealed. We and other groups have recently demonstrated that mature T cells express Notch molecules, known as a cell fate regulator, and Notch signaling regulates mature T cell activation and differentiation (17–19), although there are discrepancies among the different experimental systems. Since previous articles have revealed the involvement of Notch signaling in the lineage decision toward T cells from lymphoid progenitors (20), Notch signaling appears to govern many key aspects of immature as well as mature T cell development. Regarding the role of Notch signaling in T cell development in the thymus, there are several good reviews elsewhere (20–23). Thus, in this study we review the role of Notch signaling, focusing on mature T cell differentiation and activation.

*Notch signaling*

Notch is a single-pass transmembrane receptor protein that regulates a broad range of cell fate decisions (24). In mammals, four Notch genes (Notch1–4) and at least five of their ligands (Jagged1 and 2; Delta1, 3, and 4) are identified (24) (Fig. 1). The extracellular domain of Notch proteins consists of epidermal growth factor-like repeats and Notch/LIN-12 repeats, which are important for ligand binding and prevention of ligand-independent signaling, respectively (24) (Fig. 1). The intracellular domain of Notch proteins possesses a RAM domain, ankyrin repeats, nuclear localization sequences, a homopolymer repeat of glutamine (OPA domain), and a proline-glutamate-serine-threonine-rich domain (24) (Fig. 1). These domains have individual roles in the transduction of Notch signaling (24). When Notch receptors are stimulated by their ligands, the intracellular domain of Notch (Notch-IC) is cleaved by  $\gamma$ -secretase and Notch-IC translocates to the nucleus (23, 24) (Fig. 1). In the nucleus, Notch-IC binds to RBP-J $\kappa$  (also known as CBF-1 and CSL in mammals) and converts it from a transcriptional repressor to an activator (23) (Fig. 1). Several target genes of Notch, including Hes1, Hes5, and pT $\alpha$  (25, 26), have been

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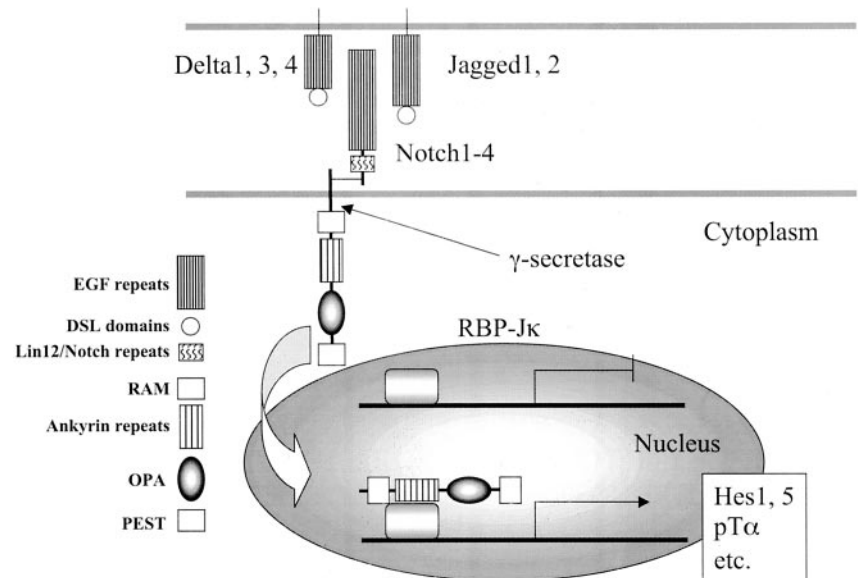
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<sup>2</sup> Abbreviations used in this paper: Treg, regulatory T cell; IC, intracellular domain; DC, dendritic cell.

**FIGURE 1.** Notch signaling. When Notch receptors are stimulated by their ligands, Notch-IC is cleaved by  $\gamma$ -secretase and translocates to the nucleus. In the nucleus, Notch-IC binds to RBP-J $\kappa$  and converts it from a transcriptional repressor to an activator.



identified, but the essential target genes in each biological event are largely unknown.

#### *Notch and T cell development*

Several lines of evidence indicate that activation of Notch1 is essential for the selection between T and B cell lineage commitment (20, 23, 27, 28). For instance, T cells were deficient and B cells were dramatically increased in the thymus of mice with conditional inactivation of Notch1 induced by IFN-regulated Cre recombinase (27, 29). Furthermore, when irradiated mice were reconstituted with bone marrow cells overexpressing Notch1-IC, those mice contained ectopic CD4<sup>+</sup>CD8<sup>+</sup> T cells and reduced B cells (28). These results suggest that Notch1 plays an obligatory and selective role in T cell lineage induction.

Notch1 was also reported to modulate the  $\beta$  selection step of thymocytes (26). Reizis and Leder (26) revealed that the enhancer region of pT $\alpha$  has a binding site for Notch and indeed, mutation of the binding sites abolished enhancer induction by Notch. This is supported by the finding that conditional inactivation of Notch1 by Lck-Cre leads to the accumulation of CD4<sup>-</sup>CD8<sup>-</sup>CD25<sup>+</sup>CD44<sup>-</sup> cells, which is also observed in pT $\alpha$ - and RAG1-deficient mice (30).

There are a number of articles suggesting that  $\gamma\delta/\alpha\beta$  and CD4/CD8 T cell fate decisions are modulated by Notch signaling (23, 31, 32). There are discrepancies among these reports about those issues, which may be explained by the mixed contribution of four types of Notch molecules or redundancy of signaling among Notch molecules in either step. Thus, it will be important to address the role of Notch signaling in terms of  $\gamma\delta/\alpha\beta$  and CD4/CD8 T cell fate decisions by crossing each Notch-deficient mice or down-regulating several Notch molecules in T cells by the RNA interference technique.

#### *Notch-related gene expression in the peripheral immune system*

In peripheral lymphoid organs, naive CD4<sup>+</sup> T cells express Notch1 and Notch2 mRNA (19, 33, 34), while they do not express Notch3 and Notch4 mRNA (19). However, mRNA expression of all Notch molecules was increased after Ag stimulation of T cells (33). T cells also express Notch ligands: Jagged1

(34), Jagged2 (35), and Delta1 (36). Regarding the expression of Notch ligands on APC, the dendritic cells (DCs) express Jagged1 and Jagged2 as well as Delta4 (19, 34, 35, 37). Amsen et al. (19) reported that the pathogen-derived products selectively up-regulated Notch ligand mRNA in DCs (19). For example, LPS induced expression of Jagged1 independent of MyD88 or Delta4 dependent on MyD88. In contrast, cholera toxin and PGE<sub>2</sub> only induced Jagged2 (19). These findings suggest that the expression pattern of Notch and Notch ligands dramatically change and are dependent on activation signals of T cells and DCs. Unfortunately, good mAbs that specifically recognize each Notch receptor or Notch ligand are lacking, which prevents analysis of the detailed expression pattern of those molecules.

#### *Effect of Notch on Th1/Th2/Treg differentiation*

According to the data from Notch expression in mature T cells, our group has addressed the role of Notch molecules in Th1/Th2 differentiation because the polarization of helper T cells represents a form of cell fate determination (17). Our group first discovered that stimulation of naive CD4<sup>+</sup> T cells with Delta1 promotes the differentiation toward Th1 partly independent of IL-12 signaling (17). At the same time, we demonstrated that overexpression of Notch3-IC, but not Notch1-IC, in activated CD4<sup>+</sup> T cells also promoted Th1, which is associated with enhanced expression of T-bet (17). Subsequently, Amsen et al. (19) also revealed that the Delta1-expressing fibroblast cells line enhanced IFN- $\gamma$  production of Ag-stimulated T cells. They also showed that stimulation by Jagged1 or the overexpression of Notch1-IC increased IL-4 production, suggesting that the Jagged1-Notch1 pathway directed Th2 cell differentiation (19). However, this study did not prove the Th2 regulation by a direct interaction between Jagged1 and Notch1. In addition, RBP-J $\kappa$ -deficient mice showed a drastic reduction in serum concentrations of IgG1 and IgE, suggesting biased Th1-immune responses in the absence of one of the major target molecules of Notch, RBP-J $\kappa$  (38). Although it should be addressed how Notch signaling is associated with many known

Th1- or Th2-inducing factors, including cytokines or transcriptional factors (9, 10, 16), the discovery of the contribution of Notch signaling in Th1/Th2 differentiation would open a new pathway about the regulation of helper T cell differentiation. Furthermore, it should be addressed whether the mode of Notch signaling induced by each Notch ligand is different in terms of helper T cell differentiation.

In contrast, several articles suggest that Jagged1 induces Ag-specific Treg instead of Th2 (34, 39, 40). Hoyne et al. (34) found that injection of Jagged1-transfected DCs into mice induced Treg, although this study does not prove the direct contribution of Jagged1 to the differentiation of naive CD4<sup>+</sup> T cells to Treg. Brenner's group (39, 40) found that the stimulation of human CD4<sup>+</sup> T cells by Jagged1 allowed induction of Treg. Since the differentiation of Th2 by the stimulation of T cells by Jagged1 was observed in mice (19), the role of Jagged1 in terms of CD4<sup>+</sup> T cell differentiation may be different between mice and humans.

#### *Effect of Notch on T cell proliferation*

There are several articles addressing the effect of Notch molecules in T cell proliferation, but so far the reports are inconsistent (Table I). Hoyne et al. (34) reported that Jagged1-expressing splenic DCs pulsed with an Ag could induce Ag-specific tolerance when injected into mice. Eagar et al. (18) reported that anti-Notch1 Ab and Jagged1- or Delta1-expressing A20 B cell lymphoma suppressed T cell proliferation *in vitro* (18). Brenner's group (39, 40) indicated that Jagged1-expressing lymphoblastoid cells induced alloantigen hyporesponsiveness of T cells. Our group also suggested that plate-bound Delta1-Fc fusion protein suppressed T cell proliferation only at a high dose (17). On the contrary, two articles indicated that chemical  $\gamma$ -secretase inhibitors, which were the inhibitors of Notch signaling, dramatically decreased T cell proliferation (33, 41). In addition, Adler et al. (33) reported that overexpression of Notch1-IC enhanced CD4<sup>+</sup> T cell proliferation and CD4<sup>+</sup> T cell proliferation was reduced in RBP-J $\kappa$ -deficient mice (38), suggesting that the activation of the Notch pathway enhanced T cell proliferation.

Two hypotheses can explain these discrepancies. The first hypothesis is that the dose of Notch ligand might be critical. As a representative data set, our results demonstrated that the plate-bound Delta1-Fc suppressed T cell proliferation only at a high dose, while it induced IFN- $\gamma$  production at a low dose without

any growth suppression (17). Furthermore, most of the experiments that observed a T cell suppressive effect by Notch signaling used overexpressed ligands on APCs (18, 34, 39, 40). In contrast, the experiments which found an enhanced effect of Notch on T cell proliferation did not use the overexpressed ligands. Thus, high concentrations of ligands might decrease T cell proliferation, whereas a low dose increases or does not affect the proliferation dependent on Notch ligands. In the future, it should be addressed whether different types of Notch ligands induce quantitative or qualitative distinct signals in terms of T cell proliferation.

The second hypothesis is that APCs might express factors, which affect T cell responsiveness, by modulating Notch signaling. Eagar et al. (18) indicated that the inhibitor of  $\gamma$ -secretase enhanced CD4<sup>+</sup> T cell proliferation, whereas other groups revealed the opposite results (33, 41). The major difference in conditions of these two experiments is that the former used purified CD4<sup>+</sup> T cells, whereas the latter used total lymph node cells or spleen cells. Indeed, Eagar et al. (18) reported that the inhibitor did not affect the proliferation in the presence of APCs. In addition, Tanigaki et al. (38) reported that RBP-J $\kappa$ -deficient T cells showed reduced proliferation in the presence of APCs, but not in the absence of APCs (38). Those results suggested that Notch signaling overcomes some inhibitory effects of APCs against T cell activation.

Although we do not have direct answers whether APCs or the dose of Notch ligands contributes to the controversial results, the examination about the precise expression pattern of Notch and Notch ligands or the pairing pattern of Notch and Notch ligands during immune responses would help to solve these issues. Furthermore, it would be essential to examine these issues using *in vivo* experimental systems to fully understand the precise role of Notch signaling in CD4<sup>+</sup> T cell responses.

#### *Effect of Notch on CD8<sup>+</sup> T cells*

Wong et al. (42) reported that constitutive expression of Delta1 on alloantigen-bearing cells induced specific unresponsiveness to a challenge with the same alloantigen. These effects could be reversed by depletion of CD8<sup>+</sup> T cells at the time of transplantation (42). They also revealed that the ligation of Notch on splenic CD8<sup>+</sup> T cells by Delta1-Fc resulted in a dramatic decrease in IFN- $\gamma$  secretion with a concomitant enhancement of

Table I. *Reported effects of Notch signaling on T cell proliferation*

Cultured Cells	Manipulation of Notch Signaling	T Cell Proliferation	Ref.
Experiments indicating positive effects of Notch signaling on T cell proliferation			
CD4 <sup>+</sup> T cells	RBP-J $\kappa$ deficient	Not affected	38
CD4 <sup>+</sup> T cells with irradiated T cell-depleted splenocytes	RBP-J $\kappa$ deficient	Suppressed	38
Mononuclear cells from DO11.10 Tg mice	Compound E ( $\gamma$ -secretase inhibitor)	Suppressed	33
CD4 <sup>+</sup> T cells	Compound E ( $\gamma$ -secretase inhibitor)	Suppressed	33
CD4 <sup>+</sup> T cells from DO11.10 Tg	Overexpression of Notch1IC	Enhanced	33
Lymph node cells	IL-CHO ( $\gamma$ -secretase inhibitor)	Suppressed	41
Experiments indicating negative effects of Notch signaling on T cell proliferation			
CD4 <sup>+</sup> T cells	Plate-bound Delta1-Fc	Suppressed (high dose)	17
T cells from DO11.10 Tg mice	Delta1-expressed A20 cells	Suppressed	18
Splenocytes	Anti-Notch1 mAb	Suppressed	18
CD4 <sup>+</sup> T cells	Compound E ( $\gamma$ -secretase inhibitor)	Enhanced	18
CD4 <sup>+</sup> T cells with APCs	Compound E ( $\gamma$ -secretase inhibitor)	Not affected	18
<i>In vivo</i> (C57BL/6)	Jagged1-expressed spleen DC	Suppressed	34
CD4 <sup>+</sup> T cells	Jagged1-expressed EB LCL cells	Suppressed	40

<sup>a</sup>Tg, Transgenic.

IL-10 production (42). Vigouroux et al. (39) indicated that cytotoxic functions of CD8<sup>+</sup> T cells were suppressed by stimulation of Jagged1. These results indicate that the activation of Notch suppressed CD8<sup>+</sup> T cell effector functions. On the contrary, Palaga et al. (41) reported that the number of IFN- $\gamma$ -producing CD8<sup>+</sup> T cells was significantly reduced by Notch1 antisense and  $\gamma$ -secretase inhibitor (IL-CHO), indicating that the activation of Notch is required for CD8<sup>+</sup> T cell function. This discrepancy might be also explained by the effects of APCs on Notch signaling similar to that in CD4<sup>+</sup> T cells because the experiments indicating the suppressive effect used overexpressed ligands and those indicating enhancement, which is seen in CD4<sup>+</sup> T cells, used total lymph nodes.

#### *Intracellular signaling in T cells induced by Notch*

The activation of Notch has been reported to induce various intracellular signals in T cells (24). The detailed relationship between Notch signaling and T cell activation/differentiation has not been established, but these signaling data will provide important clues for the elucidation of molecular mechanisms of Notch-induced mature T cell responses.

Amsen et al. (19) found three RBP-J $\kappa$  sites in the 3' enhancer region of the *IL-4* gene. They used transgenic mice in which a luciferase reporter is controlled by *IL-4* transcriptional regulatory elements, including the 3' enhancer region. Overexpression of Notch1-IC in T cells from these mice induced luciferase activity that is highly dependent on the RBP-J $\kappa$  sites in the 3' enhancer. These results indicate that the *IL-4* gene is a direct target of Notch signaling, and this pathway may contribute to Th2 differentiation.

The NF- $\kappa$ B family of transcription factors is involved in various immune responses. In mammals, five members have been identified: c-Rel, RelA (p65), RelB, NF- $\kappa$ B1 (p50/p105), and NF- $\kappa$ B2 (p52/p100). With regard to peripheral T cell differentiation, the mice lacking NF- $\kappa$ B1 failed to induce GATA3 expression in CD4<sup>+</sup> T cells and were unable to induce Th2 differentiation (43), whereas mice lacking c-Rel were defective in the Th1 response (44). In addition, several reports indicated that Notch influences NF- $\kappa$ B activation. For instance, Palaga et al. (41) indicated that  $\gamma$ -secretase inhibitor IL-CHO and Notch1 antisense inhibited NF- $\kappa$ B activation in CD4<sup>+</sup> T cells stimulated by anti-CD3/CD28 or Con A. Oswald et al. (45) revealed that transcription of NF- $\kappa$ B2 was suppressed by RBP-J $\kappa$  and activated by overexpression of Notch1-IC in Jurkat T cells. It remains unclear how these effects of Notch on NF- $\kappa$ B influence T cell differentiation. It would be necessary to address whether different Notch molecules or different pairs of Notch and Notch ligands have distinct regulation of NF- $\kappa$ B members.

The signal CD28 leads to the activation of the PI3K/AKT pathway (46, 47). This pathway has an important role in T cell activation and/or cytokine production (48, 49). Sade et al. (50) revealed that Notch1-IC formed a complex with p56<sup>lck</sup> and PI3K in Jurkat and primary T cells of mice and activated the PI3K/AKT signaling pathway in Jurkat and 2B4 mouse T cell hybridomas. In contrast, Eagar et al. (18) indicated that Notch1 activation inhibited AKT and its downstream effector GSK-3 $\beta$  phosphorylation in naive CD4<sup>+</sup> T cells of mice, but did not affect ERK1/2 phosphorylation. The types of cells or the culture conditions might cause these conflicting results. Additional experiments should be performed to examine whether

the PI3K/AKT pathway has any role in the effects of Notch on peripheral T cells.

Recently, Kamakura et al. (51) indicated that active Notch1 could induce STAT3 activation through Hes1 and Hes5, which are the target genes of Notch. They also revealed that Hes1 and Hes5 formed a complex with JAK2 and STAT3, thereby promoting STAT3 phosphorylation and activation (51). Although Amsen et al. (19) revealed that Notch-induced *IL-4* production independent of STAT6, it would be interesting to examine whether other STATs could be activated by Notch and involved in peripheral T cell differentiation.

#### *Conclusion*

Recent studies revealed crucial roles of the Notch system in mature T cell differentiation and activation, although many questions remain to be answered. For example, how does Notch signaling modulate T cell activation? What is the molecular mechanism of Notch signaling that induces Th1, Th2, and Treg cell differentiation? What are the roles of Notch-induced T cell differentiation in physiological conditions in vivo? How do T cells utilize different Notch molecules to regulate their own differentiation?

In mammals, there are four Notch molecules and at least five Notch ligands (24). Those receptors and ligands can interact with each other (52, 53) and the expression pattern of each molecule is not restricted (37), which makes it difficult to analyze the role of Notch systems in mature T cell differentiation/activation. Furthermore, Notch molecules are glycosylated by several glycosyltransferases, which is critical for appropriate interactions with the Notch ligands (54–58). The complex pattern of regulation of Notch by glycosylation rather than just expression of Notch molecules may determine the effects of cell-specific Notch signaling. Thus, in the future, the establishment of mAbs, gene-targeted mice of Notch-related molecules, or the detailed role of glycosylation of Notch receptors would facilitate the research of Notch and reveal the physiological roles of Notch signaling in mature T cell differentiation and activation.

Since the first article showing the role of Notch1 on CD4/CD8 T cell fate choice was published in 1996 (31), many articles have revealed the essential roles of Notch1 in T cell development in the thymus. In the past 2 years, we and other groups have revealed the contribution of Notch signaling in mature T cell differentiation/activation. These results suggest that Notch signaling is responsible for many key aspects during the life of T cells. Considering the broad roles of Notch signaling in many types of cell differentiation or lineage commitment, it is interesting to assess whether Notch signaling in T cells has specific target genes or whether Notch signaling affects similar gene expression independent of the cell type, and other factors specifically expressed in each cell determine the outcome of the cell activation and differentiation.

Although we have yet to reveal the detailed regulatory systems of Notch signaling in terms of mature T cell differentiation/activation, it would be interesting to determine whether defective Notch signaling is responsible for the pathogenesis of autoimmune diseases. Furthermore, Notch-related molecules might be targets for therapeutic strategies against autoimmune disease or tumors.

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