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Notch Signaling in CD4 and CD8 T Cell Development

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Summary

Because Notch often acts in concert with other signaling pathways, it is able to regulate a diverse set of biological processes in a cell-context dependent manner. In lymphocytes, Notch is essential for specifying the T cell fate and for promoting early stages of T cell differentiation. At later stages of development, Notch signaling is proposed to direct CD4 versus CD8 T lineage commitment. This hypothesis has been challenged by recent studies of conditional Presenilin-deficient mice showing that Notch promotes the selection and maturation of CD4 and CD8 T cells by potentiating TCR signal transduction in immature thymocytes. While similar conclusions have not been reported with conditional mutation of other downstream mediators of Notch activation, it appears that functional inhibition may not have been achieved at a comparable stage of development and/or analogous issues have not been addressed. The differences also question whether in thymocytes Notch signals only through the canonical pathway. Further study of conditional mutants, signaling intermediates, and transcriptional regulators are needed to elucidate how Notch facilitates TCR signaling in generating mature T cells.

Introduction

In a number of systems, Notch mediates diverse functions at successive stages of development [1–3]. Likewise, in lymphocytes, Notch regulates the T versus B cell fate decision and is required for differentiation through early DN (double negative, CD4-CD8-) stages of thymocyte development, and in conjunction with pre-TCR signaling, Notch promotes the transition to the DP (double positive, $CD4^+$ $CD8^+$) stage. Somewhat controversial are results implicating a role for Notch in pre-TCR expression, TCRβ gene rearrangement, the γδ versus γδ T lineage decision, and the generation and emigration of γδ T cells [4]. Contentious also are studies of late T cell development, some suggesting that Notch functions in DP thymocytes to specify CD4 and CD8 T cell lineages [5], while others advocate no role for Notch in generating SP (single positive, $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$) thymocytes. This review will focus primarily on studies performed over the last two years, employing alternative approaches for manipulating Notch signaling that provide new insight into how Notch functions in the selection and maturation of CD4/CD8 T cells. While the controversies are far from settled, an in-depth comparison of the model systems may help to explain some of the apparent discrepancies.

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A role for Notch in the development of CD4/CD8 SP thymocytes

Studies of Notch in T cell development have been difficult, not only because of the complexity of the Notch signaling pathway, but also because of the technical difficulties in detecting components of the pathway and assessing Notch activation in highly heterogeneous populations. There are multiple Notch receptors and ligands, but few useful reagents, since the specificity of most antisera have not been validated and few monoclonal antibodies exist. Also the redundancy in receptors and ligands and problems of embryonic lethality pose additional challenges for gene-targeting approaches.

There is the presumption that the pathway of Notch signaling operating in thymocytes and T cells is known. In the best characterized model, Notch regulates gene expression by converting a transcriptional repressor complex to an activating complex. The pathway is initiated when Notch-ligand engagement induces two successive proteolytic cleavages, the second mediated by a Presenilin-containing complex with γ-secretase activity that releases the Notch intracellular domain (NICD). This activated form of Notch translocates to the nucleus where it binds to the transcriptional regulator, CSL (CBF1/Su(H)/Lag-1; also called RBPJκ), displacing co-repressors, and recruiting co-activators, including members of the Mastermind (MAML) family [2;3]. Indicative of its importance, several crystal structures of this CSL +NICD+MAML complex have been solved [6]. While this is considered to be the canonical pathway for Notch signaling, recent data from ChIP assays question whether the model applies to all Notch/CSL regulated genes, since CSL is not always bound to the enhancer of all Notch target genes [7;8]. For some genes, CSL constitutively binds the enhancer, shifting from suppression to activation following NICD translocation to the nucleus. For other genes, there is no default repression; instead a CSL/NICD complex is assembled in the cytoplasm and then recruited to the nucleus for gene activation. Moreover, while it is certain that NICD/CSL/MAML complexes are critical for some aspects of Notch activity, there are experimental observations to suggest additional Notch pathways. For example, mutated forms of Notch that cannot bind CSL retain some functional activity [9;10]. Although Notch is thought to act primarily in the nucleus, in T cells Notch reportedly binds PI3 kinase [11] , Deltex, and the IKK complex [12]. Since Notch signaling does not always operate by the established paradigm, for the purpose of studies using gene-targeted mutations, it will be important first to determine the nature of Notch signaling and components of the pathway that are critical in thymocyte development. Obviously, in experiments where CSL or MAML function is prohibited, any non-transcriptional roles for Notch will remain intact.

Because of high turnover, very little NICD is found in the nucleus [3]; therefore, expression of target genes is most often used as a functional indicator of Notch activation. Some genes have been implicated as global Notch targets; such as Hes1 [13;14] and Herp [15]; and in T cells, there is evidence for Deltex [13;14;16;17], Nrarp [18], Ptcra (pTα) [14;17], CD25 [19], IL-4 [20;21], t-Bet [22], and GATA3 [7;23]. Nevertheless, there is often a requirement for transcriptional co-regulation which can impose cell- and tissue-restricted expression, as well as the possibility for Notch-independent regulation of putative target genes. For example, Notch signaling may stimulate pTα transcription in immature thymocytes, but not in keratinocytes or even in mature T cells. Given the technical limitations, there is no clear consensus on which/where Notch receptors/ligands are expressed in the thymus, or an unequivocal readout of the stages at which Notch is activated.

Adding to the difficulties of studying Notch is the potential for Notch itself to be regulated [2;3;24]. Notch has a number of regulators, and many are expressed in thymocytes. Overexpression of the glycosyltransferase, Fringe, can misdirect early thymocyte progenitors to the B cell lineage [24]. The adaptor Numb can negatively regulate Notch by promoting the ubiquitination and degradation of Notch at the membrane [25]. Numb and its homologue,

Numblike, are expressed in thymocytes; however, conditional deletion of these two genes has no effect on the development of T cells or any other hematopoietic lineage [26;27]. Notch can also be regulated by the ubiquitin ligases; Deltex, Itch, and Sel10 (Fbxw7). Although there is evidence for both positive and negative regulation of Notch by Deltex [28;29], Deltex1/2 - deficient thymocytes develop normally [30]. Conditional gene deletion of Fbxw7 upregulates expression of Notch and cMyc, correlating with increases in thymocyte number [31]; nevertheless, it was argued that Notch was not responsible, since the defective phenotype was maintained in double Fbxw7/CSL-deficient thymocytes. However, the timing of CSL gene/protein deletion using Cd4-Cre and the potential for CSL-independent Notch signaling could complicate this interpretation (see below).

A number of groups have used mutational approaches to investigate Notch function in the development of αβ T cells. With gain-of-function, mice expressing constitutively activated forms of Notch (NICD) exhibit decreased CD4 and increased CD8 SP thymocyte development [5]. These results are at odds with loss-of-function studies in which interference with endogenous Notch signaling in thymocytes has little or no effect on CD4/CD8 SP development. Because thymocytes express four Notch homologues, it was perhaps not surprising that DP thymocytes individually deficient for Notch 1, 2, 3, or 4 show normal SP development [32–35]. To circumvent the problem of redundancy, downstream mediators of the Notch signaling pathway were inhibited. However, in mice with conditional deletion of CSL [36] or an induced dominant negative form of Mastermind (dnMAML) [37], SP thymocytes are also generated normally. These findings are contrasted with those obtained by conditional deletion of Presenilin1/2, which is required for the activation of all Notch receptors. These mice show impaired SP thymocyte development due to defects in TCR signaling and positive selection of DP thymocytes [38]. Collectively, the results of gene targeting are difficult to reconcile, since Presenilin, CSL, and MAML are all proposed to be obligatory components of the Notch signaling pathway and in all, gene deletion is mediated by Cd4-Cre. Such discrepancies could be due to differences in the experimental models or to unique biological properties of the proteins. Of concern is whether DP thymocytes retain residual Notch activity after gene deletion. Presenilin and MAML proteins have a short half-life, but the turn-over of CSL after Notch activation is not established [39]. Although all three models employ Cd4-Cre, gene deletion of Presenilin1 is initiated at the DN2-DN3 stage and completed by the DP stage, whereas deletion of the CSL gene is later, predominantly at the DP stage [38]. As determined by GFP expression, dnMAML expression is not induced until the DP stage [37]. These differences in timing of gene deletion are functionally reflected in the numbers of DP thymocytes. Deletion of Presenilin1 by Cd4-Cre severely impacts DP thymocyte number [38], consistent with the fact that thymocytes deprived of Notch signaling before or during β-selection cannot mature past the DN3 stage [19;40]. In contrast, CSL-deficient and dnMAML models generate normal numbers of DP thymocytes [36;37], an indication that Notch signaling is abrogated later than in the Presenilin model. It was surprising, therefore, that thymocytes develop normally in another model in which a different exon of Presenilin1 was deleted by Cd4-Cre [41]. The stage of gene deletion was not examined, but the finding of normal thymus cellularity suggests that it too occurs later. Whether the timing of deletion reflects differences in background genetics and/or accessibility of the targeted locus is not clear, but these comparisons send a note of caution of the technical difficulties in attaining stage-specific gene deletion.

The reduced number of DP thymocytes generated in Presenilin-deficient mice with gene deletion mediated by Cd4-Cre more closely resembles that of CSL [36] and dnMAML [19] which use pLck-Cre, perhaps making these three models more comparable. The efficiency of generating mature thymocytes with fixed or diverse TCR repertoires have not been reported for the latter two, but the ratio of CD4 to CD8 SP thymocytes appears to be decreased [19; 36]. Similarly, ratios of CD4 to CD8 SP thymocytes are slightly decreased with germline

deletion of MAML1; however, these mice survive until birth and generate T cells, indicating that Notch activity cannot be critically reduced by this manipulation [42].

Although peculiarities of the experimental systems likely account for reported differences in generating SP thymocytes, unique functional properties of the CSL, MAML, and Presenilin proteins should also be considered. Notably, all three proteins have functions unrelated to the Notch pathway. The CSL gene family arose earlier in evolution than Notch and MAML, and CSL genes exist in organisms lacking the Notch pathway [43]. Indeed, CSL participates in Notch-independent functions in mammals, as it does with PTF-1 stimulated transcription in pancreatic development [44]. There is also the possibility that a loss of CSL could affect gene silencing in the absence of Notch signaling, since CSL often acts as a default repressor [45]. Likewise, there are Notch independent functions of MAML. MAML operates as a cofactor for transcription factors other than CSL, such as MEF2C [46] and p53 [47]. Notch-independent functions can contribute to the phenotypes associated with Presenilin mutation as well [48]. The Presenilin complex cleaves substrates other than Notch (for example; CD44, Amyloid Precursor Protein, and Syndecan3). In addition, non-enzymatic functions have been attributed to Presenilin. Therefore, although functional inhibition of these mediators may circumvent problems of Notch receptor/ligand redundancy, these caveats should be considered in interpreting the results.

Notch and TCR signaling in the generation of CD4/CD8 SP thymocytes

In invertebrates, Notch can reinforce other receptor signaling pathways, as it does with the Ras-MAPK pathway [1;49]. Similarly, in T cells, there is evidence that Notch and TCR signaling are functionally linked. Notch can modulate TCR signaling in peripheral T cells [50] and co-localizes with CD4 in TCR activation [51]. Also in DP thymocytes, Notch was found to co-cluster with Lck at the site of TCR/MHC contact [26], raising the possibility that Notch can function directly in proximal TCR signaling. Notch and pre-TCR signals are both required to mediate the DN to DP transition [40] and increased Notch activity enhances the effects of TCR simulation on gene expression [52]. DP thymocytes with a NICD transgene show enhanced calcium flux *in vitro* and a surface phenotype ex vivo suggesting that TCR signaling and thymic selection are improved by activated Notch [5;16;17]. Although enhanced Notch activity in thymocytes was reported to attenuate TCR signaling in one study [53], the conclusion is problematic since the mice succumb to a T cell leukemia of transformed DP cells. In rats, expression of transgenic NICD in thymocytes severely abrogates SP development [54], but rather than an effect on TCR signaling, this phenotype was attributed to mis-regulated expression of pre-TCR and RAG.

Recently a functional link between TCR and Notch signaling in thymocytes was demonstrated with a Notch loss-of-function mutant. Defective CD4 SP development in Presenilin-deficient thymocytes was attributed to impaired TCR signaling at the DP stage, characterized by a reduced Ca^{++} response to TCR stimulation *in vitro* and decreased CD5 expression *in vivo* [38]. Positive selection and SP thymocyte maturation were improved with higher affinity TCR or increased density of selecting MHC ligand. Forced expression of NICD restored thymus cellularity and TCR signaling, providing evidence that signaling abnormalities in Presenilin-deficient mice are due to a loss of Notch activity. Nevertheless, the stage at which Notch first mediated its effects was not obvious from the results. Since Notch and pre-TCR signaling are both required for progression from DN3 to the DP stage [40], and TCR signaling is defective in Presenilin-deficient DP thymocytes prior to selection, it was concluded that signal transduction must be impacted between the DN4 and early DP stage. Whether similar signaling defects exist in DP thymocytes of the Notch, CSL, or dnMAML models, when gene deletion is induced prior to the DP stage, has not been determined.

How Notch modulates TCR signal transduction in DP thymocytes is not clear, but Notch influences numerous signaling pathways known to be important in developing T cells; for example, TCR/co-receptors [16;17;26;51;52;55;56], Akt/P13K/PTEN/MAPK/NLK [11;57–61], PDK1 [62], calcium [38], NFAT/AP1 [53;55;63], NFκb [12;64;65], β-catenin [57;66]. Thus, it is likely that Notch interacts directly with or regulates the expression of a TCR signaling intermediate.

Notch and CD4/CD8 T lineage commitment

Early studies of NICD transgenic mice demonstrated that Notch favors the development of CD4 SP at the expense of CD8 SP thymocytes and can even redirect some MHC2-restricted thymocytes to the CD8 lineage. Collectively, these results suggested a role for Notch in the CD4/CD8 T lineage decision [67;68]. Somewhat complimentary were studies using γ-secretase inhibitors in fetal thymic organ culture, showing that blocking Notch activity has a greater impact on CD8 SP development [69;70]. Nevertheless, more recent analysis of mice bearing MHC1- or MHC2-restricted TCR transgenes provide no evidence that forced Notch activity can redirect the CD4/CD8 lineage choice; to the contrary, no SP thymocytes are generated in these mice [5;53]. Since TCR signaling is equivalent or improved in DP thymocytes with enhanced Notch activity, irrespective of TCR repertoire [5], negative selection was considered to explain the developmental block. However, reducing MHC expression by half failed to rescue any SP development. So while the differences in NICD transgenic mice with fixed versus diverse TCR repertoires go unexplained; the findings are not fully compatible with the notion that Notch functions directly in the CD4/CD8 lineage decision. Also to be considered here are the many examples in which constitutive Notch activation can have non-physiological consequences, since normally Notch activation is highly regulated, both temporally and quantitatively [71].

In contrast to the gain-of-function models, impaired development of SP thymocytes, with either fixed or diverse TCR repertoires, correlates with attenuated TCR signaling in Presenilin-deficient thymocytes [38]. MHC-dependence for positive selection is normal and although CD4 SP development is reduced, there is no evidence that CD4/CD8 T lineage commitment can be redirected. Since SP development can be partially rescued by increased TCR affinity or density of MHC, it was concluded that Notch reinforces TCR signaling, the primary determinant of CD4/CD8 T cell fate; but it plays no direct role in lineage commitment.

Conclusions

Over the last few years, a number of studies have suggested that forced Notch activity directly influences CD4/CD8 T lineage commitment. This notion began to unravel with the recent findings that activated Notch completely blocked SP thymocyte development in TCR transgenic mice; in fact, there was no indication that lineage choice could be re-directed by Notch with any TCR tested. A role for Notch in CD4/CD8 T cell fate was also disputed by a mutant model in which Notch signaling was abrogated in DP thymocytes by the loss of Presenilins. These studies suggest an alternative hypothesis, that Notch influences positive selection and the generation of SP thymocytes by modulating TCR signal transduction in DP thymocytes. The latter results have not been reported for other models mutating downstream mediators; but disruption of Presenilin/γ-secretase, MAML, or CSL function have all been found to impact mature T cell activation; perhaps suggesting that Notch function has not been inhibited at a comparable stage of thymopoiesis. Notably, TCR signal transduction and positive selection have not been specifically addressed in the CSL or dnMAML mutants. An even greater concern is identifying which of the downstream mediators are critical, since the precise mode of Notch signaling in thymocytes is yet to be established. While these are issues that can be resolved with further study, the larger goal is to determine how Notch and TCR signaling

work together to promote the differentiation of T cells both in the thymus and the periphery. Since Notch often acts in concert with other signaling pathways, the cellular response it mediates can vary widely and is highly cell context-dependent. Elucidating the signaling networks that regulate late stages of T cell maturation with its many levels of control is, indeed, a daunting challenge for the future.

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