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Synergy between the pre–T cell receptor and Notch: cementing the αβ lineage choice

Cynthia J. Guidos

Notch1 signaling suppresses B cell development and promotes T lineage commitment in thymus-seeding hematopoietic progenitors. Notch1 is also activated in early T cell progenitors, but the functions of these later Notch signals have not been clearly defined. Recent studies reveal that Notch signaling is not essential for pre–T cell receptor (TCR) expression or γδ lineage choice. Rather, pre-TCR signaling enhances progenitor competitiveness for limiting Notch ligands, leading to preferential expansion of TCRβ-bearing progenitors.

Activation of transmembrane Notch receptors regulates several binary cell fate decisions by inhibiting precursors from adopting a default "primary" cell fate (1). Notch receptors are activated by interactions with five different transmembrane Notch ligands belonging to two families: Delta-like and Jagged (also known as Serrate). In some cell types, Fringe glycosyltransferases modify the extracellular domain of Notch receptors to enhance activation by Delta-like ligands (DLs) and inhibit activation by Jagged ligands (1).

Upon ligand binding, Notch receptors become susceptible to intramembranous proteolytic cleavage by the γ-secretase protease complex, which releases the Notch intracellular domain (N^{IC}) from its membrane tether. N^{IC} then travels to the nucleus where it interacts with a transcription factor known as RBP-Jκ or CSL (for CBF-1, suppressor of hairless, and Lag-1), converting it from a repressor into an activator (2).

When Notch activation is rendered ligand independent by overexpression of N^{IC}, B cell development is profoundly inhibited, and hematopoietic progenitors generate T cell precursors in the bone marrow even in the absence of the thymus (3). Conversely, when Notch1 or CSL are conditionally inactivated in hematopoietic progenitors, the postnatal thymus completely lacks T lineage cells and contains many immature B cells (4). Furthermore, ectopic expression of the Notch ligand DL-1 (but not Jagged-1) endows bone marrow stromal cell lines (S17 or OP9) with the capacity to generate immature T cells, rather than B cells, from hematopoietic progenitors in vitro (5, 6). These findings strongly suggest that Notch1, acting via a CSL-dependent pathway, is essential to induce T lineage specification and suppress B cell development from progenitors that seed the postnatal thymus. As predicted by this model, the adult thymus contains rare progenitors that can generate both T and B cells in clonal assays (7).

Notch1 continues to be expressed (8) and activated (9, 10) after thymus-seeding progenitors lose B cell potential and progress through the CD4/CD8 double negative (DN) phases of T cell development. Progenitors that successfully rearrange *TCRγ* and *TCRδ* usually remain DN and adopt the γδT cell fate. In contrast, DN3 thymocytes that successfully rearrange *TCRβ* form pre-TCR complexes consisting of pre-Tα, TCRβ, and CD3 proteins, which signal in a ligand-independent fashion to promote DN3 survival, vigorous clonal expansion, and differentiation into αβ-committed CD4/CD8 double positive (DP) thymocytes. However, several lines of evidence indicate that TCR signals do not play a classic instructive role in the αβ/γδ lineage decision. TCRβ-deficient mice produce small numbers of DP thymocytes that harbor in-frame *TCRδ* rearrangements, and some transgenic γδ TCRs allow generation of αβ-committed DP thymocytes, particularly when they transmit relatively weak signals (11, 12). Furthermore, some transgenic αβ TCRs can promote development of γδ-like T cells.

Because Notch signaling can instruct cell fate choices (13), much interest has focused on how Notch signaling might direct early T cell progenitors to choose between the αβ and γδ T cell lineages. However, work from Robey et al. suggested an alternative stochastic/selective model in which γδ TCRs and pre-TCRs differentially influence the ability of early T cell progenitors to activate Notch1 (14). Here we discuss data from several recent papers (15, 16), and one in this issue of the *JEM* (on p. 2239 [17]), that provide further insights into the role of Notch signaling in early T cell progenitors before and after αβ/γδ lineage divergence.

T cell progenitors show stage- and lineage-specific Notch dependence

Ciofani et al. used the OP9/DL-1 culture system to define the developmental stage at which the αβ and γδ T cell lineages first diverge by assessing the clonogenic frequency of αβ-committed, γδ-committed, and αβ/γδ bipotential progenitors (15). In these in vitro analyses, all DN1 thymocytes were bipotent, in contrast to ~35% of the DN2 subset. About 60% of fetal DN2 progenitors were αβ committed, whereas <5% were γδ committed. The vast majority of DN3 cells were unipotent, with αβ progenitors out numbering γδ progenitors by about four to one. Thus, αβ/γδ T

C.J.G. is at Program in Developmental Biology, Hospital for Sick Children Research Institute, and Department of Immunology, Faculty of Medicine, University of Toronto Toronto, ON Canada M5G 1L7.

CORRESPONDENCE
C.J.G.: Cynthia.guidos@sickkids.ca

cell commitment is first evident in vitro among DN2 progenitors and is largely complete by the DN3 stage (Fig. 1). These clonal in vitro studies nicely complement the recent demonstration that γδ-expressing progenitors can first be visualized at the DN2 stage in vivo (18).

To examine the influence of Notch signaling on αβ/γδ lineage divergence, the authors cultured DN2 and DN3 thymocytes on OP9 or OP9/DL-1 stromal cells and quantified production of γδ-expressing DN versus αβ-committed DP thymocytes. Interestingly, γδ development from DN3 cells was largely Notch/DL-1 independent, and even the DN2 subset generated reasonable numbers of mature γδ T cells in the absence of DL-1. Together with the finding that γδ T cell development is not impaired by conditional deletion of all CSL-dependent Notch signaling in DN3 thymocytes (19), these findings reveal that generation of γδ-committed progenitors and their subsequent survival and maturation can occur in the absence of Notch signaling.

In contrast, survival and maturation of αβ-committed cells from both DN2 and DN3 progenitors was highly Notch dependent. Small numbers of TCRβ-expressing cells were generated in bulk cultures of DN2 or DN3 thymocytes on OP9 cells, but their progenitors were not clonogenic, demonstrating that survival and/or proliferation of αβ-committed progenitors is highly Notch dependent. Also using the OP9/DL-1 system, Taghon et al. demonstrated a distinction in the degree of Notch dependence of wild-type DN3s before and after expression of TCRβ (designated DN3a and DN3b, respectively) (20). DN3a cells could not make DP thymocytes in the absence of Notch/DL-1 signals, whereas DN3b cells could make small DP populations. In contrast, both subsets made γδ T cells in the absence of DL-1.

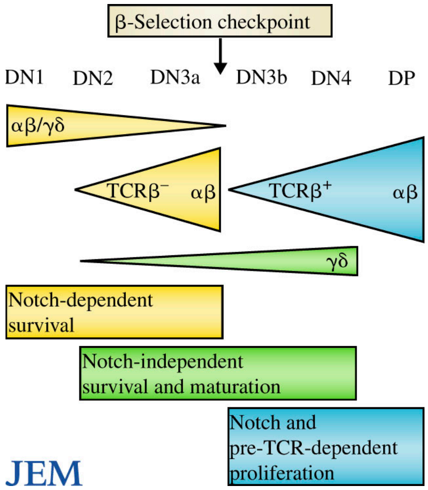

Figure 1. Requirement and role of Notch in early T cell development. The relationship between δγ/βα commitment status and Notch dependence is depicted for each stage of early T cell development. DN3a cells are TCRβ$^-$, whereas DN3b cells are TCRβ$^+$.

Collectively, these in vitro findings reveal that αβ progenitors are highly Notch dependent both before and after αβ/γδ lineage divergence, whereas progenitors committing to the γδ lineage become Notch independent (Fig. 1). Previous studies have shown that Notch signaling, perhaps dependent on the protein kinase AKT, maintains survival but does not induce proliferative expansion of *RAG-2*$^{-/-}$ DN3s, which cannot express a pre-TCR. (21). It will thus be important to determine how this Notch1-induced survival pathway overlaps with or is distinct from the γc-cytokine–mediated prosurvival pathways that also operate during the DN1-DN3 stages (22).

Pre-TCR expression is CSL and Notch independent

What could account for the exquisite and lineage-specific Notch1 dependence of αβ-committed T cell progenitors? One possibility is that CSL-dependent Notch1 signaling directly induces pre-TCR expression, which is needed for the DN3 to DP transition. Consistent with this idea, conditional deletion of Notch1 (23) or CSL (19) at the DN3 stage causes a partial block in the generation of DP thymocytes. DN3 thymocytes from these mice had abnormally low frequencies of TCRβ protein and *V* to *DJβ* rearrangements. Since expression of pre-Tα was normal, it was concluded that Notch1 and CSL regulate *TCRβ* recombination. However, since the defect was not absolute, *V* to *DJβ* rearrangement may only partially depend on Notch1 activity. Alternatively, some DN3 thymocytes may have produced pre-TCRs and undergone selection for in-frame *TCRβ* rearrangements (β-selection) before deleting Notch1. Therefore, studies to date have not clearly defined whether DN3 thymocytes require Notch1 activation upstream and/or downstream of pre-TCR signaling in vivo.

In this issue, Maillard et al. resolve the problem by targeting expression of DN Mastermind–like (DN-MAML) to DN3 thymocytes using a conditional strategy involving Lck-Cre (17). MAML transcriptional coactivators are required for CSL-dependent signaling from all Notch receptors, so DN-MAML expression inhibits transcription induced by all four mammalian Notch receptors (24). Importantly, the authors strategy also ensured that all thymocytes expressing DN-MAML were marked by coexpression of green fluorescent protein, allowing them to separately track the fate of DN-MAML$^+$ versus DN-MAML$^-$ DN3 thymocytes. Using this system, these investigators report only a partial inhibition of the DN3 to DP transition when DN-MAML is conditionally induced in DN3 thymocytes, similar to the effects of deleting CSL or Notch1 at this stage. However, purified DN-MAML$^+$ DN3 thymocytes were absolutely defective in generating DP thymocytes 10 days after intrathymic injection, whereas DNMAML$^-$ DN3 thymocytes generated substantial numbers of DP thymocytes using this in vivo assay. These findings definitively demonstrate that heterogeneity in the timing of Lck-Cre expression accounts for the incomplete block in the DN3 to DP transition when Notch1 or CSL are inactivated in DN3 thymocytes.

Notch signals influence expression of pre-TCR components earlier during T lineage specification (25), but transgenic TCRβ did not restore the DP thymocyte pool in Lck-Cre/DN-MAML mice. Although Notch1/CSL signaling could regulate TCRβ recombination before the DN3 stage, there is an absolute in vivo requirement for CSL-dependent Notch activity downstream of pre-TCR expression in DN3 thymocytes. Nonetheless, ectopic Notch activation doesn't relieve the developmental arrest of DN3s in mice lacking RAG-2 (26), although DP leukemias can eventually develop (27). These data and other findings (28) demonstrate that cooperation between Notch activation and pre-TCR signaling is absolutely necessary to promote the DN3 to DP transition (Fig. 1).

pre-TCR and Notch signaling synergize during the DN3 to DP transition

In pre-TCR–deficient mice, γδ TCRs and αβ TCRs can function as alternative pre-TCRs, but they generate a much smaller DP thymocyte pool than bona fide pre-TCRs. To investigate the basis for this difference, Garbe et al. (16) co-cultured OP9/DL-1 cells with DN3 or DN4 thymocytes that were engineered to express predominantly conventional pre-TCRs versus αβ or γδ TCRs. pre-TCRs promoted DP thymocyte differentiation and proliferation more effectively in this culture system than either αβ or γδ TCRs, similar to what has been described in vivo. Ciofani et al. also found that several γδ TCRs could induce the DN3 to DP transition in thymocytes cultured on OP9/DL-1 cells (15). As mentioned already, production of DP cells, but not γδ T cells was highly dependent on Notch/DL-1 signaling. Moreover, there was a negative correlation between the strength of γδ TCR signaling and development of DP cells. These observations are consistent with previous findings showing that strong γδ TCR signals can prevent T cell progenitors from developing into αβ-committed DP cells (11, 12). However, the new studies additionally show that these alternative pre-TCRs, like conventional pre-TCRs, promote DP thymocyte development in a Notch-dependent fashion.

Garbe et al. then titrated various amounts of γ-secretase inhibitor (GSI) into the cultures to inhibit the generation of active N^{IC}. Surprisingly, they found that DP thymocyte production from αβ- or γδ-expressing DN3 cells was highly sensitive to a given dose of GSI, much more so than DP production from pre-TCR–expressing DN3 cells (Fig. 2). Similarly, αβ-expressing DN4 cells were more sensitive to GSI than pre-TCR–expressing DN4 cells. The authors interpret these data to suggest that DN3s and DN4s expressing conventional pre-TCRs require less Notch signaling to proliferate and mature to the DP stage than progenitors expressing alternative pre-TCRs. However, an alternative interpretation is that DN3 thymocytes expressing conventional pre-TCRs are more effective at capturing Notch1 signals to promote proliferation and differentiation during the DN3 to DP thymocyte transition. Indeed, previous work from this group has shown that pre-TCR–expressing precursors profoundly out-compete αβ TCR-expressing precursors to contribute to the DP thymocyte pool in vivo (29). The new data shows that this competitive advantage can be recapitulated on OP9/DL-1 cells in vitro, and is diminished when high doses of GSI were added to the cultures. Thus, it seems likely that T cell progenitors expressing conventional pre-TCRs exhibit stronger Notch1/DL-1 interactions than progenitors expressing αβ or γδ TCRs, endowing the former cells with greater resistance to the γ-secretase–dependent generation of N^{IC}.

Potential mechanisms of pre-TCR synergy with Notch signaling

The molecular basis for the synergy between Notch and pre-TCR signaling remains to be determined. At least two nonmutually exclusive scenarios can be envisioned. One possibility is that pre-TCR signaling could more effectively down-modulate the expression or activity of molecules that specifically antagonize Notch activation, or molecules that generally inhibit proliferation. Candidates in the former category include Numb, a negative regulator of Notch activation that physically interacts with the TCR in mature T cells (30). Candidates in the latter category include the E47 and Gfi-1 transcription factors, which both restrain proliferation of DN3 thymocytes (31, 32).

Alternatively or in addition, pre-TCR signaling could enhance the efficiency or avidity of Notch1 interactions with DLs, perhaps by modulating expression or activity of Fringe proteins (1). Lunatic Fringe is highly expressed in DN3 and DN4 thymocytes, where it enhances competition for limiting intrathymic niches in vivo to homeostatically regulate the size of the DP thymocyte pool (33). Moreover, Lunatic Fringe enhances the ability of DN3 and DN4 thymocytes to bind DL-1 without affecting Jagged-1 binding (33). T cell progenitors lacking Lunatic Fringe can respond to OP9/DL-1, but they are more sensitive to GSI than wild-type progenitors (unpublished data). Collectively these findings reveal that Lunatic Fringe–Notch1 interactions regulate T cell progenitor competition for limiting DLs in vivo. Thus, both the pre-TCR and Lunatic Fringe enhance Notch1 interactions with DLs, increasing their resistance to GSI and their competitive fitness. It will therefore be important to determine whether pre-TCR signals directly regulate Lunatic Fringe expression in DN3 and DN4 thymocytes.

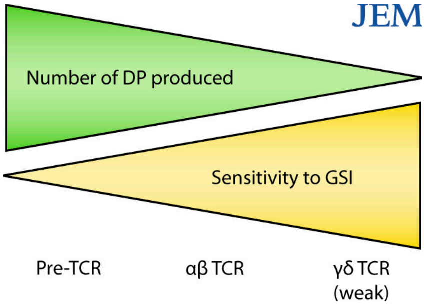

Figure 2. TCR isotype determines sensitivity of DP thymocyte production to GSI. The figure depicts the inverse correlation between the number of DP thymocytes produced by DN3 cells expressing each type of TCR and the degree to which DP cell production is inhibited by GSI (15).

Conclusions

In summary, these findings complement previous work showing that DN1, DN2, and DN3 thymocytes must continuously compete for limiting Notch1 signals in vivo (10) and further suggest that Notch1 activation has different functions during these early stages of intrathymic T cell development. Notch activation is needed to maintain survival of αβ/γδ bipotent and αβ-committed progenitors before TCRβ expression. Commitment to the αβ or γδ T cell lineages, which likely occurs stochastically, is first evident at the DN2 stage and can occur in the absence of Notch1/DL interactions. However, αβ-committed cells remain highly dependent on Notch signals, which act cooperatively with pre-TCR signals to induce vigorous proliferation and maturation to the DP stage (Fig. 1). In contrast, γδ-committed cells become Notch independent. Since weak γδ TCR signals can promote maturation to the DP stage in a Notch-dependent fashion (Fig. 2), strong γδ TCR signals may be needed to terminate Notch1 dependency and promote full γδ T cell maturation. Alternative TCRs are inefficient at promoting the Notch-dependent generation of DP thymocytes because they do not synergize effectively with Notch signals, but the molecular basis of this effect is currently unknown. Importantly, the highly effective synergy between pre-TCR signals and Notch1 activation provides a selective mechanism to prevent αβ-committed progenitors that express αβ or γδ TCRs from effectively competing with pre-TCR–expressing progenitors for access to limiting DL niches in vivo.

The importance of Notch-induced survival and proliferation throughout the early stages of αβ T cell development likely explains why activating Notch1 mutations are found in >50% of T cell acute lymphoplastic leukemias (34). In future work, it will be important to identify the targets of Notch1 at each developmental stage and to determine how Notch signaling interacts with other pathways regulating early T cell development, as this may provide new candidates for targeted therapy of T cell leukemia.

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