The Journal of Immunology

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J Immunol 2014; 192:4017-4023; ;
doi: 10.4049/jimmunol.1302259
http://www.jimmunol.org/content/192/9/4017

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Brief Reviews

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An Overview of the Intrathymic Intricacies of T Cell Development

Divya K. Shah,* and Juan Carlos Zúñiga-Pflücker†

The generation of a functional and diverse repertoire of T cells occurs in the thymus from precursors arriving from the bone marrow. In this article, we introduce the various stages of mouse thymocyte development and highlight recent work using various in vivo, and, where appropriate, in vitro models of T cell development that led to discoveries in the regulation afforded by transcription factors and receptor–ligand signaling pathways in specifying, maintaining, and promoting the T cell lineage and the production of T cells. This review also discusses the role of the thymic microenvironment in providing a niche for the successful development of T cells. In particular, we focus on advances in Notch signaling and developments in Notch ligand interactions in this process. ***The Journal of Immunology*****, 2014, 192: 4017–4023.**

The thymus is the site of T cell development, providing a microenvironment that supports and guides the generation of a diverse T cell repertoire, which is self-restricted and self-tolerant (1). Thymic seeding progenitor cells (TSPs), or early thymic progenitor cells (ETPs), arrive from the adult bone marrow (BM), enter the thymus at the corticomedullary junction (2), and undergo T-lineage specification, followed by a series of well-characterized developmental checkpoints (3–7). These cells lack the expression of CD4 and CD8 and are termed double-negative (DN) cells [or triple negative if it is not already presumed that these cells are also CD3/TCR negative (8)]. They are subdivided by the expression of the cell surface markers CD44 and CD25. DN1 cells ($CD44^+CD25^-$) are heterogeneous and have the potential to give rise to αβ T cells, γδ T cells, NK cells, dendritic cells, macrophages, and B cells (1). Further characterization of the DN1 subpopulation into five subsets based on CD117 and CD24 expression (DN1a–e) revealed that the DN1a and DN1b subsets, which express CD117, are the most potent at giving rise to T-lineage cells (9). Fig. 1 provides an overview of T cell development.

After specification to the T cell lineage, DN2 cells ($CD44^+$ $CD25^+$) migrate through the cortex and begin TCR-β, TCR-γ, and TCR-δ gene segment rearrangements, which are mediated by RAG1 and RAG2. Full commitment to the T cell lineage occurs following the transition from the DN2 to the DN3 stage of development (1). DN3 cells ($CD44^-CD25^+$) that have successfully rearranged their TCR β-chain associate with an invariant pre-TCR α-chain and CD3 signaling molecules to form the pre-TCR complex, which enforces β-selection, rescuing cells from apoptosis, mediating allelic exclusion at the TCR β-chain locus, and initiating cellular proliferation, permitting passage along the αβ lineage (10). In the subcapsular region, β-selection also leads to CD25 downregulation, producing an apparent and transient DN4 population ($CD44^-CD25^-$) that upregulates expression of CD4 and CD8 to yield double-positive (DP) cells, which usually progress through an immature cycling $CD8^+$ intermediate SP population (11), and initiates TCR-α gene rearrangements (1).

DP cells that have successfully rearranged their TCR α-chain, to produce an αβ-TCR, undergo positive and negative selection in the cortex, as well as further negative selection in the medulla, and typically become MHC class I or MHC class II restricted (12). During positive selection, thymocytes bearing "useful" TCRs undergo differentiation to the $CD4^+$ helper or $CD8^+$ cytotoxic lineage, and mature $CD4^+$ or $CD8^+$ single-positive (SP) cells exit the thymus and circulate in the periphery (13). γδ T cells do not undergo a selection checkpoint in the same manner as αβ T cells (10, 14), but they adopt this lineage from the strength of the γδ TCR signal (15). Other nonclassical lineages that develop in the thymus include innate NKT cells and natural T regulatory cells (16, 17).

The Notch-signaling pathway

The Notch-signaling pathway is critical for the specification, commitment, and development of thymocytes (18, 19). Notch is a heterodimeric receptor (in mammals Notch1–4) that binds to two families of Notch ligands: the Delta family (in mammals, Delta-like [Dll]–1, Dll3, and Dll4) and the Serrate family (in mammals, Jagged [Jag]-1 and Jag2). Upon ligand–

*Anthony Nolan Research Institute, Royal Free Hospital, London NW3 2QG, United Kingdom; and †Department of Immunology, Sunnybrook Research Institute, University of Toronto, Toronto, Ontario M4N 3M5, Canada

Received for publication September 23, 2013. Accepted for publication February 19, 2014.

This work was supported by funds from the Canadian Institutes of Health Research. D.K.S. was supported by a Canadian Institutes of Health Research Fellowship award, and J.C.Z.-P. is supported by a Canada Research Chair in Developmental Immunology.

Address correspondence and reprint requests to Dr. Juan Carlos Zúñiga-Pflücker or Dr. Divya Shah, University of Toronto, Sunnybrook Research Institute, 2075 Bayview Avenue, Room M7-619, Toronto, ON M4N 3M5, Canada (J.C.Z.-P.) or Anthony Nolan Research Institute, Royal Free Hospital, Pond Street, London NW3 2QG, U.K. (D.S.). E-mail addresses: jczp@sri.utoronto.ca (J.C.Z.-P.) or Divya.Shah@anthonynolan.org (D.S.)

Abbreviations used in this article: BM, bone marrow; Dll, Delta-like; DN, double negative; DP, double positive; ETP, early thymic progenitor cell; HSC, hematopoietic stem cell; ICD, intracellular domain; Jag, Jagged; Mib, Mindbomb; SP, single positive; TCF, T cell factor; TEC, thymic epithelial cell; TSP, thymic seeding progenitor cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302259

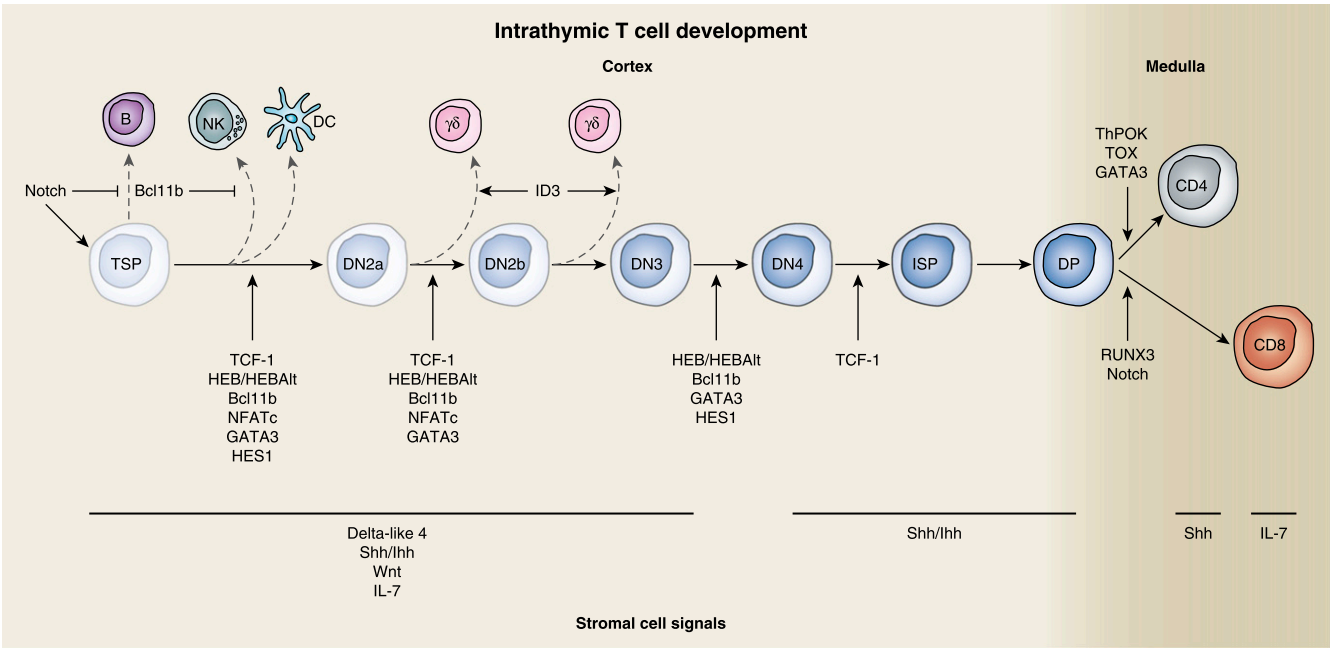

FIGURE 1. Overview of thymocyte development, highlighting role of key transcription factors and signaling molecules at specific developmental points. The main stages of thymocyte development are depicted, with transcription factors marked with arrows and signals provided by thymic stromal cells, including receptor–ligand interactions, shown at the bottom (see text for details). Notch–Dll4 signaling is required for specification of TSPs to the T cell lineage and for instructing transcription factors to adopt and commit to the T cell pathway at specific stages during differentiation. Thymic epithelial signals, such as those from the Hh- (Shh and Ihh), Wnt-, and IL-7–signaling pathways, aid in the commitment to the T cell lineage and continued proliferation and survival of developing thymocytes.

receptor interactions, the Notch receptor undergoes two proteolytic cleavage events, the first mediated by ADAM metalloproteases and the second mediated by γ-secretase activity; this causes the release of the intracellular portion of Notch, which translocates into the nucleus and, in cooperation with coactivators, induces target gene transcription (20).

Unlike other hematopoietic lineages, there is no set master regulator that instructs cells to adopt the T cell lineage; rather, cells that enter the thymus lose the potential to give rise to other lineages and sequentially acquire the ability to choose their ultimate T cell fate through the expression of T-lineage genes (specification and commitment). Notch comes close to encompassing and enforcing all of these outcomes; however, other transcriptional regulators are required for Notch to instruct T-lineage adoption (21).

TSP to DN2 stages

Several transcription factors have been identified recently as critical regulators of T cell specification, some of which are thought to act in concert with Notch signaling to promote T cell specification and limit other alternative fates. Notch signaling activates multiple transcription factors in thymocytes, including *Gata3*, *Tcf7*, and *Hes1*.

GATA3 is a zinc-finger transcription factor that has long been known to play a role at various stages of T cell development (22). GATA3 expression increases from the ETP to DN3 stages of T cell development. GATA3 hypomorphic mutant embryos, adoptive transfer of GATA3-null hematopoietic stem cells (HSC) and mice conditionally deleted for GATA3, conclusively showed that GATA3 is required for the development of functional ETPs (23). Recent work confirmed the requirement for GATA3 at the transition from DN1 to DN2 and its essential role in promoting a Notch-induced T cell program. *Gata3*$^{-/-}$ progenitors cultured on OP9-DL cells did not progress beyond the DN2 stage, although molecular analysis confirmed the T cell identity of these cells. There was also an increase in the production of CD19^{+} B cells, suggesting a role for GATA3 in inhibiting latent B cell potential (24). Beyond the specification stage, recent evidence showed that the positive and negative effects of GATA3 levels must be balanced at the DN2 stage to allow for further differentiation along the T cell pathway (25). The E protein, E2A, seems to play a critical role in limiting GATA3 expression specifically in DN2 cells. In the absence of E2A, excess GATA3 causes a block in T cell development, whereas E2A$^{-/-}$ multipotent progenitors knocked down for *Gata3* by small interfering RNA were able to generate DN3 cells (25). The role of E proteins, including E2A and E2-2, in thymocyte development is covered in a recent review (26). The relationship between Notch and Gata3 remains complex; however, both are clearly required for the development of ETPs and thymocytes.

TCF-1 (T cell factor, also known as *Tcf7*) is a member of the HMG family and is highly expressed in ETPs, with expression increasing upon Notch–Dll signaling (27). TCF1$^{-/-}$ mice have a severe reduction in the ETP subset but do not exhibit an increase in B cells (28). TCF-1$^{-/-}$ cells are able to upregulate Notch target genes in response to Notch–Dll signals; however, they are unable to upregulate T-lineage genes. Ectopic expression of human TCF-1 in progenitor TCF1$^{-/-}$ cells rescued T cell development, even in the absence of Notch–Dll signals, and it specifically upregulated T-lineage–specific genes (*Bcl11b* and *Gata3*), TCR genes, including *Tcf7* itself, as well as Notch1 target genes (*Deltex1* and *Ptcra*) (29). Notch binds the *Tcf7* gene locus, and ectopic expression of Notch1 leads to transcriptional upregulation of *Tcf7* (28), indicating that Notch signaling is upstream of *Tcf7*.

HES1, a bHLH transcriptional repressor, is a major component of Notch1-induced signaling during T-lineage com-

mitment (30) and is important for constraining myeloid cell fate outcomes (31, 32). ETPs and DN cells have high *Hes1* expression, whereas *Cebpα* is reduced in ETPs and absent from DN2 and DN3 cells. Ectopic expression of HES1 in BM Lin$^-$ Sca1$^+$ ckit$^+$ progenitors resulted in downregulation of *Cebpα*, and HES1 is suggested to directly regulate the *Cebpα* promoter, because it has several putative Hes1 binding sites. When cultured on OP9-DL4, *Hes1*$^{-/-}$ cells gave rise to myeloid cells, whereas *Hes1*$^{-/-}$ cells deleted for *Cebpα* restored T cell development (31), indicating an important role for HES1 in inhibiting *Cebpα* expression.

Cre-mediated deletion of *Hes1* in BM cells results in a reduction in the DN and DP cell compartments in competitive situations, and intrathymic transfer of these cells gave rise to T cells; however, there was an increase in the DN1 compartment, which was composed of immature B cells. The investigators hypothesized that this was a consequence of HSCs receiving weak Notch signals, because *Hes1*-deleted HSCs receiving suboptimal Notch signals were unable to adopt the T cell lineage, indicating that *Hes1* is essential to induce a Notch1-dependent genetic program for efficient T cell commitment (30).

NF-AT proteins are transcription factors that are activated by pre-TCR signaling. NF-ATc1 was recently shown to have an indispensible role during early thymocyte development, before β selection. NF-ATc1 activation increases as DN1 cells differentiate into DN3 thymocytes, and NF-ATc1 cooperates with STAT5 in response to IL-7 signals to contribute to the survival of early DN thymocytes. Conditional deletion of NF-ATc1 results in reduced cellularity; although these cells expressed key genes, such as *Notch1* and *Tcf7*, an arrest at the DN1 stage was observed (33).

DN2a/b to DN3 stages

The DN2 population recently has been subdivided, based on the expression of CD117, into DN2a (CD44$^+$ CD25$^+$ CD117^{hi}) and DN2b (CD44$^-$ CD25$^+$ CD117^{lo}) subsets. Gene expression, cell-proliferation profiles, and dependency on Notch–Dll signals show that the DN2b population is more committed to the T cell lineage than is the preceding DN2a subset, but it is not as fully locked-in as DN3 cells (34). High, continuous Notch signals are required to promote and support early thymocyte populations and inhibit NK cell development, whereas low Notch signals are sufficient to inhibit the development of B cells (35).

Several articles (36–38) recently highlighted the role of the tumor suppressor Bcl11b in the commitment of progenitors to the T cell lineage. Bcl11b is required to suppress the NK cell lineage, by repressing NK-promoting genes (*Id2* and *Il2rβ* [CD122]) (37). Thymocytes conditionally deleted for Bcl11b and cocultured on OP9-DL1 cells give rise to Nkp46$^+$ CD3$^-$ NK-lineage cells (38) and exhibit decreased expression of T-lineage genes (*Notch1*, *Hes1*, *Gata3*, *Tcf7*) and increased levels of genes normally associated with NK cells (*Id2*, *Il2rβ*, *Zfp105*) (38). Bcl11b is also a downstream target of both TCF-1 and Notch signaling, where it is directly upregulated upon Notch–Dll interactions (29).

αβ and γδ lineages

The γδ lineage is less sensitive to Notch signals (14), and Id3 has been suggested to play a role in commitment and differentiation to the γδ T cell lineage by integrating Notch and TCR signals (39). Strong TCR signals promote the γδ lineage, whereas weak pre-TCR signals, in collaboration with Notch, cause the repression of the E protein, E47, and promote the αβ lineage (39).

Notch signaling is absolutely required at the β-selection checkpoint (40) for its functional outcomes, including survival, proliferation, and differentiation, by regulating cellular metabolism, involving the PI3K/Akt pathway (41, 42). Notch signals mediate these trophic effects through HES1, PTEN, and cMyc (32). Notch signaling withdrawal from Rag2$^{-/-}$ DN3 cells resulted in decreased *Hes1* transcription and an increase in *Pten* expression, whereas cells transduced with dominant-negative Hes1, or knockdown for Hes1, showed an increase in PTEN protein levels, indicating that HES1 represses PTEN. In the absence of Notch signals, Pten conditionally deleted DN3 cells are able to differentiate to the DP stage, but they fail to undergo proliferation unless they overexpress cMyc. Thus, HES1 and PTEN are responsible for supporting differentiation, survival, and metabolism of pre-T cells at the β-selection checkpoint by bridging Notch signals to the activation of the PI3K/Akt pathway through PTEN, whereas cMyc drives proliferation of β-selected cells that reach the DP stage. In support of its mitogenic role at this stage, a recent global transcriptome analysis of the entire αβ T cell pathway revealed that cMyc has a steady expression pattern in DN cells, which decreases abruptly in DP thymocytes (43). cMyc protein levels increased after β selection but were absent by the DP stage. Ectopic expression of cMyc in DP cells resulted in a decrease in small DP cell maturation, indicating that cMyc regulation may be a key molecular event that drives maturation to the small DP cell.

HEB proteins are bHLH family transcription factors implicated in several stages of thymocyte differentiation: β-selection, TCRβ and TCRα gene rearrangements, regulation of pre-TCRα, and CD4 expression (26, 44). Separate transcriptional start sites in HEB give rise to canonical and shorter alternate (HEBAlt) forms (26). A developmental block in HEB$^{-/-}$ cells observed at the β-selection checkpoint can be partially restored with transgenic expression of HEBAlt, bypassing β-selection, to the DP stage, thus implicating HEBAlt as a critical regulator of early T-lineage genes (45). In addition, HEBAlt limits myeloid cell outcomes by collaborating with intracellular Notch (46). Furthermore, HEB$^{-/-}$ cells that have compromised Notch1 activity retain lineage plasticity, producing a DN1-like phenotype that could be induced to develop into NK cells and expresses *Gata3* and *Id2* but had lower levels of *Bcl11b*, a putative Notch target gene (47), suggesting that the canonical form of HEB is important for inhibiting NK cell fate.

RUNX1 is a transcription factor that is highly expressed at the DN stage and downregulated by the DP stage; however, until recently, the significance of this downregulation had not been resolved. Runx deficiency leads to severe defects in DN thymocyte differentiation, whereas overexpression of the distal isoform of RUNX1 has an inhibitory effect that results in a smaller thymus and a reduction in DP cells due to decreased proliferation of the DN4 population (48).

DP to SP stages

The process of CD4/CD8 lineage choice involves a series of transcription factors—ThPOK (*Zbtb7b*), TOX, and

GATA3—important for the CD4 lineage choice, and RUNX3 is required for the development of the CD8 lineage (13). The role of Notch signaling in the CD4/CD8 lineage decision is controversial, with Cre-mediated deletion of Notch1 under the CD4 promoter not affecting CD4 or CD8 SP T cell development (49), in contrast to a previous report (50) that suggested a role for Notch signaling. Recent data showed that the Notch pathway and TCR signaling may work together to influence positive selection and CD4/CD8 T cell development, as evidenced in mice conditionally deleted for Presenilin1/2 (51) and with in vitro–generated CD8 T cells (52). A decrease in CD8 SP cells developing from MHC class I–restricted TCR Tg^+ DP cells was observed when cocultured in the presence of the Notch-signaling inhibitor (52), indicating that Notch signaling is required for the positive selection of CD8 SP cells.

The thymic microenvironment

Reciprocal interactions between thymocytes and thymic epithelial cells (TECs) are essential for the development of T cells and the maturation of the thymic epithelium itself. The thymus is organized into distinct cortical and medullary regions, and the development and differentiation of the TECs depend on the transcription factor FoxN1 (53). Several chemokines, cytokines, and ligands are produced or expressed by the thymic epithelium, which interact with the developing progenitors. Below we describe some recent advances in this field and concentrate on Notch–Dll interactions.

Recruitment and homing of TSPs to the thymus involve adhesion molecules, such as P-selectin, which signals to PSGL1 (54), and chemokines, such as CCL25 and CXCL12, and their receptors, CCR9 and CXCR4, respectively, which have been implicated in the migration of progenitors from the corticomedullary junction to the outer cortex (55, 56). Mice deficient in CCL25 or CCR9, exhibit normal T cell development (55, 57, 58), whereas mice deficient in CXCR4 or CXCL12 have a reduced number of DP cells (59). CXCR4, along with the pre-TCR, functions to mediate survival and proliferation at the β-selection transition (60). Positive selection of DP cells mediates downregulation of CXCR4 (61) and upregulation of CCR7 (62), which is required for SP cell entry into the medulla (63–65).

Adult mice lacking both CCR7 and CCR9 have a largely normal thymus but exhibit a decrease in the proportion of ETPs, suggesting an additional role for CCR7 in settling of progenitors in the thymus (66, 67). Thymocyte development is not completely abolished in $CCR9^{-/-}$ $CXCR4^{-/-}$ mice, but it is severely reduced in combination with CCR7 deficiency because of the lack of chemotactic effects regulating the distribution of progenitor cells in and around the thymic anlage (68). When CXCL12, CCL25, stem cell factor (cKit ligand), and Dll4 are reintroduced into FoxN1-deficient thymic epithelium, there is an accumulation of $CD45^+$ T-lineage cells in and around the thymic anlage. Surprisingly, only CXCL12 and Dll4 are required for the production of DP cells (69), in keeping with what was shown using simple in vitro systems (70); where CXCR4:CXCL12 (71), together with Notch signals (41, 42), were required for DN3 cells to transit through the β-selection checkpoint to the DP stage.

IL-7 is produced by TECs (72) and is needed to promote proliferation, survival, and differentiation of DN thymocytes (73). Cells are refractory to IL-7 signaling during the preselection DP stage (74) and at the CD4 lineage–commitment stage (75); however, postselected DP cells restore cytokine responsiveness and induce upregulation of IL-7Rα and downregulation of SOCS1 (76). IL-7 signaling is required to express the transcription factor RUNX3, for specifying the CD8 lineage choice (75), and, in combination with IL-15, for the development of CD8 cells. Mice conditionally deleted for IL-7Rα in preselected DP cells fail to induce a RUNX3-dependent cytotoxic lineage gene-expression profile (77). IL-7Rα is also needed for the development and proliferation of Tregs, as well as for proliferation and maturation of innate NKT cells (78).

Hh-signaling pathway

The role of Hh signaling during thymocyte development remains controversial. Data for the differentiation, proliferation, and survival of the earliest DN thymocyte subsets, the transition from DN to DP, as a negative regulator of pre-TCR–induced thymocyte expansion (79–83), the transition from DP to SP, for TCR repertoire selection, at the CD4/CD8 lineage decision (84, 85), and negative selection (83) all support a role for Hh signaling in T cell development. In contrast, other work showed that the Hh signal transducer Gli1 is not needed to mediate the effects of Hh signaling at early stages of thymocyte differentiation (86), and gain- and loss-of-function genetic models of Smoothened show that it is not required for adult BM HSC self-renewal or differentiation or for thymocyte development (87). Although previous results for Ptc deletion in hematopoietic cells suggested a role in T cell development (82), subsequent experiments showed that it is dispensable for T cell development (88); the previously observed developmental block, resulting in a reduced number of ETPs (82), could be due to a defect in thymic-homing progenitors (88).

Wnt-signaling pathway

Like Hh, the role of Wnt signaling in thymocyte development is controversial. Wnt signaling is important for proliferation and survival (89, 90). Pre-TCR signals are important in inducing β-catenin/TCF activity for β-selection (91, 92), and β-catenin can be induced in response to αβ-TCR signaling and can affect DP thymocyte selection and survival (93–95). Surprisingly, conditional deletion of β-catenin does not perturb the hematopoietic compartment or reduce thymocyte development (96), and this was not due to redundancy with γ-catenin (97). The effects observed in $TCF1^{-/-}$ mice (98, 99) point to it acting through a Wnt-independent pathway.

Notch and Notch ligands in T cell development

Although developing thymocytes express Notch receptors (100), recent work found Notch3 to be dispensable for T cell development (101, 102), indicating that Notch1 is the key player during T cell development.

In vivo, Dll1, Dll4, Jag1, and Jag2 are expressed in the embryonic and adult thymus (103). FoxN1 seems to regulate the expression of Dll1, Dll4, and Jag2 expression in TECs (104). Conditional deletion of Dll4 in TECs revealed that it is the relevant physiological ligand for T cell development, because these mice were unable to support T cell development, in contrast with conditional deletion of Dll1, which did not

abrogate T cell development (105–107). A recent report suggested a cell-autonomous role for Dll3 in the thymus. Dll3 is expressed in DN and SP thymocytes, with highest expression in the DN3 subset, and Dll3$^{-/-}$ mice exhibited an increase in *Hes5* transcription at the DP stage (108). αβ and γδ T cell development can be supported by overexpression of Jag2, Dll1, and Dll4 in vitro (70, 109, 110), but Dll4 is uniquely better suited to support T cell development in vitro compared with Dll1 when ectopically expressed in OP9 cells at levels similar to those seen in TECs (103).

Notch ligand endocytosis

Endocytosis of the Notch ligands is thought to be important for initiating cleavage of the receptor to release the intracellular domain of Notch, which is necessary for the induction of target gene transcription (100) and could explain some of the above differences. Two models have been proposed to explain the purpose of Notch ligand endocytosis. The Recycling Model suggests that Notch ligands, present in an "inactive" state on the signal-sending cell, undergo endocytosis and unknown posttranslational modifications to become "activated" and are recycled back to the cell surface and are better able to interact with Notch receptors (111). The Mechanotransduction Model proposes that, upon ligand–receptor interactions, the "pulling" force generated by endocytosis of the Notch ligand into the signal-sending cell exposes the ADAM cleavage site and allows for Notch receptor cleavage and activation in the signal-receiving cell. The extracellular portion of the Notch receptor is "transendocytosed" into the signal-sending cell (112).

Notch ligand endocytosis and T cell development

The intracellular domain (ICD) of the Notch ligands is the target for the E3 ubiquitin ligases Mindbomb (Mib) and Neuralized, which induce their endocytosis and subsequent activation of the Notch-signaling pathway (113). Through the generation of constructs that modified or deleted the ICD of Dll4 and Dll1, it was shown that this domain is necessary for efficient development of T cells in vitro and highlighted some unique features of Dll4 function (114). Absence of the ICD caused a reduction in T cell development and Notch target gene induction, presumably as a result of reduced binding of Notch1-Fc, and it decreased internalization/recycling of Dll4 (114). Furthermore, when Dll1 is mutated at all of its potential lysine residues (Dll1K17) and overexpressed in OP9 cells, it is unable to support T cell development to the DP stage or *trans*-endocytose the Notch extracellular domain (115), indicating that ubiquitination is a requirement for ligand endocytosis and Notch activation for progenitors to adopt the T cell lineage. We (114) and other investigators (116) showed that Dll1 and Dll4 have strong Mib1 interactions; although all E3 ubiquitin ligases (Mib1, Mib2, Neur1, and Neur2) are expressed in both the stromal and hematopoietic compartments, only Mib1 is necessary for T cell development and, therefore, is the relevant physiological E3 ubiquitin ligase in the thymus (114, 117). Conditional deletion of Mib1 led to the abrogation of T cell development in these mice, and knockdown of Mib1 in OP9-DL1 cells caused a block in development at the DN1 stage, indicating that Mib1 can interact with Dll1 (117). Because Dll4 is the physiological ligand, and Mib1 seems to be the functional E3 ubiquitin ligase in the thymus, it would be interesting to investigate the role of Mib1 on Dll4; presumably, Mib1 interacts with and ubiquitinates Dll4 in vivo, but this remains to be investigated.

Conclusions

A critical step in thymocyte development is specification and commitment to the T cell lineage. Recent advances in trying to identify a master regulator of thymocyte development revealed that no one factor is responsible; rather, it is their interplay and cooperative actions that specify the T cell fate, and proliferative and survival signals from other molecules, provided by the thymic epithelium or the developing thymocytes themselves, support and aid in their continued development. Notch–Dll signaling remains critical in instructing cells to adopt the T cell lineage at the expense of other lineages and induces the expression of many key regulators of T cell development. In addition, recent evidence has shed some light on how activation of the Notch-signaling pathway via its ligands can also influence T cell development.

Disclosures

The authors have no financial conflicts of interest.

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