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The Thymus Chapter in the Life of Gut-Specific Intra Epithelial Lymphocytes

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Summary

The intestinal intraepithelial lymphocytes (IEL) represent multi-lineage T cell populations. In addition to a major γδTCR$^+$ T cell subset, many IEL express αβTCRs and they can be separated into αβ sub-lineages. Some TCRαβ$^+$IEL have characteristics in common with conventional TCRαβ$^+$T cells whereas others share an unconventional phenotype with their TCRγδ$^+$ counterparts. Because the latter are enriched for autoreactive TCRs and can be generated in the absence of a thymus, it has long been postulated that some IEL subsets develop locally in the intestine. Several new data however, indicate that under physiological conditions, IEL require a thymic education that directs lineage commitment and functional differentiation. This review will discuss the contributions of the thymus in shaping the various intestinal IEL sub-lineages.

Introduction

The intestinal epithelium is colonized by IEL that are numerous and diverse. Based on their phenotype and developmental origin, the IEL have been classified into two distinct groups [1]. One group consists of CD4$^+$ or CD8αβ$^+$TCRαβ$^+$IEL, which are thought to have followed conventional thymic selection and reached the gut after antigenic stimulation in the periphery [2] (Figure 1). The other group of IEL encompasses gut resident T cells that either express a γδ- or αβTCR and that have adapted an antigen-experienced phenotype during their initial education in response to self-antigens [1,2] (Figure 1). IEL in the latter group do not express a TCR co-receptor but typically express homodimers of CD8α (CD8αα), which has been used as a hallmark to identify and distinguish these IEL from the conventional subset [3]. The frequent association of CD8αα expression with the gut microenvironment, led to the belief that CD8αα marks those IEL that developed locally within the intestine [3]. The identification of cryptopatches (CPs) which harbor ckit$^+$, IL-7Rα$^+$, Thy1$^+$, CD44$^+$ and lin$^-$ lymphoid precursor cells that under experimental conditions can differentiate to CD8αα$^+$TCRγδ$^+$ and TCRαβ$^+$ T cells, further fueled the idea of extrathymic development for these cells [4-6]. Nevertheless, the appearance of CD8αα$^+$TCRγδ$^+$ and TCRαβ$^+$IEL well before the initial development of CPs [4], together with the absence of CPs in any other organism other than mice, render the CPs as the exclusive differentiation niche for CD8αα$^+$IEL unlikely. The most compelling evidence, that questions the intestine as the primary lymphoid tissue for CD8αα$^+$ IEL, is the fact that congenitally athymic mice have much reduced γδTCR IEL and almost no αβTCR IEL [7,8]. Although the number of IEL in athymic mice does increase with age, it still remains far less

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than one would expect if CD8αα$^+$ IEL developed extrathymically. Collectively these observations indicate that CD8αα expression is not consistent with extrathymic development and that the thymus serves an indispensable role in the differentiation of CD8αα$^+$IEL. CD8αα on T cells is not a typical lineage marker but correlates more with an activated phenotype [9]. Even though CD8αα shares the same CD8α chain with CD8αβ TCR co-receptor, which marks MHC class I restricted T cells, CD8αα does not function as a stable co-receptor but rather negatively regulates TCR activation independently of MHC restriction [9,10]. Furthermore, CD8αα can also be induced on CD4$^+$ or CD8αβ$^+$TCRαβ$^+$T cells upon antigenic stimulation or as part of their adaptation to the gut environment [2,11,12] indicating that CD8αα cannot be used as a faithful lineage determinant. Therefore, we will refer in this review to the CD8αα$^+$TCRαβ$^+$IEL as the CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL instead.

DN seeding from the thymus to the gut

Recent evidence suggested that the thymus may provide a critical source of immature T-committed precursors that egress the thymus before rearrangement of the *Tcr* genes and complete their further sublineage differentiation locally under the specific conditions of the gut microenvironment [13••]. Although this is a reasonable hypothesis that ties together the essential role of the thymus with the lymphopoietic capacity of the gut, it also raises new ambiguity. T cell differentiation in the thymus is a complex process that involves multiple checkpoints that direct T versus B, γδ versus αβ and among αβ progenitors, CD4 versus CD8 commitment in addition to conventional or specialized T cells differentiation [14]. It is not known whether under normal physiological conditions, undecided thymocytes can leave the thymus prematurely without transitioning through these different checkpoints. Furthermore, several developmentally more advanced and already lineage committed thymocytes have been identified that are direct progenitors of TCRγδ$^+$ and TCRαβ$^+$IEL subsets [15••,16] and the observation that immature TCRαβ thymocytes may act like LTi cells that can influence the gene expression profile of γδ thymic progenitors [16], all suggest that the thymic chapter for the development of IEL is likely to involve multiple stages and checkpoints.

γδ/αβIEL lineage checkpoints in the thymus

All T lineages develop from multipotent double negative (DN) (that express neither CD4 nor CD8), precursors. In the murine thymus these DN progenitors can be further subdivided into four maturation stages (DN1 to DN4) determined by the variable expression of CD44 and CD25 [17] (Figure 1). The γδ/αβ lineage decision occurs at these DN stages but the exact time point is debatable [18,19]. Furthermore, γδ T cell progenitors may not follow the same DN1 to DN4 pathway as their αβ counterparts and they basically branch off before *Tcra* gene recombination. Initial rearrangements of the TCRγ, TCRδ and TCRβ loci can be detected at the DN2 stage and are completed during the DN3 stage [20]. DN precursors that express a full γδTCR can commit to the γδ lineage, whereas DN3 cells that rearrange a functional TCRβ and form a pre-TCR together with the invariant pre-Tα chain and members of the CD3 complex, further mature along the αβ lineage [19] (Figure 1). Although successful *Tcr* gene rearrangements undoubtedly impact the eventual αβ/γδ-lineage commitment, several data indicate that DN2 cells may display a biased potential already before *Tcr* rearrangements [21]. Specific transcription factors might pre-set the stage and in that aspect various WNT signaling events have been identified that critically regulate expression of genes involved in lineage diversification. The WNT induced β-catenin controlled T cell factor-1 (TCF-1) and lymphoid enhancer factor-1 (LEF-1) were shown to play important albeit partially redundant roles in controlling development of αβ-lineage progenitors [22,23] (Figure 1). Nevertheless, in addition to TCRαβ$^+$T cells also TCRγδ$^+$IEL but not TCRγδ$^+$splenocytes, were drastically reduced in *Tcf1*$^{-/-}$ mice pointing at a unique role for TCF-1 in the generation and/or maintenance of gut-specific TCRγδ$^+$T cells [24]. T cell differentiation is also critically

dependent on signals transduced through cytokine receptors belonging to the family of common γ-chain receptors. Especially IL-7R plays a non-redundant role during the initial survival and expansion of bipotent T cell progenitors [25] (Figure 1). Although differential expression of the IL-7R at the DN2 stage can be used to identify lineage bias [21], and although IL-7R signals promote TCRγ rearrangements [26], there is no direct evidence suggesting that IL7R signals play a decisive role in the TCRαβ/γδ lineage commitment. However, continuous IL7R signaling in IL-7Rα transgenic thymocytes does suppress the expression of the transcription factors TCF-1/LEF-1 and retinoic acid orphan nuclear receptors RORγ/RORγt, and prevents survival and further differentiation of TCRαβ progenitor cells [27] (Figure 1). In contrast transgene-driven local expression of IL-7 in the intestine of IL-7 deficient mice restored the TCRγδ$^+$ IEL [28], suggesting that this IL-7 transgene-driven differentiation of progenitors in the gut might be different from the TCF-1 dependent TCRγδ$^+$ IEL development.

Notch-ligand interactions have decisive roles for the initial T lineage commitment [29] and *Notch-1*$^{-/-}$ BM cells accumulate as immature B cells in the thymus of BM chimeric mice [30]. In sharp contrast however *Notch-1*$^{-/-}$ BM derived progenitor cells in the CPs of these animals remain arrested at the CD117$^+$lin$^-$ stage, suggesting that either the intestine does not support development of immature B cells or alternatively that immature DN *Notch*$^{-/-}$ thymocytes do not migrate to the gut prematurely [30].

The defined lineage split of γδ versus αβ at the level of TCRγδ or pre-TCR expression respectively, has focused on TCR signal strength and duration for the instruction or confirmation of pre-determined lineage fate [31,32]. New evidence has indicated that in addition to phenotypic changes the quality of signals received through a full TCR versus a pre-TCR, has important implications for survival, migration and functional differentiation of thymocytes [33•, 34•, 35,36]. The fact that in TCRγ transgenic mice few αβ TCR T cells develop [37] and in TCRβ transgenic mice most TCRγδ development is suppressed [38], indicate that signals generated by a full TCR or a pre-TCR are crucial elements in the αβ/γδ lineage choice. Several observations support the notion that strong signals from engaged full γδ- or αβTCRs expressed at the immature DN stages favor the γδ lineage, whereas reduced signaling through a γδTCR redirects development along the αβ lineage [33•, 34•]. The γδTCR-driven αβ lineage differentiation is insufficient and together with the constant association of pre-TCR with αβ lineage choice, they point to unique qualities of the pre-TCR for specific αβ lineage differentiation. Recently it was shown that some DN thymocytes express a full αβTCR [39]. It is possible that early expression of αβTCRs before the pre-TCR, may mimic γδTCR ligation signals rather than pre-TCR signaling and drive the precursor cells into γδ-like IEL [40]. However, the fact that non-transgenic CD8αα$^+$TCRαβ$^+$IEL strictly dependent on pre-TCR expression, indicate that early TCRαβ$^+$ precursors are not major progenitor cells for CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL and that these TCRαβ$^+$IEL are not part of the γδ lineage but represent genuine pre-TCR dependent αβ lineage cells.

γδTCR IEL precursor checkpoints in the thymus

The TCR recombination system creates the potential to generate a repertoire of almost unlimited diversity. Despite this, TCRγδ$^+$T cells display a very restricted repertoire that is tissue specific and strongly correlates with particular variable gene segments of the TCRγ (Vγ) [41]. Furthermore, various γδ subsets develop with different time kinetics that are in part programmed by stem cell subpopulations [42]. In mice, TCRγδ$^+$IEL populate the intestine around birth and sometime thereafter and they almost all express Vγ5* [43*, 44]. Because Vγ5 is rarely expressed by DN4 thymocytes and productive *Vg5* gene segments are underrepresented at the DN3 stage in C57BL/6 mice, it suggests that TCRγδ$^+$IEL precursors might exit the thymus on or before the DN3 stage [45]. Although genetically programming is involved in *γδ* gene rearrangements, selective TCRγδ-associated signals may control in part

the migration ability of the TCRγδ$^+$thymocytes. For example, G8-TCRγδ$^+$thymocytes specific for the MHC class Ib molecules, T10^b/T22^b, differentiate in the presence of their cognate antigen and readily migrate to the gut but not elsewhere [46]. In that aspect it was shown that unlike the conventional selected TCRαβ precursors, functional S1P1 was not required for thymic egress of γδ-selected progenitors of small intestine TCRγδ$^+$IEL [47••]. The existence of γδ-selection was also demonstrated using GFP-*Tcrd* reporter mice [48••], which indicated that DN3 thymocytes require a full γδTCR together with CD3ε and functional linker for activation of T cells (LAT) for their differentiation to mature γδTCR$^+$ T cells. A gene expression analysis of TCRγδ$^+$IEL identified a γδ-biased profile that distinguishes them from conventional selected TCRαβ$^+$T cells [49]. Interestingly, rather than typical for TCRγδ expressing IEL this profile was also shared in part by the CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL [49,50•, 51•], which also require agonistic selection [35] and which also egress from the thymus in a S1P1 independent manner [47].

The specific loss of TCRVγ5$^+$IEL in mice with defects in IL-15R mediated signals [52] and the increase of Vγ5$^+$IEL in IL-15 transgenic mice [53•], suggest a central role for IL-15 in the generation and/or maintenance of TCRγδ$^+$IEL. In support of this, enforced expression of Bcl-2 partially restored the numbers of Vγ5$^+$IEL in IL-15 deficient mice [54]. Nevertheless, Bcl-2-rescued Vγ5$^+$IEL did not display normal effector's functions indicating that IL-15 may also serve important roles for the functional differentiation of Vγ5$^+$IEL. In addition, the selective reduction of Vγ5$^+$thymocytes in *IL-15*$^{-/-}$ or IL-15 signal transducer *stat5*$^{-/-}$ newborn mice [53•], indicated that IL-15 specifically influences development and/or survival of Vγ5$^+$thymocyte precursors. Consistent with this, it was shown that, prior to *Tcrg* rearrangement, IL-15 is able to direct biased *Vg5* transcription by specifically regulating chromatin domain modifications and accessibility in the exclusive vicinity of the *Vg5* gene [53•]. Nevertheless, the absence of Vγ5 transcripts among *IL-7r*$^{-/-}$ thymocytes whilst they were detectable among *IL-7r*$^{-/-}$ precursors in the gut [53•], suggests that *Vg5* rearrangements controlled by IL-15 are not a major mechanism during thymic differentiation but it might operate in the small intestine to allow for specific Vγ5 differentiation when thymic selected IEL are limiting.

Thymic checkpoints for TCRαβ$^+$ IEL precursors

Whereas co-receptor expressing TCRαβ$^+$IEL mainly differentiate from conventional positively selected CD4$^+$ and CD8αβ$^+$ T cells that have encountered a cognate non-self antigen in the periphery [1,2], CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL have acquired their antigen experienced phenotype during agonist selection in the thymus [35,55] (Figure 1). In contrast however to TCRγδ$^+$ thymocytes, which commit to the γδ lineage upon ligation of their mature γδTCR, αβ progenitors pass through additional checkpoints that involve a pre-TCR stage followed by a full αβTCR-based selection process (Figure 1). Although CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL display phenotypic and functional similarities with the γδ subset, this is likely not due to a shared lineage commitment but rather it coincides with the full TCR-based agonist selection process in the thymus. Some DN thymocytes can commit to the γδ-lineage upon expression/ligation of a full αβTCR, as is the case in male H-YTCR transgenic mice [40,56], however the nearly absence of CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL in *pre-Tcr*$^{-/-}$ animals [57,58], makes it unlikely that this pre-TCR independent pathway is contributing significantly to the CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL sublineage in normal mice. Consistent with this, H-YTCR transgenic mice in which the transgenic TCR was driven by the CD4 promoter, do not generate DN H-YTCRαβ$^+$ thymocytes and male mice do not generate significant numbers of CD4$^-$CD8αβ$^-$H-YTCR$^+$IEL [59•]. Furthermore, the functional capacity of the transgenic CD4$^-$CD8αβ$^-$H-YTCRαβ$^+$IEL are distinct from their natural occurring counterparts and suggests that true CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL may depend on a pre-TCR pathway for their lineage and functional differentiation.

Pre-TCR also promotes the transition of the DN4 to the double positive CD4CD8αβ$^+$ (DP) stage together with a bust of proliferation (Figure 1). At the DNA level productive pre-TCR signals coincide with β-selection and cessation of further TCRβ rearrangements together with initiation of TCRα rearrangements. Upon expression of a full TCR, DP thymocytes pass through a second checkpoint where αβTCR-based selection events eliminate self-reactive TCRs from the conventional repertoire and allow for functional differentiation of positively selected thymocytes. The observation that the TCR repertoire of CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL is greatly enriched for self-reactive TCRs is inconsistent with negative selection of CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL precursor cells in the thymus. Instead, the enhanced accumulation of CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL in the presence of cognate antigen suggests the existence of an alternative pathway for thymic differentiation that operates in parallel with negative selection and that preserves and differentiates some TCRαβ$^+$thymocytes with high affinity for self-antigens [35]. In support of this, when DP thymocytes from H-YTCR female mice were exposed to H-Y antigen *in vitro*, a large number of them survived and differentiated to CD8αα$^+$TCRαβ$^+$T cells with the typical gene transcription signature of CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL [36]. This indicated that precursors of CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL might be positively selected by self-agonist at the DP stage. Direct evidence for this was provided by genetic cell-fate mapping using expression of green fluorescent protein (GFP) driven by the CD4 promoter, which confirmed that CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL thymic progenitors transition through the DP checkpoint [60]. These observations imply that DP thymocytes might be heterogenic and contain precursors for conventional T cells as well as CD4$^-$CD8αβ-TCRαβ$^+$IEL. We recently identified a new subset of DP thymocytes that co-express CD8αα [15••]. Using TCR transgenic cells, we further demonstrated that, in contrast to DP thymocytes, CD8αα expressing, triple positive (TP) thymocytes, survived, expanded and adapted to the CD4$^-$CD8αβ-TCRαβ$^+$IEL phenotype in response to *in vitro* stimulation with their cognate antigen [15••]. Intrathymic injection of TP but not DP thymocytes generated a significant population of CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL *in vivo*, indicating that TP thymocytes are indeed immature thymic precursors of CD4$^-$CD8αβ-TCRαβ$^+$IEL (Figure 1). Consistent with this, thymocytes that survived agonist selection conditions *in vitro* acquired an antigen experienced phenotype as well as innate-like features and gut-specific homing receptors [36, 51•]. A gene array analysis of *ex vivo* CD8αα$^+$TCRαβ$^+$IEL indicated an almost identical transcription signature [50•] and further underscores the importance of the thymic agonist selection process as a central mechanism for the unique differentiation and migration of CD4$^-$CD8αβ-TCRαβ$^+$IEL.

The initial induction of CD8αα is consistent with a pre-TCR signal since it does not depend on a full αβTCR or MHC ligation [15••]. Not all pre-TCR triggered thymocytes, however, induce CD8αα indicating that diversity in thymocyte precursors may in part be determined at the pre-TCR level before full αβTCR expression. It is not known how TP thymocytes survive agonist selection conditions but it is likely that CD8αα may function as a repressor to reduce the signal strength and allow for survival [9].

We identified mature TCRαβ$^+$DN thymocytes as post agonist selected precursors and showed that these cells differentiate to CD8αα$^+$CD5$^-$TCRαβ$^+$IEL *in vivo* without the need for further checkpoints in the thymus [15••]. The co-receptor negative phenotype of agonist selected thymocytes is consistent with the accumulation of co-receptor independent high affinity TCRs among CD4$^-$CD8αβ$^-$TCRαβ$^+$IEL. The acquisition of CD8αα and the downregulation of CD5 are post thymic events that occur locally in the intestine and that are actively promoted by IL-15 [15••].

Conclusions

T cells do not absolutely require a thymus for their development and many tissues with lymphoietic capacity, including BM, spleen, LNs and without a doubt, the intestinal epithelium can effectively support T cell poiesis. The thymus however has evolved as a specific T cell organ equipped with a three dimensional network of stromal and epithelial cells that interact closely with the developing thymocytes. It is this exclusive microenvironment that provides the unique combination of cellular interactions together with cytokines and chemokines that guide thymocytes through defined TCR-dependent “self”-based selection processes that lead to the functional differentiation of self- and nonself-reactive T cells. The gene profile that is uniquely shared by agonist-selected γδ- and αβ-lineage IEL as opposed to their conventional selected counterparts, importantly underscores that the functional diversity and homing capacity of the various IEL subsets is directly linked to the specific selection process of their thymic precursor cells.

This however does not exclude lymphopoiesis in the gut and it is possible that RORγt$^+$ lymphoid tissue inducer (LTi)-like cells may transform CPs to lymphoid follicles [61] that together with cytokines such as IL-15, promote a transient burst of non-selective T cell poiesis in the gut in response to challenges imposed by infections or local tissue damage.

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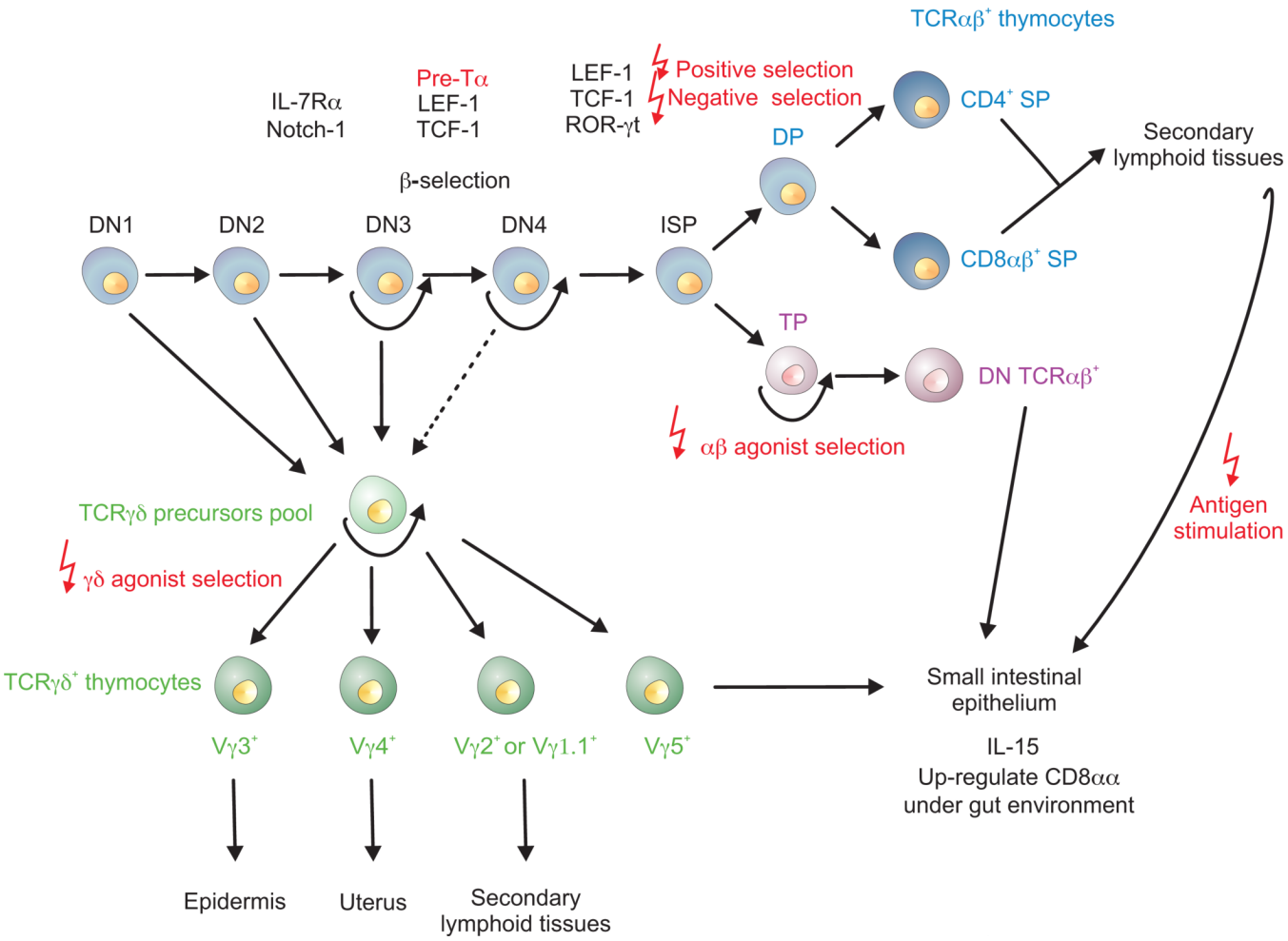

Figure 1. Differentiation pathways and checkpoints of TCRαβ and TcRγδ T cells

DN thymocytes are subdivided in 4 populations (DN1 to DN4) that contain precursors belonging to the αβ- and γδ-lineage. DN TcRγδ precursors transition through agonist TCRγδ selection and give rise to waves of TCRγδ subsets with different homing capacity. Agonist selected TCRγδ IEL precursors migrate to the gut epithelium independently of functional S1P1. At the DN3 stage, precursors that successfully rearranged the β-chain will undergo pre-TCR driven β-selection and transition to the DP ($CD4^+CD8\alpha\beta^+$) or TP ($CD4^+CD8\alpha\beta^+CD8\alpha\alpha^+$) stage. The DP population contains αβ lineage committed precursors that will undergo positive and negative conventional selection and commit to the CD4- or CD8αβ-SP sublineage. Positive selected SP thymocytes egress the thymus in a S1P1-dependent fashion and reside in the periphery as naïve T cells. Antigenic stimulation further differentiate these cells to effector cells that gain the capacity to migrate to the intestinal epithelium. TP thymocytes can proceed through agonist selection and give rise to mature DN TCRαβ thymocytes that home to the intestinal epithelium in a S1P1-independent way. Molecules that have important roles during T cell differentiation were added. Solid black arrows show established pathways whereas dashed arrows indicate uncertain transitions.