Development of γδ T cells in the thymus – a human perspective

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Abstract

γδ T cells are increasingly emerging as crucial immune regulators that can take on innate and adaptive roles in the defence against pathogens. Although they arise within the thymus from the same hematopoietic precursors as conventional αβ T cells, the development of γδ T cells is less well understood. In this review, we focus on summarising the current state of knowledge about the cellular and molecular processes involved in the generation of γδ T cells in human.

Keywords

γδ T cells, T cell development, lineage commitment, human, thymocyte, thymus

1 Introduction

Like other unconventional T cells, γδ T cells seem to combine both innate and adaptive characteristics in their phenotype and behaviour. The γδ T cell receptor (TCR) is generated through somatic gene rearrangements and exhibits junctional diversity similar to the αβ TCR; however, the overall γδ TCR repertoire is restricted and its functionality appears to resemble that of a pattern recognition receptor [1]. Moreover, γδ T cells display rapid responses to pathogen encounters without the need for extensive proliferation and effector differentiation. In contrast to conventional αβ T cells, γδ T cells can recognise unprocessed peptide antigens but also a range of different non-peptide antigens such as lipids and phosphoantigens from exogenous and endogenous sources [2,3]. Their ability to respond to tumour-derived antigens and their cytolytic capacities, in combination with their lack of HLA restriction, has made them attractive tools for cancer immunotherapy [4–6]. Most clinical studies to date have focussed on *ex vivo* expansion or *in vivo* activation of mature γδ T cells [4,7] but these approaches come with several limitations, such as T cell exhaustion. Instead, *de novo* generation of γδ T cells, for instance from induced pluripotent stem cells (iPSCs), holds great promise to overcome these issues and may be suited to address the need for off-the-shelf therapies [8–10]. To further develop these technologies, a detailed understanding of human γδ T cell differentiation and maturation is crucial. Many exceptional reviews on the development of γδ T cells in the mouse thymus have been published [11–20], however, insights into this process in the human context are still more limited. This gap is not surprising given the experimental limitations in studying human samples and the elegant ways in which mouse models and perturbation approaches have been able to advance the field. Nevertheless, there is ample evidence of prominent differences between human and mouse γδ T cell development (Table 1), which has direct and critical consequences for translational work. For this reason, we use this review to specifically discuss the current state of knowledge with regard to the establishment of γδ T cells in the human thymus.

2 Early development of bipotent T cell progenitors in the thymus

The regulatory intricacies of early T cell development, during which thymus immigrating multipotent precursors undergo sequential T lineage specification and commitment, apply to all conventional T cells as well as the unconventional T cell subsets discussed in this issue and have been reviewed extensively in mouse [21–27] and human [28–31]. Nevertheless, we will provide a brief overview of the earliest stages of T cell development in the human thymus.

2.1 Commitment to the T lineage

Although their differentiation and maturation takes place in the thymus, all T cells develop from hematopoietic stem and progenitor cells (HSPCs) that originate from the bone marrow or fetal liver. These HSPCs migrate to and colonise the thymus as multipotent thymus seeding progenitors (TSPs), that then rely on the thymic microenvironment to direct their complex and gradual differentiation into a broad range of different T cell subsets (Figure 1).

Human HSPCs and TSPs not only possess T lineage potential but can also develop into Natural Killer (NK) cells and Dendritic Cells (DC), as well as other lineages under permissive culture conditions *in vitro* [32–36]. As a result of their engagement with Notch ligands expressed on thymic epithelial cells (TECs), TSPs experience Notch signalling, which leads to progression to the early T cell precursor (ETP) stage and upregulation of genes with essential roles in T lineage development, such as *GATA3* [37] and IL7 receptor (*IL7R*) [38,39]. ETPs already exhibit a reduced potential to develop into other hematopoietic lineages [39–41] and the transcription factors BCL11B and GATA3 subsequently further extinguish non-T lineage potential by suppressing alternative cell fates and inducing the T lineage transcriptional programme [34,42] (Figure 2A). Upregulation of CD1 on the cell surface marks the irreversible commitment of thymocytes to the T lineage, although loss of CD44 has recently been identified as a more precise marker of this developmental milestone [43]. Induction of IL7R signalling via TEC-derived IL7 is then thought to promote proliferation and survival of T lineage cells [39,44] and thereby represents another essential driver of early T cell development as shown by the prominent lack of T cells that IL7R-deficient patients present with [45]. Following T lineage commitment, cells are often referred to as bipotent precursors to denote their ability to differentiate into αβ and γδ lineage T cells but lack of non-T lineage potential.

2.2 Rearrangement of the T cell receptor loci

Generation of a diverse TCR repertoire is achieved through V(D)J recombination of the four TCR loci, which takes place in the CD34+ stages for *TRB*, *TRG* and *TRD*, and later in the double positive (DP, CD4+CD8+) stage for *TRA* (Figure 1). Immature thymocytes that successfully rearrange their *TRG* and *TRD* loci and are thus able to assemble a functional γδ TCR, can commit to the γδ lineage, whereas an in-frame rearrangement of the β-chain allows the generation of a pre-TCR, which drives cells through β-selection and towards the αβ lineage. Rearrangement of the TCR loci is initiated already prior to T lineage commitment since expression of RAG and initial *TRD* rearrangements have been observed at the CD34+CD1- stage [32,46–48]. Importantly, the rearrangements do not preclude alternative fates as these cells can still be directed towards myeloid and erythroid lineages *in vitro* [32]. Rearrangement of the *TRG* and *TRB* loci is only fully initiated after T lineage commitment [43,46–48], whereby in-frame *TRB* rearrangements seem to arise mainly at the immature single positive (ISP) stage [46]. In fact, several studies point towards a late onset of *TRB* recombination: Rearranged *TRG* loci were observed in the majority of peripheral αβ T cells and αβ lineage-committed thymocytes, whereas in most γδ lineage T cells and thymocytes the *TRB* locus was still in germline configuration [49–51]. Moreover, rearrangement of both *TRG* alleles was detected in a large proportion of γδ T cells [51], which indicates that V(D)J recombination is initiated and potentially also completed much earlier at the *TRG* compared to the *TRB* locus. This implies sequential albeit overlapping rearrangement windows in the order *TRD-TRG-TRB* in human thymocytes.

The human *TRG* locus comprises seven functional *TRGV* elements and two *TRGJ* arrays which are each associated with one *TRGC* element (Figure 3). During fetal development cells predominantly rearrange the central *TRGV* elements, which are recombined with the central J array and paired with *TRGC1*, whereas postnatal thymocytes mainly use distal *TRGV* and

TRGJ segments in combination with *TRGC2* [52,53]. The *TRD* locus contains eight *TRDV* segments, of which *TRDV4-8* also have *TRA* designation due to the nested localisation of *TRD* within the *TRA* locus. In addition, three *TRDD*, four *TRDJ* and a single *TRDC* segment can be utilised during V(D)J recombination. The downstream *TRDV* and *TRDJ* elements are predominantly rearranged in fetal thymocytes and a shift towards more upstream elements occurs later in life [52,54,55] (Figure 3). This differential use of V-segments is reflected in the distinct γδ T cell types that arise during certain developmental windows, for example, Vγ9+Vδ2+ cells represent the main subset generated in early gestation whereas Vδ1+ cells dominate after birth, as discussed below.

In addition, repertoires of embryonic, fetal and postnatal γδ thymocytes differ in their incorporation of nucleotides during V(D)J recombination. This is a consequence of delayed induction of terminal deoxynucleotidyl transferase (TdT, encoded by the *DNTT* gene) in fetal thymocytes at around 20 weeks post conception (wpc) [56], resulting in highly invariant germline-encoded CDR3 sequences in γδ T cells generated during early development [54]. A likely regulator of TdT expression is the RNA-binding protein LIN28B, which is highly expressed in fetal but not postnatal γδ T cells and causes inhibition of TdT, a reduction of N additions and more invariant CDR3 sequences upon overexpression in cord blood-derived HSPCs [54]. Surprisingly, increased LIN28B levels in peri-/postnatal HSPCs also result in preferential usage of *TRDV2* over *TRDV1*, which mimics fetal thymocyte development and indicates a direct impact of LIN28B on the regulation of V(D)J recombination [54]. Moreover, it has been suggested that in absence of TdT, short homology repeats at certain V/D/J segments can drive recombination and thereby promote the generation of specific germline-encoded sequences in fetal γδ thymocytes [54,57]. These findings imply that certain aspects of V(D)J recombination differ by gestational age due to intrinsic properties of the progenitor cells and this has a phenotypic and functional impact on the generated γδ T cells.

2.2.1 Allelic exclusion of the γ- and δ-chain

Allelic exclusion describes the process of achieving monoallelic expression of a gene, which, in the T cell context, critically determines if a cell can express one or multiple different TCRs on the cell surface. Studies investigating the frequencies of in-frame *TRG* and *TRD* rearrangements in γδ T cells from thymus and peripheral blood reported a large proportion of cells with functional rearrangements at both *TRG* alleles [50,53,58,59]. This suggests allelic inclusion for this locus and therefore the possibility that two different γ-chains can be expressed on the same cell, which has indeed been demonstrated in γδ T cell lines [60]. In contrast, although biallelic rearrangements have been observed at the TRD locus, these are less frequent and overwhelmingly appear to represent incomplete or out-of-frame rearrangements [50,58,59], indicating allelic exclusion for the δ-chain. Of note, productive rearrangements at both the *TRD* and the *TRG* locus may not guarantee the successful assembly of a functional γδ TCR due to incompatibility between certain γ- and δ-subunits. This has only been confirmed for murine but not human γδ T cells [61,62], but such a pairing restriction might partially account for the described biallelic in-frame rearrangements. That being said, a small proportion of cells with surface expression of multiple different δ-chains has been observed in cell lines [63], which suggests that allelic exclusion is incomplete and may simply vary in stringency between different loci. Curiously, analyses in mice suggest an

inverse allelic exclusion pattern, with *TRG* but not *TRD* being allelically excluded [64–66]. This bears a resemblance to the general understanding that for human γδ T subsets the δ-chain seems to be the distinguishing feature, whereas murine γδ T subtypes can be grouped by their γ-chain, and might therefore imply a stricter exclusion control for the phenotype-determining TCR subunit.

2.2.2 Regulation of *TRD*/*TRG* rearrangement

The factors and molecular processes that initiate and control V(D)J recombination at the *TRD* and *TRG* loci in human are still largely unknown, but some hints might be found in mouse studies. For instance, the role of IL7R signalling in the regulation of *Trg* rearrangement in murine thymocytes has been well documented. IL7R-deficient mice exhibit defects in γδ T cell development, which seem to be at least partially inflicted by impaired recombination at the *Trg* locus [67–71] since a rearranged *Trg* transgene is able to rescue the phenotype [72]. IL7 signalling has been linked with increased histone acetylation, chromatin accessibility, transcription and rearrangement at the *Trg* locus [70,72–76], which has been attributed to IL7-induced recruitment of STAT5 to the *Trg* enhancer Eγ [73,77] (Figure 2B). Evidence for IL7-mediated *TRG* rearrangement in human thymocytes is more sparse, but it has indeed been demonstrated that IL7R signalling can activate STAT5 [44], which binds to conserved STAT consensus motifs at *TRG* regulatory regions and drives germline transcription at the locus [78]. Moreover, γδ T cells from patients with instability-inducing mutations in *IL7R* exhibit an unusually restricted *TRG* repertoire and reduced activation of STAT5 in response to IL7 stimulation [79], which further supports a role of IL7 in *TRG* rearrangement during human γδ T cell development. Surprisingly, while knock-out experiments in mice targeting various IL7 pathway components, such as *Il7ra*, *Jak3* and *Il2rg*, resulted in disrupted γδ T cell development regardless of age [68,80,81], STAT5 deficiency only perturbed *Trg* rearrangement and γδ T cell differentiation in fetal but not adult mice [76]. This indicates that while IL7R signalling is generally required for *Trg* rearrangement in mouse thymocytes, the downstream mediators may vary throughout development. In contrast to the *Trg* locus, IL7R deficiency in mice does not seem to disrupt the generation of a functional δ-chain [67,68], although a recent study demonstrated IL7/STAT5-dependent induction of germline transcription at the Eδ enhancer [82], which suggests a previously overlooked role of IL7 at this locus. In human, Eδ activity was shown to be induced by IL7 treatment of Jurkat cells [82], suggesting a possible involvement of IL7R signalling activity in *TRD* rearrangement. Furthermore, a xenograft study with HSPCs derived from IL7R-deficient severe combined immunodeficiency (SCID) patients reported that cells were able to mature until the ETP stage but did not initiate *TRD* rearrangements [83]. This could indicate a potential role for IL7 in the recombination of the human *TRD* locus, but the absence of *TRD* rearrangements could also merely be a consequence of the early developmental block and general defects in thymocyte development.

E proteins, in particular HEB and E2A, have been found to play a crucial role in the regulation of V(D)J recombination at the *TRG* and *TRD* locus. In mice, E2A has been implicated in the stage-specific control of *Trg* rearrangement by selectively promoting or repressing the recombination of certain *Trgv* elements during fetal or adult γδ T cell development [84,85]. Similarly, two independent transfection studies demonstrated that E2A

and HEB can induce V(D)J recombination at the human *TRG* and *TRD* loci in non-lymphoid cells [86,87]. The detected rearrangements were limited to certain segments, which seems to confirm that E protein-mediated control of recombination is targeted to specific *TRG/D* regions. Moreover, based on elevated levels of germline transcription upon E protein transfection, it was hypothesised that E2A and HEB regulate accessibility at recombination signal sequence (RSS) sites [87], which supports a local rather than global role of E proteins in the regulation of TCR locus rearrangement. Recent transcription factor footprinting analyses from our lab have predicted a large number of E2A binding sites throughout the *TRD* and *TRG* loci, which were associated with open chromatin and permissive histone marks in CD34+ thymocytes and could thus indicate involvement of the E protein in controlling chromatin state and germline transcription at the loci in developing thymocytes [88] (Figure 2B). Furthermore, using single cell data, we found that *E2A* RNA levels were positively correlated with those of *TRGC2*, which points towards a role of E2A in *TRG* germline transcription or expression of the rearranged γ-chain [88]. Notably, the rearrangements reported from transfection experiments appear to be comparable to those that are normally observed in thymocytes prior to T lineage commitment and only involve D-D and V-D but not D-J recombinations, which implies that E proteins might participate in the initiation but not necessarily progression and completion of *TRG*/*TRD* rearrangement [86,87]. E proteins have also been shown to induce *RAG* expression [89,90], which constitutes an additional mechanism by which this subset of transcription factors can control V(D)J recombination (Figure 2B).

Notch signalling has also been implicated in the control of TCR rearrangement in human thymocytes, as cord blood-derived HSPCs cultured in the presence of the Notch ligand DLL4 showed recombination of certain *TRGV* elements [37]. Mechanistically, it has been reported that Notch signalling can induce the expression of *IL7R* under certain conditions [82,91], which consequently promotes TCR rearrangement as described above. In addition, Notch upregulates the transcription factors MYB and RUNX1 [91], which both bind at regulatory elements at the *TRG* and *TRD* loci [77,92–96] (Figure 2B). RUNX factors have been suggested to act as pioneering factors that are required to promote chromatin accessibility and thus permit recruitment of MYB, GATA3 and STAT5 to the TCR loci [77,95], which subsequently leads to activation of the Eδ and Eγ enhancers and germline transcription [91,92,97–99]. Of note, while RUNX1 appears to be responsible for this pioneering activity in immature thymocytes, its paralog RUNX3 most likely fulfils this role in mature γδ T cells [100,101]. Curiously, in addition to this positive impact of Notch signalling on *TRD* and *TRG* rearrangement, it also seems to exhibit some degree of negative control over the process: Notch activity can restrain E protein function via induction of the inhibiting factor ID3 [102–104] and is further able to initiate degradation of E2A via ERK signalling-mediated phosphorylation [102], which might consequently interfere with TCR locus accessibility and V(D)J recombination. In addition, reduction of Notch signalling leads to increased *RAG* expression [101,105], which implies a potential Notch-induced suppression or termination of TCR rearrangement via downregulation of *RAG* (Figure 2C).

Importantly, the discussed pathways and transcription factors appear to be closely connected, as shown by Notch-mediated induction of *IL7R* expression and RUNX-dependent STAT5 recruitment, as well as the negative control of E protein activity by Notch signalling. This

demonstrates the intricacies of the molecular processes governing the rearrangement of *TRG* and *TRD* loci and illustrates the pitfalls of studying either of the pathways in isolation. Moreover, the previously described roles of Notch and IL7 in thymocyte survival and T lineage commitment complicate their investigation at a later developmental stage and make it difficult to distinguish TCR rearrangement-dependent and -independent effects on maturation and differentiation of γδ T cells progenitors. Hence, further investigation is needed to establish which factors drive recombination of the *TRG* and *TRD* loci and how changes in V, D and J element usage are regulated through human ontogeny.

Rearrangement of the *TRD/TRG* loci is suspended upon commitment to the γδ lineage (Figure 2C). This is triggered by TCR signalling, which blocks E protein activity by inducing the expression of Id3 [106]. In addition, TCR signalling was shown to downregulate Notch targets, including *MYB* and *RUNX1*, and thereby inhibits Eδ activity [91]. Lastly, the upregulation of c-Jun in response to TCR activity seems to suppress *IL7R* expression [107], which is expected to interfere with its control of TCR rearrangement. Hence, the assembly of a functional γδ TCR triggers a negative feedback loop that prevents further rearrangement of the TCR loci.

3 Commitment to the γδ lineage

The exact point at which bipotent progenitors fully commit to the αβ or the γδ lineage has been difficult to determine and may in fact not be associated with a certain developmental stage (see section 3.2). Furthermore, the definition of these two lineages has been somewhat ambiguous. Thus far, no unique surface marker for γδ T cells other than the γδ TCR itself has been identified, which has therefore been used as indicator of γδ fate in most studies. On the other hand, since no TCR is expressed on the surface of early αβ committed cells, the DP stage has often been described as an αβ lineage-specific characteristic. Especially in experimental settings these markers often do not hold up, for instance, premature expression of a transgenic αβ TCR leads to TCRαβ+ cells with a clear γδ lineage phenotype, including effector behaviour and lack of CD4/CD8 expression [108–110]. Similarly, TCRγδ+ cells that develop in the absence of DP thymocytes do not adopt a γδ lineage gene expression programme [111] and TCRγδ-transgenic RAG-deficient mice develop TCRγδ+ DP thymocytes [112,113]. Even in the physiological context γδ TCR expression and DP phenotype do not seem to be mutually exclusive, since human γδ lineage cells appear to be able to differentiate via a transient DP stage [114,115]. Establishment of unambiguous lineage gene expression profiles and identification of additional lineage-specific surface markers are required to resolve this issue, but in the meantime, caution is required in the interpretation of studies that rely on DP vs. TCRγδ+ phenotype as a means of lineage distinction.

3.1 Molecular mechanisms involved in the fate decision

3.1.1 TCR signalling and its impact on lineage commitment

How the TCR plays into the αβ vs. γδ fate decision has long been a matter of debate. Initially, two scenarios were proposed; an instructive model, according to which cell fate is informed by the type of assembled TCR, and a stochastic model, whereby lineage fate is pre-

programmed and only cells with a matching TCR survive. In support of a stochastic principle, some indication of hard-wired γδ cell fate in mice has been presented. For instance, DN thymocytes with high levels of SOX13 or IL7R were found to exhibit a higher potential to develop along the γδ lineage [116–118], although concerns have been voiced that some of these results might be biased by the positive impact of IL7 on *Trg* rearrangement (see section 2.2.2). On the other hand, an instructive fate decision model is currently well supported by several lines of evidence. Curiously, it was shown that the lineage choice is not informed through a qualitative difference in the signals transduced by pre-TCR vs. γδ TCR but rather through differential signal strength. Initial experiments revealed higher Ca2+ mobilisation, mitogen-activated protein kinase (MAPK) signalling, and stronger induction of the transcription factor EGR in γδ compared to αβ lineage cells [113,119,120], suggesting greater signalling activity through the γδ TCR than through the pre-TCR. Two landmark studies from the labs of David Wiest and Paul Love then demonstrated that although under normal conditions the γδ TCR conveys strong signals and this quantitative signal difference constitutes the decisive element, it does not seem to be categorically linked with the TCR type. Instead, dampening of γδ TCR signalling strength by manipulation of ITAMs or Lck deficiency was sufficient to divert cells away from γδ fate and towards the αβ lineage, whereas elevated signal strength achieved through ligand engagement or CD5 deficiency resulted in a reduction of the DP thymocyte fraction in favour of γδ development [113,120]. The signal strength hypothesis was further corroborated by lineage tracing experiments in individual cells, which verified that DN thymocytes can be diverted to the γδ lineage through induction of strong TCR signals, even if the cells have already reached the β-selection stage [121]. Moreover, cells co-expressing pre-TCR and γδ TCR develop along the γδ lineage and adopt a γδ gene expression profile, which implies that strong signalling activity by the γδ TCR dominates over weak pre-TCR signals [122]. Additional studies attempted to elucidate the means by which the two types of receptors achieve different signal intensities and found discrepancies in the use of downstream pathway components, membrane localisation and cytoplasmic domain structure between pre-TCR and γδ TCR [123–127]. Moreover, it has been suggested that a higher absolute abundance of γδ TCR on the cell surface could be responsible for the stronger signalling intensity [20,128], which is supported by the finding that reduction of γδ TCR expression levels in transgenic mice promotes the development of TCRγδ+ DP thymocytes [120].

For both γδ TCR and pre-TCR the main signalling cascade that is triggered downstream is the MAPK pathway, which stimulates the phosphorylation and activation of ERK. This in turn mediates the upregulation of EGR transcription factors, which induce expression of their target *Id3* [106,129,130]. Importantly, the levels of ERK phosphorylation and EGR/ID3 induction are proportional to the TCR activity and therefore higher in TCRγδ+ cells compared to pre-TCR+ cells [106,113,119,120,129,130]. Further research by Lee at al. has provided additional insight into how the differences in ERK activation might be translated into distinct gene expression programmes in αβ and γδ lineage cells. The authors observed that in cells adopting γδ fate, ERK phosphorylation was not just stronger but also maintained for a longer period of time after removal of the TCR stimulus [131]. This prolonged ERK activity enabled the interaction and posttranslational stabilization of the transcriptional target EGR1 through the ERK DEF binding pocket. Such non-canonical ERK signalling has

previously been described for other immediate early genes [132,133] and implies that certain DEF domain-containing proteins might be stabilised only upon γδ TCR signalling, whereas pre-TCR-mediated ERK activation may be too transient to mediate stabilisation of its transcriptional targets. This could represent a way in which a differential gene expression programme can be established in response to pre-TCR vs. γδ TCR signalling [133]. Importantly, the role of TCR signalling in the fate decision process has primarily been studied in the mouse context and therefore the abovementioned mechanisms have yet to be substantiated in human thymocytes. Recent work froFergm our lab revealed that in the human thymus commitment to the γδ lineage is associated with extensive changes in the chromatin landscape and that the differentially accessible regions in TCRγδ+ thymocytes are enriched for motifs of AP-1 family transcription factors [134], which represent known downstream targets of TCR signalling [135]. AP-1 activity has indeed been shown to play a crucial role in chromatin opening during TCR-induced T cell activation [136], which substantiates the idea that changes in chromatin accessibility in cells differentiating along the γδ lineage are mediated by TCR signalling. Importantly, no such alterations in the chromatin landscape were observed in β-selected thymocytes that are predicted to have received a weak pre-TCR signal. TCR stimulation of such immature αβ lineage ISP thymocytes prevented transition to the DP stage and instead resulted in the development of CD4-CD8+CD73+ thymocytes, which resemble immature γδ T cells. Moreover, the TCR-stimulated cells displayed wide-spread chromatin opening, similar to the previously observed changes in γδ lineage cells [134]. Finally, thymocytes committing to the γδ but not the αβ lineage were found to upregulate *EGR* transcription factors as well as *ID3* [134], which constitute downstream targets of TCR signalling as discussed above. While more research is needed to definitively link TCR signalling intensity and αβ vs. γδ cell fate in human thymocytes, these observations strongly suggest that the signal strength-based instructional model holds true in the context of human T cell development.

3.1.2 The role of Notch signalling in the lineage decision

Notch signalling in the context of T cell development has been studied extensively; however, several discrepancies between mouse and human have made the interpretation of experimental results rather complicated (Table 1). Specifically, Notch signalling in mouse thymocytes favours development of the αβ over the γδ lineage, whereas several lines of evidence suggest the opposite in human T cell development. Overexpression of ICN1 or ICN3 – the active intracellular domains of Notch1 and Notch3, respectively – in human thymocytes was shown to skew T cell development towards the γδ lineage in a proliferation-independent manner [33,41,137]. The ICN1-mediated skewing was more prominent when CD34+ thymocytes were used as starting population, whereas only a mild effect was observed with ISP thymocytes and cells past the β-selection point could not be reverted to γδ fate via ICN1 overexpression [41], which is in line with the gradual loss of γδ potential throughout development. These findings were further corroborated in two studies that employed γ-secretase inhibitor (GSI) to block cleavage-mediated activation of Notch1, which resulted in impaired γδ lineage development from CD34+ precursors in the OP9-DLL co-culture system and in human/mouse Fetal Thymic Organ Cultures (FTOCs) [41,101]. The studies disagreed on the requirement of Notch signalling for αβ lineage development, with

increased frequencies of DP and TCRαβ+ cells in the OP9 system but reduced DP numbers in the FTOCs. This discrepancy can most likely be explained by differing GSI concentrations and therefore different residual levels of Notch activity, given that subsequent analyses in *Dll4*$^{lox/lox}$/*Jag2*$^{lox/lox}$ FTOCs demonstrated a dose-dependent reduction of γδ lineage cells but normal αβ lineage development as long as at least one *Dll4* or *Jag2* allele was intact [138]. This substantiates a higher Notch dependency of differentiating γδ T cells compared to αβ lineage cells during human thymocyte development. In fact, high levels of Notch activity seem to impede αβ T cell development as observed by the beneficial effects of low doses of GSI and the negative impact of ICN1 overexpression on the αβ lineage [39,41,101].

When assessed in isolation using the OP9 co-culture system, JAG2 appears to be the Notch ligand that best supports γδ T cell development from CD34+ thymocyte precursors [33]. This has been attributed to its ability to interact with both Notch1 and Notch3 on immature thymocytes, which leads to strong Notch signalling activity [33]. DLL1 and DLL4 can also support γδ T cell development, yet in OP9 co-cultures they also promote the differentiation of a higher proportion of αβ lineage cells [33,101,137,139]. These assays demonstrate that either of the three ligands is sufficient to permit γδ T cell development, but due to their redundancy, neither of them is absolutely required for this process. This is further illustrated by *Jag2*$^{-/-}$ FTOC experiments, in which γδ T cell development is reduced but not completely abrogated [33]. Similarly, γδ lineage cells can still develop to a certain extent in *Dll4*$^{-/-}$/*Jag2*$^{wt/-}$ or *Dll4*$^{wt/-}$/*Jag2*$^{-/-}$ FTOCs and only *Dll4/Jag2* double-deficient thymic lobes entirely disrupt γδ T cell differentiation. In contrast, JAG1 is not able to support efficient differentiation of γδ lineage cells due to its weak interactions with Notch1 and Notch3 [33] and *Jag1*-deficient FTOCs exhibit normal γδ T cell development [138]. Importantly, the expression of the different Notch ligands is not homogenous throughout the thymus lobes, with DLL4 mostly found in cortical TECs, whereas JAG2 is expressed in the cortex and medulla [139,140]. It has been hypothesised that this results in the generation of distinct niches, which permit the regulation of specific Notch-dependent developmental decisions such as T lineage commitment or αβ vs. γδ cell fate specification [140]. Based on this, 2D or 3D cell culture systems with homogenous ligand distribution may exhibit some technical bias and not reflect the thymic microenvironment in full accuracy. Similarly, the DLL4 and JAG2 expression levels and localisation differ between human and mouse thymus [140] (Table 1), which might affect timing and intensity of Notch signalling in the FTOC system. These limitations need to be considered during the interpretation of experimental findings.

While the Notch targets that mediate T lineage commitment are well known [105], the molecular mechanism through which strong Notch signalling favours γδ lineage fate remains largely unclear. This gap may be due to certain obstacles in the analysis and interpretation of Notch activity, for instance, divergent Notch1 and Notch3 expression windows (Figure 1), a lack of information about target gene redundancy between these two receptors [33] and an apparent difference in dose-sensitivity for certain Notch targets [101]. Recently, it was shown that Notch signalling counteracts the expression of BCL11B, a transcription factor that is essential for αβ lineage development but less crucial for the γδ lineage [138]. Moreover, as previously mentioned, IL7R appears to be upregulated in response to Notch signalling [38,82] and might support the development of γδ T cells through its involvement in *TRG/TRD* rearrangement. The normal use of *TRGV* and *TRDV* elements in γδ T cells derived

from *Jag2*$^{-/-}$ FTOCs argues against this idea [33], but an in-depth analysis of the repertoire would be required to draw a definitive conclusion. Moreover, TCRγδ+CD73- thymocytes that have not yet fully committed to γδ fate are still dependent on Notch signalling for their maturation, which indicates a role of Notch signalling in γδ lineage development beyond TCR rearrangement [138].

3.1.3 IL7R signalling during γδ commitment

Similar to Notch signalling, studies in different species have indicated differing requirements for IL7 signalling in γδ T cell development. Mice deficient in IL7 pathway components, such as IL7R, JAK3, or STAT5, display significant impairments in the γδ lineage while αβ T cells are only moderately affected [73,81,141,142]. Likewise, in medaka, where γδ T cell development in the thymus is spatially separated from αβ development, IL7 is predominantly expressed by cells in the γδ niche and IL7 overexpression promotes γδ T cell development [143]. In contrast, IL7-mutant zebrafish show no substantial disruption of αβ and γδ lineage differentiation [144]. Contradicting findings have been reported in the context of human γδ T cell development: Pallard and colleagues showed that when CD34+ thymocytes cultured in FTOCs were treated with IL7R inhibitory antibodies, αβ lineage development was completely disrupted but γδ T cells could still differentiate, albeit at reduced frequencies [44]. In addition, a dominant negative form of STAT5B resulted in impaired αβ T cell development but γδ lineage expansion in FTOCs [44], suggesting a much stronger dependence of αβ lineage cells on IL7R signalling activity. On the other hand, increasing concentrations of IL7 in CD34+ OP9-DLL1 co-cultures promoted both αβ and γδ lineage development but the beneficial impact was more prominent in the γδ lineage [101]. Analysis of prenatal and paediatric thymocyte samples also revealed a γδ lineage-specific upregulation of IL7R signalling components, such as *IL7R*, *STAT5A*, *STAT5B* and *JAK3* [134,145], and chromatin opening at the *IL7R* locus [134], which implies a beneficial effect of IL7R signalling on human γδ T cell development. Insights from patients with IL7R mutations into the role of IL7 in γδ lineage differentiation are difficult to obtain due to the role of IL7 in T lineage commitment and the resulting early arrest of thymocyte development. However, STAT5B gain-of-function mutations seem to be very common in γδ T cell lymphoma [146,147], which indicates that this downstream mediator of IL7R signalling might provide a growth advantage in γδ T cells. Due to these limited and partially conflicting data, definitive conclusions about the involvement of IL7R signalling in human γδ lineage commitment cannot be drawn. Moreover, the impact of the IL7R pathway on TCR rearrangement is difficult to separate from any additional roles of IL7 in the fate decision and further research is therefore needed to address this question.

3.1.4 Transcriptional regulation of γδ lineage commitment

Despite the advances in high-throughput sequencing approaches, the identification of transcription factors that are required and sufficient to establish γδ fate has been surprisingly difficult. A transcriptional signature of mouse γδ thymocytes, including *ICER*, *Sox13*, *Runx3*, *Id3* and several NR4A nuclear receptor factors, was described by the Hayday lab [111]. However, most of these factors were already detectable in DN thymocytes and were later selectively downregulated in the αβ but not in the γδ lineage. Moreover, many were found to

be expressed in other unconventional T cell types, such as TCRαβ+ CD8αα cells, and are therefore not specific to the γδ lineage [111,148].

The prime candidates as γδ-promoting regulators in the lineage fate decision are perhaps EGR1-3 and ID3, which are upregulated in response to TCR signalling. As discussed above, their induction levels are proportional to the intensity of TCR activity and therefore especially high in γδ thymocytes, yet pre-TCR signalling is also able to induce *Egr/Id3* expression in αβ lineage cells [106,113,119,120,129,130]. Analyses from our lab showed that these findings from mouse studies are mirrored in human thymocytes, with immature TCRγδ+CD1+ thymocytes displaying much higher levels of *EGR1-3* and *ID3* compared to β-selected ISP thymocytes [134]. In addition, using single cell data we discovered a strong but highly transient upregulation of *ID3* in cells undergoing β-selection [88], which further confirms that *ID3* is expressed in the course of commitment to both αβ and γδ lineage.

Ectopic expression of ID3 in CD34+ fetal liver cells or CD34+CD1- HSPCs in the FTOC system has been shown to prevent T lineage commitment and interferes with the induction of *TRD* rearrangements [149,150], which can probably be attributed to ID3-mediated inhibition of E protein activity as well as the downregulation of *RAG1/2* [149] (Figure 2C). In contrast, overexpression of ID3 in cells that have already committed to the T lineage and initiated TCR rearrangements only impairs the development of αβ lineage cells, whereas γδ T cells differentiate normally [149]. Importantly, the elevated ID3 levels do not seem to divert cells to the γδ fate as indicated by stable frequencies of TCRγδ+ thymocytes. This resembles findings in mice, where ID3 overexpression is unable to promote increased γδ lineage commitment [113] and implies that ID3 is insufficient for induction of γδ fate.

Mechanistically, in mouse thymocytes ID3 interferes with the E protein-induced initiation of gene expression programmes that are required for γδ effector differentiation [151]; however, this specification does not seem to apply in the human thymus (see section 4.1) and therefore the mechanisms through which ID3 contributes to γδ T cell development in the human context remain unclear.

Another transcriptional regulator with potential involvement in γδ T cell differentiation is SOX13. Initial mouse experiments showed impaired γδ but not αβ lineage development in *Sox13*-deficient mice and increased TCRγδ+ but reduced DP thymocyte frequencies upon SOX13 overexpression [117]. Moreover, SOX13 expression in DN thymocytes was found to be heterogeneous and high SOX13 levels were predictive of increased γδ lineage potential [116]. However, subsequent studies demonstrated that SOX13 is not expressed in all γδ T cells [152] but appears to be required solely for differentiation of the γδT17 subtype via the induction of the γδT17 transcriptional programme [153,154]. In human thymocytes, *SOX13* is also upregulated in γδ lineage cells, whereas in the αβ lineage the locus exhibits increased levels of repressive H3K27me3 marks [134,155]. Since there is no human equivalent of the murine γδT17 subset, it still needs to be established if and how SOX13 could play a role in human γδ T cell development.

RUNX3 is a transcription factor primarily known for its role in establishing CD8+ cytotoxic T cells through repression of alternative T helper fate [156] but it is also upregulated during γδ lineage commitment and downregulated in cells undergoing β-selection [101,134]. Interestingly, analysis of chromatin accessibility and histone modifications at the *RUNX3* locus throughout thymocyte differentiation revealed high levels of H3K27ac at the

transcription start site of a short *RUNX3* isoform in TCRγδ+ but not CD8+ SP thymocytes [134], which suggests that the two cell types might use different isoforms of RUNX3 or at least engage in different mechanisms of transcriptional regulation. As described earlier, RUNX3 may act as a pioneering factor at the *TRG/TRD* loci to facilitate γδ TCR expression in mature γδ T cells [100], but further functions in this context remain to be elucidated. Several other transcription factors identified in mouse γδ T cells also seem to be selectively upregulated in human γδ lineage thymocytes but repressed in the αβ lineage, including *NR4A1-3, ETV5, KLF2, RELB, HES1 and ZBTB16* [111,134,157,158]. *TBX21* (encoding T-bet), which has previously been implicated in murine γδ T cell development and function [159,160], was expressed at elevated levels in human TCRγδ+ thymocytes and shut down during β-selection via promoter histone methylation [134]. TBX21 motifs were found to be enriched in accessible chromatin regions in γδ T cells, suggesting high activity of the transcription factor in this lineage [134]. On the other hand, a subset of transcriptional regulators with known involvement in mouse γδ T cell differentiation seems to be expressed at low levels in human TCRγδ+ thymocytes [111,134,153], which highlights that not all γδ-biased transcription factors are conserved between species. Importantly, in mouse thymocytes, many of the listed genes have been linked with the adoption of a specific γδ T cell effector fate in the thymus, which does not seem to apply in the human context and therefore creates difficulties in translating molecular functions between species.

Epigenetic regulation of γδ T cell development and the αβ vs. γδ fate decision has not been studied in much detail. Our lab recently described widespread chromatin remodelling, which occurred specifically in γδ lineage committed thymocytes, whereas αβ lineage cells retained a stable chromatin landscape throughout β-selection [134]. This was despite extensive transcriptional changes in both lineages, which implies that gene expression is regulated through different mechanisms in αβ vs. γδ committed cells. Differentially accessible regions in TCRγδ+ cells were mostly located in intronic and intergenic regions, suggesting that differential gene expression is predominantly controlled by changes at distal enhancers [134]. Given the complexities associated with enhancer annotation, this may explain difficulties in the establishment of γδ-specific gene regulatory networks. In contrast, αβ lineage thymocytes displayed upregulation of components of the Polycomb Repressive Complex 2 (PRC2), such as *SUZ12* and *EZH2*, and consistent with this, a global increase in H3K27me3 levels was detected [134]. The repressive histone mark was found in αβ thymocytes at the regulatory regions of many γδ-biased genes, including *SOX13, ZBTB16, TBX21, RUNX3, EGR1* and *NT5E* (encoding CD73), which implies that transcription of these is shut down in the αβ lineage via PRC2 complex activity. TCRγδ+ cells on the other hand exhibited upregulation of the H3K27me3 erasers *KDM6A* and *KDM6B* and consequently a very limited increase in H3K27me3 [134]. To our knowledge, no studies have been published addressing DNA methylation in connection with the αβ vs. γδ fate decision in human thymocytes. However, we found that commitment to the γδ lineage and maturation of γδ cells is associated with upregulation of *TET2* and *TET3* and consequently with large-scale CpG demethylation. In contrast, αβ lineage cells displayed few changes in DNA methylation until the DP-to-SP transition and upregulated the DNA methyltransferase *DNMT1*, which might be required for maintenance methylation during proliferation (unpublished observations, data from [134,161]).

Information on the role of miRNAs in γδ lineage differentiation is similarly sparse. miRNA profiling of human thymocytes indicated a downregulation of many miRNAs in ISP thymocytes compared to bipotent progenitors, whereas few miRNAs were differentially regulated in cells committing to the γδ lineage [138]. Analysis of miRNA changes in response to Notch signalling identified miR-17 as a direct Notch target and overexpression of miR-17 in Artificial Thymic Organoid (ATO) cultures resulted in an increased γδ:αβ thymocyte ratio. This resembles the effects of Notch signalling on αβ vs. γδ development and miR-17 was indeed able to rescue Notch inhibition-induced defects in γδ T cell differentiation [138]. Mechanistically, miR-17 was found to inhibit the expression of the αβ lineage transcription factor BCL11B. Although knockdown of miR-17 did not selectively affect the γδ lineage, the findings suggest a possible role of the miRNA miR-17 in promoting γδ over αβ fate via the repression of BCL11B [138]. In contrast, the microRNA miR-181a was found to have a negative effect on γδ T cell differentiation through inhibition of Notch2 and MAP3K2 and a resulting Notch/MAPK signalling reduction [162], which is expected to counteract γδ lineage commitment.

3.2 The fate decision window

The bifurcation point of the αβ and γδ lineages and the window within which thymocytes are bipotent and retain some alternate lineage potential have remained remarkably ill-defined. As discussed above, rearrangement of the *TRD* and *TRG* loci commences very early in human thymocytes and, assuming that fate is not intrinsically pre-programmed, cells should be able to commit to the γδ lineage as soon as they have successfully rearranged one *TRD* and *TRG* allele each. The observation that the *TRB* locus is still in germline configuration or incompletely rearranged in most γδ T cells [49–51] suggests that many thymocytes in fact adopt the γδ fate before initiating the generation of a β-chain. This portrays the bifurcation process as a sequence of decisions, where in the majority of cells the possibility of becoming a γδ T cell is tested first and only in case this is unsuccessful or too slow, do thymocytes attempt to adopt the αβ fate. Curiously, based on clonal cultures from CD34+ thymocytes it was concluded that a sizeable fraction of cells already loses its bipotent properties around the T lineage commitment step, but this may represent a side-effect of the OP9-DLL1 *in vitro* culture system since partial inhibition of Notch signalling was shown to increase the frequency of bipotent cells [101]. Two things can likely be responsible for extinguishing γδ potential under physiological circumstances: biallelic out-of-frame rearrangements for *TRG* or *TRD* or β-selection and concomitant commitment to the αβ lineage. The timing of β-selection has been a matter of debate, with different studies placing it in the ISP or DP stage based on the expression of *RAG1/2*, *PTCRA*, CD3 and the TCR β-chain itself [48,149,163–165]. It is therefore likely that β-selection does not coincide with a specific CD4/CD8 surface profile [29,166] but can take place throughout the ISP and up until the CD4+CD8αβ+ DP stage. Consistent with this, it has been shown that γδ lineage potential drops rapidly from the ISP stage onwards. However, some γδ T cells can still arise from ISP and CD3- DP thymocytes in FTOCs and OP9 co-cultures [49,115], indicating an extensive window during which thymocytes can adopt γδ fate. Interestingly, a small number of DP TCRγδ+CD1+ cells also seem to give rise to CD3+TCRαβ+ thymocytes *in vitro* [115], which implies that immature γδ T cells still retain some αβ lineage potential. Overall, thymocytes experience a

long period, during which cells can choose to commit to the αβ or γδ lineage, and the exact decision point will vary immensely between individual cells. For this reason, novel single cell approaches may prove useful to further investigate and quantify the lineage potential of thymocytes throughout differentiation. Nevertheless, progenitors seem to start with a high potential to adopt γδ fate, which then gradually drops as the cells differentiate further, as recently predicted based on single cell thymic data using the STEMNET algorithm [134].

3.3 Thymic selection of γδ T cells

For the αβ lineage, it is well established that cells undergo several selection steps that confirm the functionality and lack of autoreactivity of their TCR. Whether a similar procedure takes place in the γδ lineage is not entirely clear. To our knowledge, no evidence has been published supporting the presence of a negative selection mechanism in γδ T cells [51] and it is known that γδ TCRs frequently recognise self-antigens. Peripheral tolerance mechanisms most likely exist to prevent auto-reactivity [167], but this remains to be demonstrated. A positive selection step similar to the αβ lineage is unlikely to apply in γδ T cells due to their HLA independence. However, it has been speculated that TCRγδ+ thymocytes engage in ligand-dependent TCR signalling, which could represent a mechanism to test antigen specificity and promote the development of specific subsets as shown in mouse [168,169]. Analysis of human fetal γδ T cells has indeed shown that these already exhibit a phosphoantigen-reactive TCR repertoire, but since the enriched TCR CDR3 sequences appear to be germline-encoded, it is unclear whether endogenous phosphoantigens are responsible for the enrichment [170]. While it is certainly possible that γδ thymocytes respond to endogenous or exogenous antigens in the thymic environment, it has been suggested that the γδ TCR may be able to spontaneously dimerise and thereby induce TCR signalling to promote γδ lineage commitment and proliferation in a ligand-independent manner, similar to the pre-TCR during β-selection [171,172]. However, γδ TCR dimers have so far only been demonstrated in human but not mouse [173], so this mechanism of action remains speculative.

4 Mature γδ T cells in the human thymus

The maturation of TCRγδ+ thymocytes can be traced based on several surface markers. Upregulation of CD73 has been described as an indicator of full commitment to the γδ lineage [138,174]. Moreover, like in the αβ lineage, CD1 levels drop in mature γδ T cells whereas CD27 is upregulated [114] (Figure 1).

4.1 Acquisition of γδ T cell effector functions in the thymus

For murine γδ T cells, it is well known that the adoption of different effector phenotypes already takes place in the thymus and mature γδ T cells can be broadly grouped into IFNγ- and IL17-producing subtypes [157,171,175–177]. This grouping does not directly apply to human γδ T cells as the vast majority are IFN producers and IL17-producing cells have been shown to arise only under certain inflammatory conditions and in very small numbers [178,179] (Table 1). Human paediatric γδ thymocytes also seem more naïve compared to peripheral blood γδ T cells based on their surface marker profile, ability to produce TNFα and

IFNγ upon TCR stimulation, and cytolytic activity against leukemic cells [180]. On the other hand, they can react to certain cytokine stimuli with proliferation and initiation of a cytotoxic response [114,180], which indicates a higher level of maturity than observed in αβ lineage thymocytes. Their ability to develop into effector cells and carry out cytotoxic functions without the requirement for TCR stimulation represents one of the key reasons why γδ T cells can be considered innate-like. IL7 plays an essential role in the induction of effector differentiation in mouse thymocytes [181], but it is unable to accomplish this in human thymic γδ T cells [180].

Importantly, recent work from the Vermijlen lab was able to demonstrate that adoption of effector function may occur in fetal thymic TCRγδ+ cells. They found that the transcriptional programme of CD1- γδ T cells from fetal thymus and liver was comparable to activated T and NK cells, which was not the case for postnatal γδ thymocytes [54]. Moreover, fetal thymic γδ T cells were able to produce effector cytokines, such as IFNγ, TNFα and granzymes, without the need for stimulation [54,182]. scRNA-seq analysis of fetal thymus samples revealed effector programming of a considerable fraction of γδ thymocytes and the size of this subset was negatively correlated with gestational age [183]. In contrast, in paediatric thymi effector programming of γδ T cells was exceedingly rare and limited to a minor subset of Vγ9+Vδ2+ cells [183]. This suggests that fetal and postnatal γδ T cells in the human thymus exhibit distinct differences with a prominent level of effector programming before but not after birth.

4.2 Development of human γδ T cell subtypes

Similarly to the age-dependency of effector pre-programming, different human γδ subtypes arise during distinct developmental windows throughout pre- and postnatal life. Mature γδ T cells are commonly distinguished based on *TRDV* usage with the main two subtypes being Vδ2+ and Vδ2- γδ T cells, which differ in their target antigens, adaptive vs. innate behaviour and tissue localisation (reviewed in [167,184,185]). *TRDV2* most frequently pairs with *TRGV9* and the resulting Vγ9+Vδ2+ T cells are the first γδ T cells to arise in embryonic development (Figure 4). This phosphoantigen-responsive subtype can be detected in the embryonic liver as early as 5 wpc and shortly after from 8 wpc in the thymus [186,187], where they represent the main γδ T cell type until mid-gestation [54,55]. Consequently, they also make up the majority of γδ T cells in the fetal blood but there their frequencies continuously decrease until birth [170,188,189], which has been attributed to their emigration into various tissues in combination with reduced thymic output. Soon after birth, circulating Vγ9+Vδ2+ cells start to expand rapidly in response to pathogen exposure and eventually represent the predominant γδ T subtype in the blood throughout childhood, adolescence and adulthood [189–193] (Figure 4). It was long thought that circulating Vγ9+Vδ2+ cells are exclusively fetal-derived and maintained by proliferation and clonal expansion [59,185,189], but recent evidence has made a strong case for postnatal thymic development of Vγ9+Vδ2+ cells: Earlier studies claiming a complete absence of Vγ9+Vδ2+ thymocytes after birth [59,180,187,189] were disproven by the discovery of a small population of *TRDV2*/*TRGV9* co-expressing cells in the postnatal thymus [55], indicating the possibility of ongoing differentiation of this cell type beyond the initial fetal wave. Moreover, Vγ9+Vδ2+ cells can successfully regenerate after stem cell transplantation in adults [194,195], suggesting that

their development does not strictly require the fetal thymic environment. Finally, fetal and postnatal Vγ9+Vδ2+ T cells in the blood show clear repertoire differences with divergence in the use of *TRDJ1/2/3* [55,191,196] and age-correlated increase in the number of N insertions in the γ- and δ-chain CDR3. Importantly, these differences are mirrored in the thymic Vγ9+Vδ2+ repertoire at the corresponding developmental stage [55]. *In vitro* stimulation of fetal Vγ9+Vδ2+ cells did not result in repertoire changes or selective expansion that would support an exclusively fetal origin of peripheral Vγ9+Vδ2+ cells in adult blood [55]. Hence it is likely that this cell type is continuously produced throughout life and contributes to the maintenance of the peripheral Vγ9+Vδ2+ T cell pool. The observed differences in nucleotide addition and CDR3 sequence length in fetally and postnatally generated Vγ9+Vδ2+ cells are probably the result of different TdT levels throughout gestation [54,56]. This results in very limited insertion of nucleotides during V(D)J recombination in early development and hence in a highly invariant fetal repertoire [54]. This seems to be an intrinsic property of the precursor cells and independent of the developmental environment since HSPCs derived from fetal liver and cord/peripheral blood still exhibit differences in N insertions when cultured *in vitro* under identical conditions [55] and fetal but not adult HSPCs can give rise to certain invariant TCRs [54]. The invariant repertoire of fetal γδ T cells has been associated with a more innate-like character, which can represent a vital first-line defence in absence of a mature adaptive immune system. Therefore, the deliberate downregulation of TdT in fetal thymocytes might represent a protective mechanism against infections *in utero* and in infancy when the αβ lineage is still naïve [170,185,197,198].

A much smaller, less well-studied subgroup of Vδ2+ T cells uses γ-chains other than *TRGV9* to assemble the γδ TCR. These Vγ9-Vδ2+ cells possess a private TCR repertoire and are not responsive to phosphoantigens, which suggests a more adaptive behaviour and thus higher similarity to Vδ1+ rather than Vγ9+Vδ2+ T cells [196]. They represent the second wave of γδ T cells in the human thymus and are the most abundant population at mid-gestation [54,55] (Figure 4). In contrast, only low numbers of Vγ9-Vδ2+ cells are detected in the postnatal thymus [54,55], which indicates that their differentiation may continue throughout life but most likely at low frequencies. The proportion of Vγ9-Vδ2+ cells in the blood appears to be negligible throughout fetal and postnatal life [54,170,188], which implies rapid migration to their main sites of residence, for instance the subcutaneous adipose tissue [199].

The Vδ2- subset of γδ T cells is mainly composed of Vδ1+ cells and smaller proportions of Vδ3+ and Vδ5+ cells, all of which pair with a diverse mix of γ-chains. These γδ T cells are considered to be more adaptive-like and exhibit a private repertoire [167,195]. Vδ1+ thymocytes seem to be generated from mid-gestation onwards and remain the dominant thymocyte population throughout late fetal, postnatal and adult life [54,189] (Figure 4). Their emigration towards their main target tissues, e.g. gut, liver and epithelial sites, is evident from increased Vδ1+ fractions in late fetal and cord blood [170,182,188,189], and high proportions of Vδ1+ thymocytes postnatally combined with moderate frequencies in peripheral blood suggest continuous thymic output after birth [54,188,200]. Similar to Vγ9+Vδ2+ cells, the repertoire of Vδ1+ γδ T seems to undergo age-related focussing due to pathogen exposure as evident from the presence of few highly expanded clones in adult blood, which do not yet occur in cord blood [195]. Of note, it has been shown in *in vitro* co-cultures that HSPCs derived from fetal liver/thymus or cord/adult blood are intrinsically programmed to

preferentially give rise to Vδ2+ and Vδ1+ T cells, respectively [54], which suggests that differences in the precursor cells rather than changes in the thymic environment are responsible for the age-dependent development of γδ subtypes.

Little is known about the thymic development of Vδ1-Vδ2- γδ T cells but they were shown to be relatively abundant specifically in late-fetal and neonatal blood, which suggests that this subtype predominantly arises around birth [170,200] (Figure 4).

Curiously, effector programming of γδ T cells in the fetal thymus was observed for both Vγ9+Vδ2+ and non-Vγ9+Vδ2+ cells and no preference for a certain effector fate was detected between the two subsets [183]. This is in contrast to observations in mice, where different effector fates are normally associated with distinct TRGV usage. Furthermore, the presence or absence of specific Notch ligands does not seem to affect the proportions of Vγ9+, Vδ1+, Vδ2+ and Vδ3+ cells developing from human thymocytes [33], whereas mouse γδ effector subtypes exhibit varying dependence on Notch signalling activity [201]. This lack of conservation in γδ T cell subtypes and effector differentiation mechanisms between mouse and human illustrates the need for further research into the processes that govern γδ T cell development specifically in the human context.

5 Conclusion

The existence of γδ T cells has been known for close to 40 years and research efforts have provided us with remarkable insights into the functional roles and modes of action of this fascinating cell type. Yet the details underlying the development of γδ T cells in the human thymus are still incompletely understood. Only few signalling pathways have been linked with γδ lineage commitment and their contribution to γδ differentiation remains ill-defined due to their changing roles throughout development and the conflicting findings in mouse and human (Table 1). Similarly, little is known about the transcription factors controlling adoption of the γδ fate. However, recent technological advances, such as the increasing accessibility of single cell sequencing techniques and the vast improvements in *in vitro* gene editing and iPSC-derived T cell differentiation, hold great promise and will undoubtedly be extremely valuable to tackle the remaining open questions in this field of research.

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Declaration of interest

The authors declare no conflicts of interest.

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Figure 1: T cell development in the human thymus

Schematic representation of human thymocyte differentiation. The main developmental stages are depicted and the timing of T lineage commitment and β-selection are indicated. The expression of key surface markers, receptors and rearrangement windows of the TCR genes are shown below, whereby green corresponds to bipotent cells, blue to the γδ lineage and yellow to the αβ lineage. Dashed colouring implies selective expression of the gene in either CD4+ or CD8+ SP thymocytes. The arrow thickness represents the assumed potential of thymocytes to differentiate along the αβ or γδ lineage; for further details on this also refer to section 3.2. HSPCs and TSPs are marked by the expression of CD34 but lack of CD1, whereas ETPs are characterised by the additional upregulation of CD7. The initiation of CD1 expression and a decrease in CD44 levels signifies the commitment to the T lineage. CD4+ ISP thymocytes show a gradual reduction in γδ lineage potential and their maturation is associated with loss of CD34 expression and upregulation of CD28. The subsequent DP stage, during which the *TRA* locus is rearranged, can be divided into early (CD3-, proliferating) and late (CD3+, quiescent) DP thymocytes, which then further differentiate into either CD4+ or CD8+ SP cells. In the γδ lineage, commitment to the γδ fate is marked by the induction of CD73 and their subsequent maturation is indicated by downregulation of CD1 and upregulation of CD27. Abbreviations: HSPC: Hematopoietic stem and progenitor cell, TSP: thymus seeding progenitor, ETP: early T cell progenitor, DC: Dendritic Cell, NK cell: Natural Killer Cell, ISP: Immature Single Positive, DP: Double Positive, SP: Single Positive, TRA/B/G/D: T cell receptor α/β/γ/δ locus.

Figure 2: Signalling pathways during γδ T cell development

Schematic depiction of Notch, IL7R and TCR signalling at different stages of thymocyte differentiation. The direct and indirect effects of signalling activity on gene expression, V(D)J recombination, and viability are indicated. Of note, some of these mechanisms have only been reported in mouse thymocytes and are not yet confirmed in human. Abbreviations: TSP: thymus seeding progenitor, ETP: early T cell progenitor, ICN: intracellular domain of Notch, TCR: T cell receptor, TRG/D: T cell receptor γ/δ locus.

Figure 3: The human *TRD* and *TRG* loci

Illustration of the structure of the human *TRD* and *TRG* loci (not to scale). Variable (V), diversity (D), joining (J) and constant (C) segments as well as the main enhancers (Eδ and Eγ) are shown. The *TRD* locus is nested within the *TRA* locus and several V-segments are shared between both genes. Interspersed *TRAV* segments are not depicted but their location has been indicated by dotted lines. Unfilled segments represent pseudogenes according to [202]. At the *TRD* locus, the *TRDV3* segment exhibits an inverted orientation as implied by the arrow. Certain V(D)J rearrangements predominantly take place during specific gestational windows, which are depicted below each locus. Of note, the constant segments do not undergo V(D)J recombination but are included during transcription and joined with the rearranged V(D)J segments via mRNA splicing. In this context, *TRGC1* is mainly used during early gestation, whereas *TRGC2* predominates in later development. Furthermore,

during rearrangement of the *TRD* locus, two *TRDD* segments can be joined to a *TRDJ* segment, which further helps to increase the δ-chain diversity.

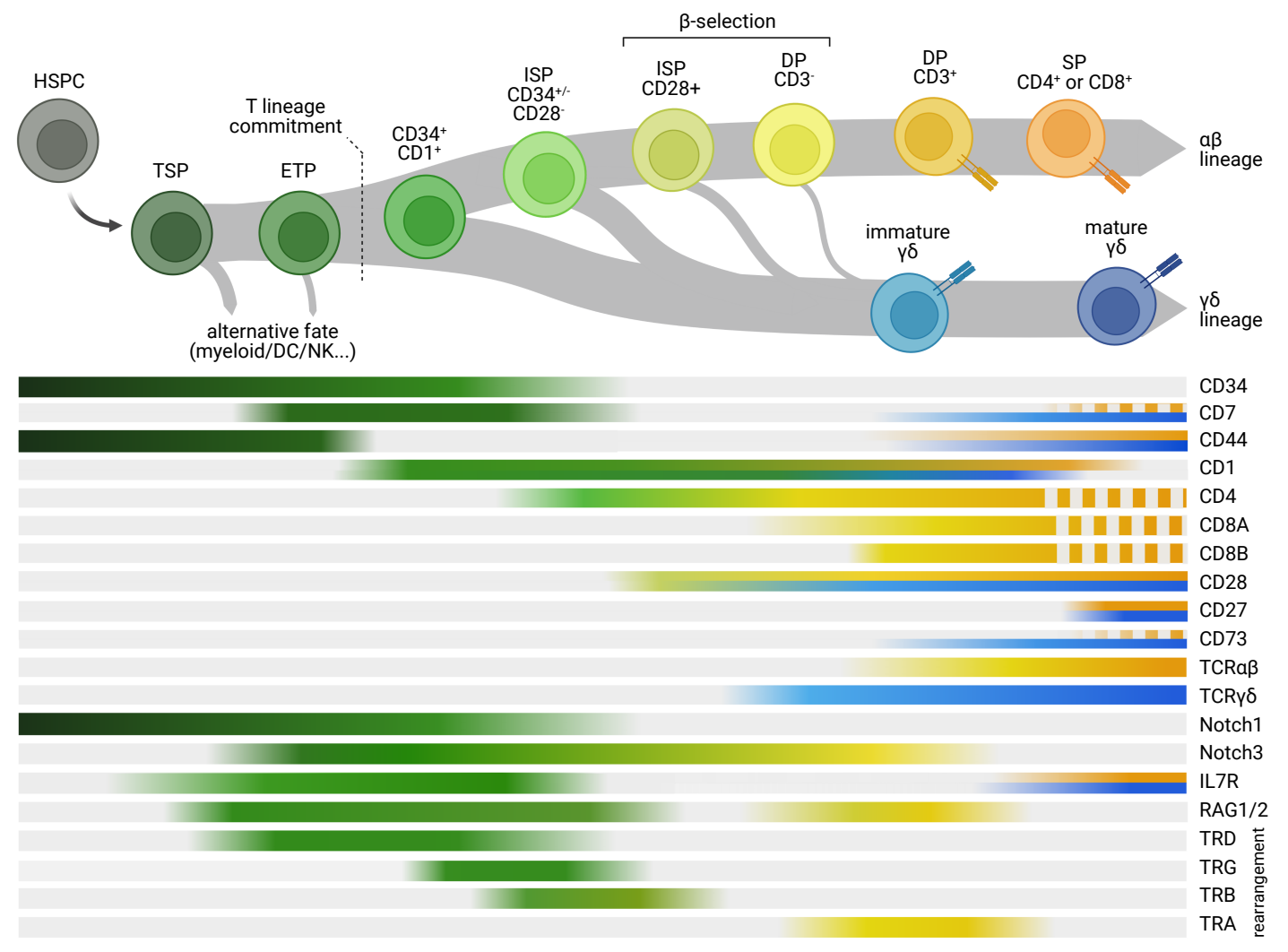
Figure 4: Proportions of different human γδ T cell subsets in thymus and blood throughout development

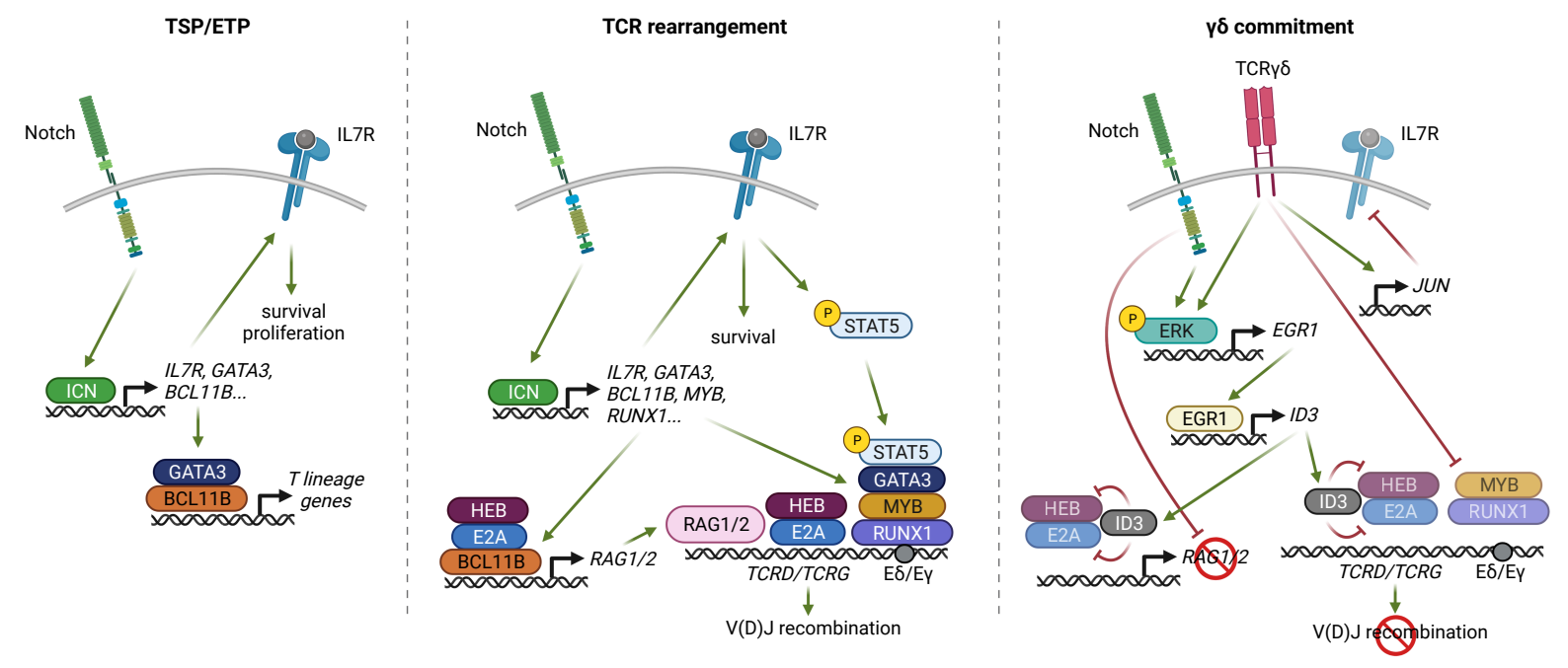
Overview of the frequencies of certain γδ T cell subsets throughout gestation, shown as proportion of total γδ T cells. Frequencies were gathered from a range of sources [54,55,186–190,200], which reported on embryonic, mid-gestation fetal, neonatal and paediatric thymus samples and cord or peripheral blood samples from mid-/late-gestation fetal, neonatal and paediatric donors.

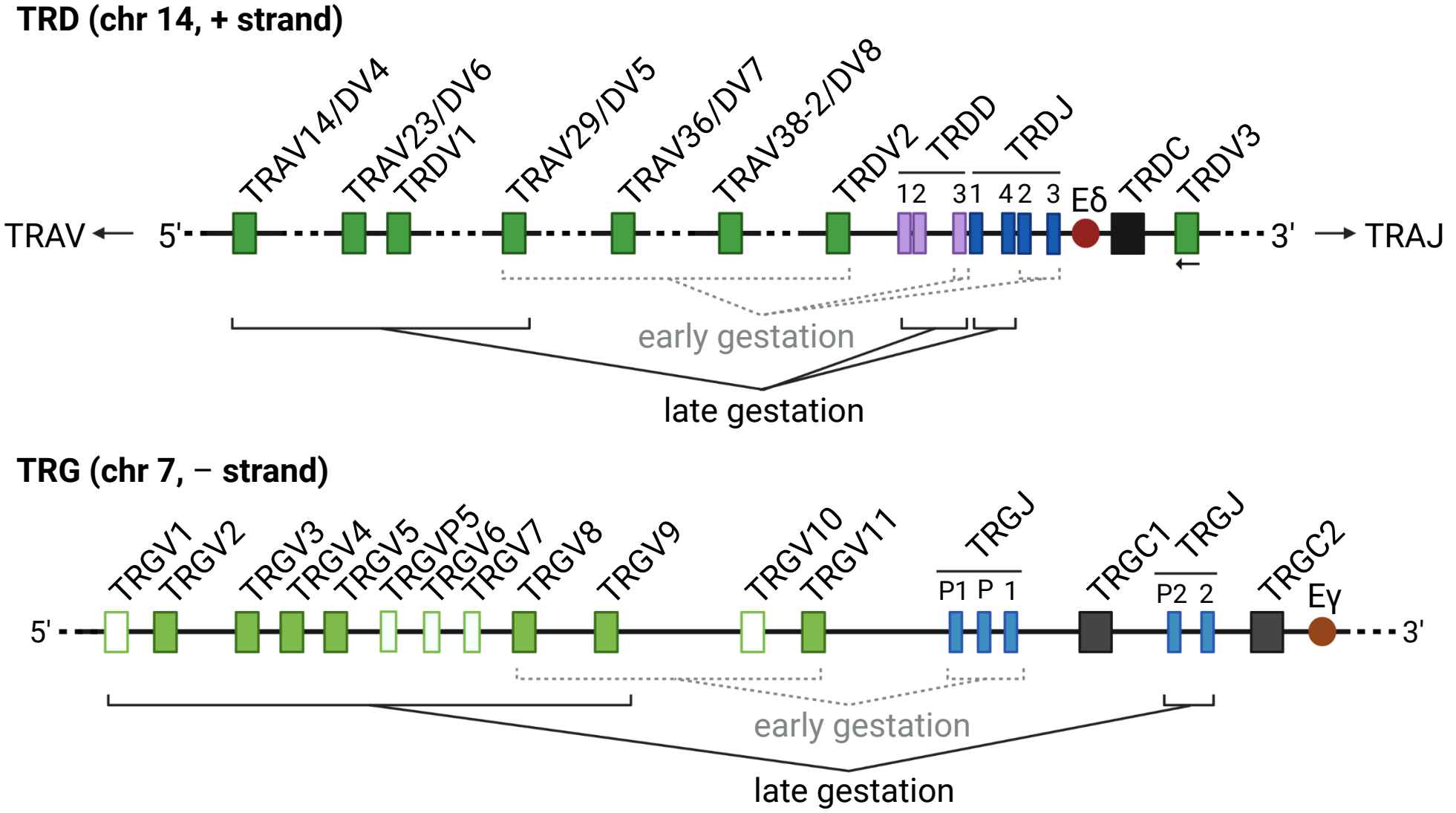
Feature	Mouse	Human
TRGV conservation	No phylogenetic homology [203]	
TRD locus conservation	Some sequence conservation but no evidence for functional similarities [203]	
Timing of *TRB* and *TRG* rearrangement	A notable proportion of thymic γδ T cells have complete *Trb* rearrangements, suggesting parallel rearrangement of *Trb* and *Trg* [204–206]	Most thymic and peripheral αβ T cells carry a rearranged *TRG* locus, but only a minor fraction of γδ T cells/thymocytes have a rearranged *TRB* locus [49–51]
Allelic exclusion	Yes for *Trg*, no for *Trd* [64–66]	Yes for *TRD*, no for *TRG* [50,53,58–60]
Notch activity	Increase up until β-selection [112,207,208]	Decrease from T cell commitment onwards [101]
Notch requirement	αβ lineage development depends on Notch signalling, whereas in γδ lineage Notch is just needed for proliferation [112,207–209]	High Notch signalling promotes γδ lineage, whereas αβ lineage development requires Notch signalling reduction [33,39,41,101,137,138]
JAG2 expression	High in cortex, lower at cortico-medullary junction (CMJ) [140]	High at CMJ, lower in cortex [140]
IL7 in the fate decision	Impairment of IL7 signalling affects γδ lineage severely but has little impact on αβ lineage [141,142,210]	Contradicting findings, see section 3.1.3
Pre-programming of γδ fate	IL7R/SOX13-high DNs are biased towards γδ lineage [116–118]	No evidence for pre-programming
TCRγδ+ DPs	Have been observed in transgenic mice [112,113] but do not seem to arise during normal development	γδ T cells can go through a DP stage under physiological conditions [114,115]
γδ TCR complex stoichiometry	TCRγδ + 2x CD3γε + 2x TCRζ [211]	TCRγδ + 1x CD3γε + 1x CD3δε + 2x TCRζ [211]
Requirement of CD3δ	γδ T cells develop normally in CD3δ deficient mice but knock-out of CD3γ or CD3ε affects αβ and γδ T cells [212–215]	Patients with CD3δ deficiency don't have γδ T cells but those with CD3γ deficiency do, i.e. CD3δ is required and can substitute for CD3γ [211]
Fate decision point	Lineage decision is probably completed by the DN3 stage [121,207]	Cells retain γδ potential up until DP stage [49,115]

CD27	Identifies γδT1 committed cells [176]	Identifies naïve Vγ9+Vδ2+ cells [216]
Effector specification in the thymus	Specification of IFNγ and IL17 producing subsets [157,171,175–177]	Some degree of effector programming in the fetal thymus [54,182,183]
IL7 in effector programming	Essential role of IL7 in effector differentiation [181]	Does not result in effector differentiation in the thymus [180]
Distinguishing feature of subtypes	*Trgv* [203]	*TRDV* [203]
Phosphoantigen-reactive γδ T cells	Do not exist [185]	Vγ9+Vδ2+ subtype
Dendritic epidermal T cells (DETCs)	Epidermis-colonising subset with invariant γδ TCR, which is the first subset to arise in the fetal thymus [217]	No equivalent subtype
Occurrence of IL17 producing γδ T cells	Physiological subset, which is programmed in the thymus [153,175,181]	Can develop under pathological conditions in the periphery [178,179]
TCR composition of IL17-producing γδ T cells	IL17-producing γδ T cells display preferential use of certain *TRGV* elements [218]	IL17 production is found in both Vδ1 and Vδ2+ γδ T cells [178,219]

Table 1: Non-exhaustive list of differences between mouse and human in the context of γδ T cell development in the thymus


β-selection
HSPC
T lineage commitment
TSP
ETP
CD34+ CD1+
ISP CD34+/- CD28-
ISP CD28+
DP CD3-
DP CD3+
SP CD4+ or CD8+
αβ lineage
immature γδ
mature γδ
γδ lineage
alternative fate (myeloid/DC/NK...)
CD34
CD7
CD44
CD1
CD4
CD8A
CD8B
CD28
CD27
CD73
TCRαβ
TCRγδ
Notch1
Notch3
IL7R
RAG1/2
TRD
TRG
TRB
TRA
rearrangement


TSP/ETP
Notch
IL7R
survival
proliferation
ICN
IL7R, GATA3,
BCL11B...
GATA3
BCL11B
T lineage
genes
TCR rearrangement
Notch
IL7R
survival
STAT5
ICN
IL7R, GATA3,
BCL11B, MYB,
RUNX1...
STAT5
GATA3
MYB
RUNX1
HEB
E2A
BCL11B
RAG1/2
RAG1/2
HEB
E2A
TCRD/TCRG
Eδ/Eγ
V(D)J recombination
γδ commitment
TCRγδ
Notch
IL7R
JUN
ERK
EGR1
EGR1
ID3
HEB
E2A
ID3
RAG1/2
ID3
HEB
E2A
MYB
RUNX1
TCRD/TCRG
Eδ/Eγ
V(D)J recombination


TRD (chr 14, + strand)
TRAV14/DV4
TRAV23/DV6
TRDV1
TRAV29/DV5
TRAV36/DV7
TRAV38-2/DV8
TRDV2
TRDD
TRDJ
12
31
42
3
Eδ
TRDC
TRDV3
TRAV
5'
3'
TRAJ
early gestation
late gestation
TRG (chr 7, − strand)
TRGV1
TRGV2
TRGV3
TRGV4
TRGV5
TRGVP5
TRGV6
TRGV7
TRGV8
TRGV9
TRGV10
TRGV11
TRGJ
P1
P
1
TRGC1
TRGJ
P2
2
TRGC2
Eγ
5'
3'
early gestation
late gestation

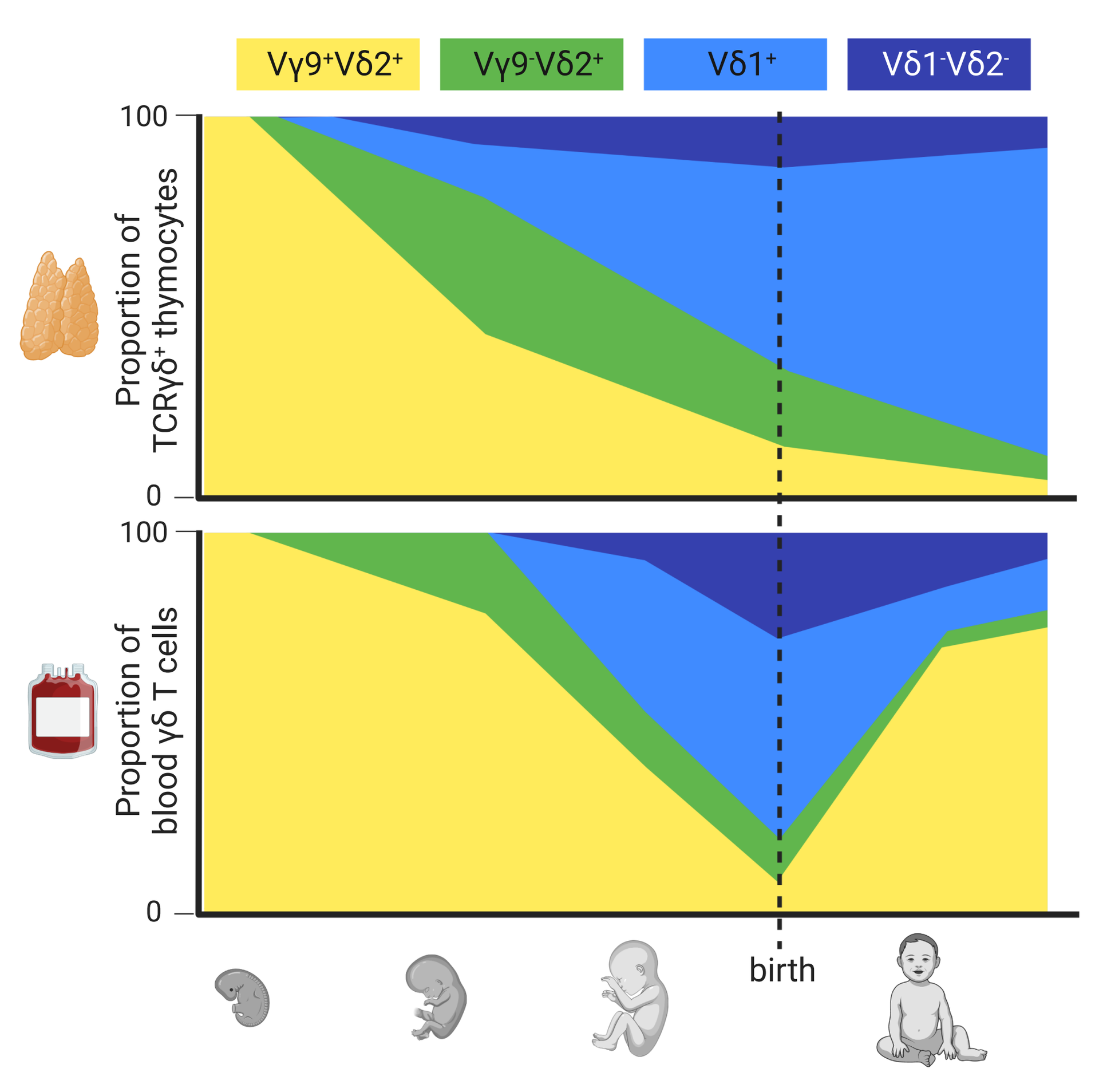

Vγ9+Vδ2+
Vγ9-Vδ2+
Vδ1+
Vδ1-Vδ2-
100
Proportion of TCRγδ+ thymocytes
0
100
Proportion of blood γδ T cells
0
birth

Figure 1

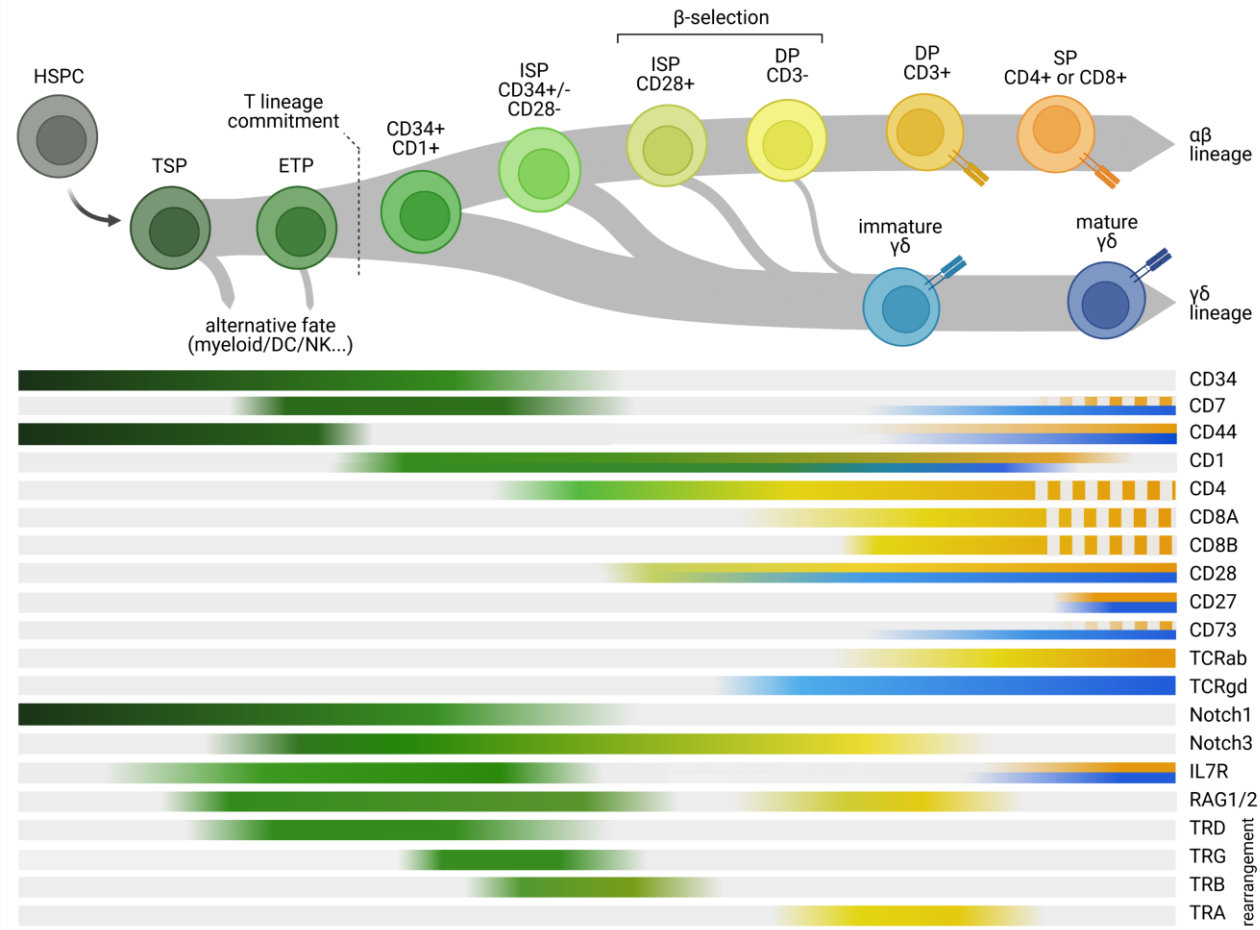

β-selection
HSPC
T lineage commitment
TSP
ETP
CD34+ CD1+
ISP CD34+/- CD28-
ISP CD28+
DP CD3-
DP CD3+
SP CD4+ or CD8+
αβ lineage
immature γδ
mature γδ
γδ lineage
alternative fate (myeloid/DC/NK...)
CD34
CD7
CD44
CD1
CD4
CD8A
CD8B
CD28
CD27
CD73
TCRab
TCRgd
Notch1
Notch3
IL7R
RAG1/2
TRD
TRG
TRB
TRA
rearrangement

Figure 2

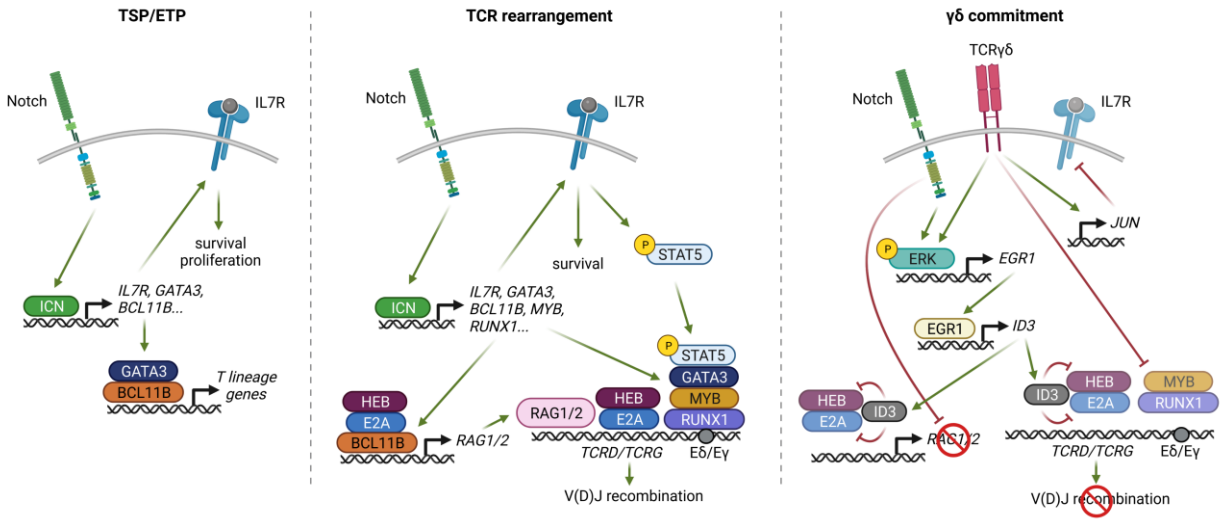

TSP/ETP
Notch
IL7R
survival
proliferation
ICN
IL7R, GATA3,
BCL11B...
GATA3
BCL11B
T lineage
genes
TCR rearrangement
Notch
IL7R
survival
STAT5
ICN
IL7R, GATA3,
BCL11B, MYB,
RUNX1...
STAT5
GATA3
HEB
E2A
BCL11B
RAG1/2
RAG1/2
HEB
MYB
E2A
RUNX1
TCRD/TCRG
Eδ/Eγ
V(D)J recombination
γδ commitment
TCRγδ
Notch
IL7R
JUN
ERK
EGR1
EGR1
ID3
HEB
E2A
ID3
RAG1/2
ID3
HEB
MYB
E2A
RUNX1
TCRD/TCRG
Eδ/Eγ
V(D)J recombination

Figure 3

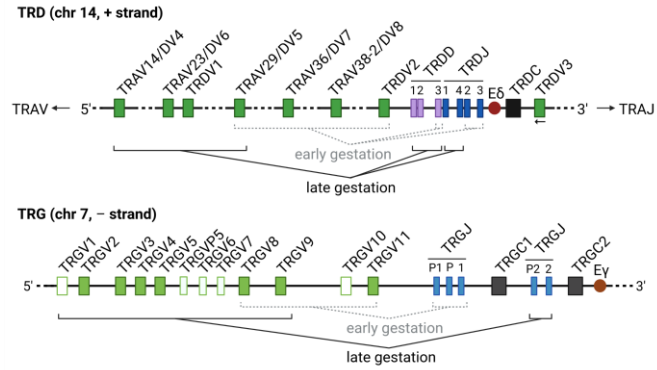
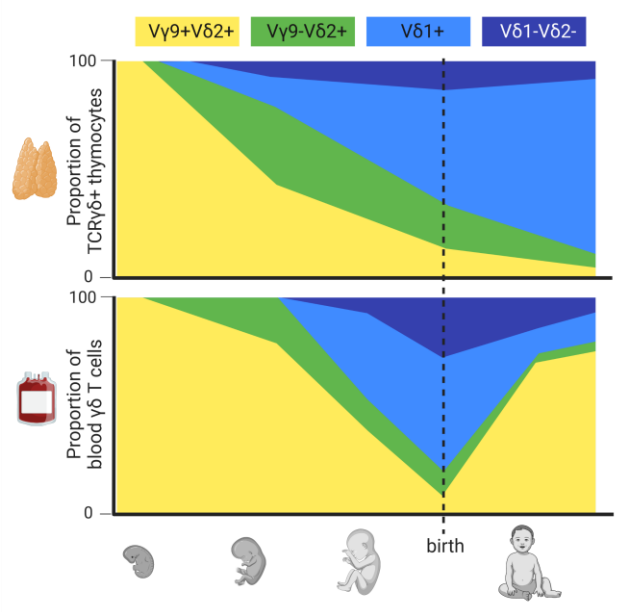

TRD (chr 14, + strand)
TRAV14/DV4
TRAV23/DV6
TRDV1
TRAV29/DV5
TRAV36/DV7
TRAV38-2/DV8
TRDV2
TRDD
TRDJ
12
31
42
3
Eδ
TRDC
TRDV3
TRAV
5'
3'
TRAJ
early gestation
late gestation
TRG (chr 7, − strand)
TRGV1
TRGV2
TRGV3
TRGV4
TRGV5
TRGVP5
TRGV6
TRGV7
TRGV8
TRGV9
TRGV10
TRGV11
TRGJ
P1 P 1
TRGC1
TRGJ
P2 2
TRGC2
Eγ
5'
3'
early gestation
late gestation

Figure 4


Vγ9+Vδ2+
Vγ9-Vδ2+
Vδ1+
Vδ1-Vδ2-
100
Proportion of TCRγδ+ thymocytes
0
100
Proportion of blood γδ T cells
0
birth