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INVITED REVIEW

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Surfing on the waves of the human γδ T cell ontogenic sea

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Summary

While γδ T cells are present virtually in all vertebrates, there is a remarkable lack of conservation of the TRG and TRD loci underlying the generation of the γδ T cell receptor (TCR), which is associated with the generation of species-specific γδ T cells. A prominent example is the human phosphoantigen-reactive Vγ9Vδ2 T cell subset that is absent in mice. Murine γδ thymocyte cells were among the first immune cells identified to follow a wave-based layered development during embryonic and early life, and since this initial observation, in-depth insight has been obtained in their thymic ontogeny. By contrast, less is known about the development of human γδ T cells, especially regarding the generation of γδ thymocyte waves. Here, after providing an overview of thymic γδ wave generation in several vertebrate classes, we review the evidence for γδ waves in the human fetal thymus, where single-cell technologies have allowed the breakdown of human γδ thymocytes into functional waves with important TCR associations. Finally, we discuss the possible mechanisms contributing to the generation of waves of γδ thymocytes and their possible significance in the periphery.

KEYWORDS
gammadelta, human, wave, fetus, thymus, TCR

1 | INTRODUCTION

γδ T cells are, like αβ T cells and B cells, lymphocytes that can rearrange gene segments at the DNA level with the potential to generate a set of highly diverse antigen receptors.[1,2] This tripartite division of immune cells possessing somatically diversified antigen receptors (γδ T cell receptor (TCR), αβ TCR and B cell receptor (BCR)) is a fundamental pillar of the immune system in jawed vertebrates (Gnathostomata).[3] Indeed, this division is traced back even in distant species with cryptic lymphocyte lineages, such as cartilaginous fish (Chondrichthyes),[4] and a similar division pattern can be observed in jawless vertebrates (Agnatha), despite arising from a distinct somatic recombination system that originates around 500 million years ago.[5] This striking conservation emphasizes the non-redundant roles of γδ

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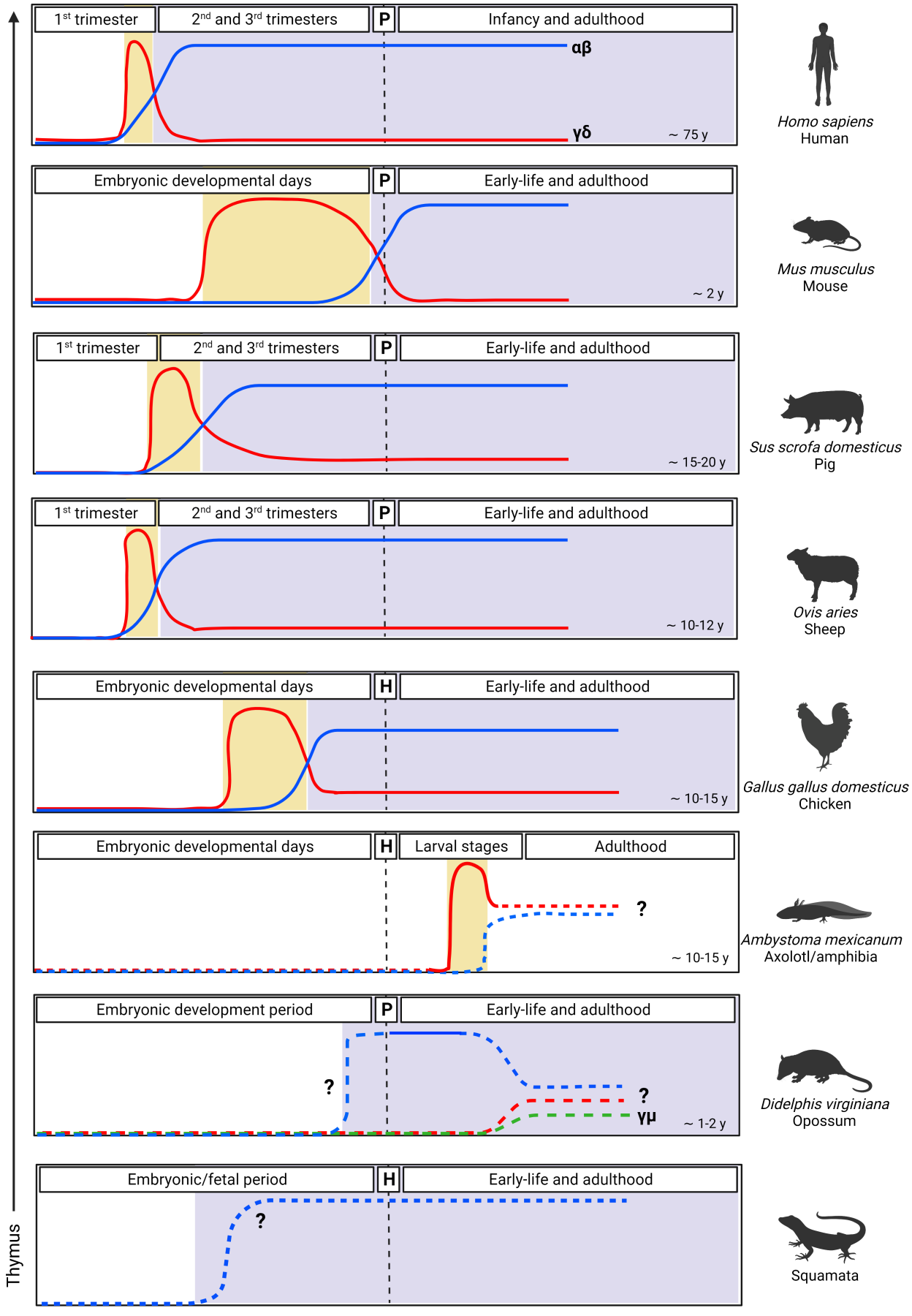
T cells besides αβ T cells, which is likely related to the fundamental different antigen recognition mechanisms between these two T cell lineages.[2,6,7] The absence of the γδ T cell lineage in squamates[8] and of the CD4+ αβ T cell subset in Atlantic cod[9] indicates that in certain species, selection processes may have favored overlapping functions in different lymphocyte lineages. γδ T cells can play various roles in the immune system including protection against cancer and infection, and they are increasingly being studied as a cellular target in cancer immunotherapy, where their MHC-independent activation provides a main advantage over αβ T cell-based approaches.[1,10–15]

The rearrangements of the TRG (gamma chain) and TRD (delta chain) loci take place in the thymus during the development of γδ T cells from a common αβ/γδ T cell precursor.[16] During TRG or TRD rearrangement, a V (variable), D (diversity; only for TRD), and J (joining) gene segments are joined to form a final TR chain. The variability created during the V(D)J recombination is significantly enhanced by the junctional diversity, which includes the introduction of random nucleotides (N additions), a process mediated by the enzyme Terminal deoxynucleotidyl Transferase (TdT). The most variable domain, that includes these N additions and is usually accountable for antigen recognition, is found in the complementarity determining region 3 (CDR3) of the TRG and TRD chains. The pairing of a single TRG with a TRD chain at protein level results in the final TCR expressed on the surface of the γδ T cell. In mice and humans (and other primates), γδ T cells are usually divided into subsets based on the type of V region used (encoded by a TRGV or TRDV gene segment) in the surface-expressed γδ TCR.[1] In humans, γδ T cells can be divided into the innate-like phosphoantigen-reactive Vγ9Vδ2 T cells (V parts of the gamma and delta chains encoded by TRGV9 and TRDV2 gene segments, respectively), and the more adaptive-like nonVγ9Vδ2 γδ T cells.[1,11] Despite the increasing knowledge about the effector functions and TCR recognition modalities,[17–20] only little is known about the thymic development of human γδ T cells. Indeed, most of the information regarding the thymic development of γδ T cells unsurprisingly comes from mice, given the availability of experimental tools to create sophisticated mouse models to study the complex pathways of γδ T cell development in vivo and also the limited availability of human tissue samples. While these mouse model studies have provided important insights into the biology of γδ T cell development,[21–25] the translation of these findings in humans is hampered by the lack of conservation of the TRG and TRD loci.[19] This is reflected in the generation of different types of γδ T cells in different species such as murine dendritic epidermal T cell (DETC) subset in the skin epidermis that is not observed in human, and human phosphoantigen-reactive Vγ9Vδ2 T cells that are absent in mouse.[26] Thus, there is clearly a need to shed more light into human γδ T cell development before any translation to the clinic. For example, data in this research line could contribute to *de novo* generation of human γδ T cells which may overcome some of the limitations, such as T cell exhaustion, encountered during clinical trials targeting γδ T cells.[14,27,28] In addition, such studies can highlight the "core characteristics" of γδ thymocyte development shared among different species along the identification of species-specific features.

Here, we review different pieces of evidence to reconstruct the puzzle of human γδ T cell ontogeny. We put first the human fetal thymic γδ T cell wave into context of the other early-life waves observed in a series of different vertebrate species. Then, we break down this human γδ thymocyte wave into their different constitutive components with a focus on recent insights obtained with single-cell technologies, and compare it with postnatal thymic γδ T cell development. Finally, we propose possible mechanisms involved in the generation of fetal γδ thymocyte waves and discuss their significance in the human periphery.

FIGURE 1 γδ T cells appear early in ontogeny in the vast majority of vertebrate species. Since their discovery in mice, γδ T cells have been described to develop in the thymus early in ontogeny and this event has been reported to be strikingly conserved across the animal world (jawed vertebrates). Animals with short periods of gestation display a T cell compartment development, and consequently a γδ T cell window (period of time where γδ T cells are the only mature T cell subset), shifted toward mid-late gestation compared with other species such as human. In mice (*Mus musculus*), γδ T cells appear first in thymus at embryonic day (E) 13 while mature αβ T cells are reported to arise during last days of gestation and first days of life.[31] In a similar fashion, chicken (*Gallus gallus domesticus*) γδ thymocytes have been detected at E12 while αβ thymocytes become the major subset at E17 close to hatching (E21).[35,36] Despite having a different life cycle, the amphibian axolotl (*Ambistoma mexicanum*) has also been reported to generate first the γδ T cell lineage in the thymus. In the work of Fellah and colleagues, rearranged TRD sequences were reported by RT-PCR at 7-week posthatching in larval head extracts (which contain the thymus), while TRB sequences could not be detected until 8 weeks.[33,34] Besides this, no further information is known about the abundance of the αβ and γδ T cell lineages in axolotl (depicted by dotted lines). Opossum (*Didelphis virginiana*) is an exception in the short-gestation group of species, as rearranged α and β chains have been detected during the first day of life while δ, γ and μ sequences appear in the following days after birth (μ is a constitutive chain of the γμ TCR that is exclusively present in some species from the marsupial lineage). As this observation is based on RT-PCR experiments, we do not know yet the abundance of the different T cell subsets in the opossum thymus (depicted by dotted lines). CD3ε+ cells that have been detected in the thymus of opossums at 48-24h prior to birth, based on the previously described PCR results, might belong to the αβ T cell lineage[212] (depicted by dotted lines before partum). A more extreme exception is represented by the squamata lineage (squamates), as this family of reptiles has been reported to have lost the TCR γ and δ loci during evolution.[8] When αβ T cells appear in squamate ontogeny is not known. The ontogeny of the T cell compartment in swine (*Sus scrofa domesticus*) and sheep (*Ovis aries*) displays similarities with humans, with adult-like levels of γδ thymocytes and a diverse TCR repertoire before birth. In both species the first γδ thymocytes are detected at ~40 days of gestation and remain at high levels until day ~55 when αβ thymocytes take the lead.[48,49] For more details of human T cell ontogeny see the section in the text "γδ T cell ontogeny, the first T cell wave across evolution." Estimated life span of species can be found at the bottom right of the boxes. y: years; P: Partum; H: Hatching. Red line: γδ thymocytes. Blue line: αβ thymocytes. Green line: γμ thymocytes. Figure created with BioRender.com website.



2 | γδ T CELL ONTOGENY, THE FIRST T CELL WAVE ACROSS EVOLUTION

γδ T cells are widely reported as the first T cells detected in the thymus gland (Figure 1), despite the interspecies differences concerning their TCR conformational structure[26] and respective genomic loci,[19,29] as well as their relative abundance.[30] This implies that for a (short) period, mature γδ T cells dominate the T cell compartment. Although this observation was first reported in eutherian mammals,[31,32] a similar ontogenic pattern has been described in species belonging to distant clades such as Amphibia[33,34] (Figure 1). Similarly, γδ T cells in chickens are present earlier in the thymus (embryonic day 12), while αβ T cells cannot be detected in significant numbers until late development.[35,36] In fact, avian and murine T cell ontogeny bear an interesting resemblance as in both species γδ T cells arise from mid-to-late gestation thymus[31,32,37] (Figure 1).

In humans, early reports using cloned T cells or detection of rearrangements by reverse transcription polymerase chain reaction (RT-PCR)/Southern blotting, describe the appearance of γδ thymocytes around 8-9 postconceptional weeks (PCW, corresponding to 10-11 weeks of gestation),[38,39] thus within the first trimester and well before the γδ T cell wave in mice (and birds and amphibians) (Figure 1). This is probably related to the timing of the organogenesis of the thymus, which begins in humans at 4-5 PCW, and the thymic primordium is colonized by hematopoietic progenitor cells (HPCs) by weeks 7–9.[40,41] Recent findings from single-cell transcriptomic studies have provided a comprehensive and detailed picture of the organogenesis and cellular landscape of the human thymus throughout gestation,[42–44] with one study reporting the presence of γδ thymocytes as early as 7 PCW.[43] Conversely, αβ T cells become prevalent in the thymus around 10 PCW, while the first naive αβ T cells egress the gland and migrate toward the periphery around 11 or 12 PCW.[43] Even-toed ungulates (Artiodactyla), which include several TCR γδ high (γδ-hi) species such as pig and goat,[30,45,46] appear to follow an "early" pattern of γδ thymocyte wave like in human (Figure 1), achieving adult-like levels of γδ thymocytes and a diverse TCR repertoire before birth.[46–48] Intriguingly, even though γδ-hi species possess high levels of γδ T cells in the periphery during mid-late gestation and the first year of life, the proportion of γδ thymocytes remains relatively low than their conventional αβ counterparts[30,49] (Figure 1). Finally, so far, Opossum and European Sea Bass are "the exception that proves the rule," since in these species γδ T cells do not appear first during the development in the thymus[50,51] (Figure 1).

This apparently conserved early appearance of γδ T cells during ontogeny could be possibly attributed to their unrestricted, thus faster, developing nature, which unlike their conventional counterparts does not require selection mechanisms based on complex antigen-processing machinery with associated presentation on major histocompatibility complex (MHC) class I or MHC class II molecules. Strikingly, this situation can be recreated using *in vitro* "thymus-like" culture systems such as OP9-DL1[52] or artificial thymic organoid (ATO),[53,54] further supporting the notion that γδ T cells are poised to develop before αβ T cells, even in artificial culture environments.

BOX 1 What constitutes a thymic γδ T cell wave?

Embryonic development of the immune system is characterized by an orchestrated layered development of distinct members of the hematopoietic compartment. This behavior is the basics of the model of layered development of the immune system, which proposes that timely controlled waves of progenitors (and their corresponding progenies) are produced to support distinct needs during the development of the organism. Some of the cell subsets produced in early ontogeny (embryonic, fetal and perinatal period) have been described to persist in most adult tissues and include diverse cell types such tissue-resident macrophages, mast cells or ILCs.[134,201–204] The described ontogenic waves in the non-lymphoid compartment are defined by two fundamental concepts: (i) they originate from time and/or niche-specific pools of precursors; (ii) precursors give rise to cell types with specific associated functions. The thymic development of distinct (mouse) γδ T cell subsets adds an additional layer on top of this "common" ontogenic wave features, namely the presence of wave-specific T cell receptor gene rearrangements, that are associated with particular functional profiles.

However, we propose that these early γδ T cells waves are not just a "side effect" of T cell developmental mechanisms but have been selected through evolution in order to serve critical functions in early life, either within the thymus itself[55] or in peripheral tissues after their export from the thymus.[56–67] In line with this, in several γδ-hi species (chicken, sheep and medaka) thymocytes follow distinct intrathymic trajectories of (compartmentalized) development without intermixing with αβ T cells, which may indicate a specific process of selection.[35,68,69]

3 | SEEKING FOR THE HUMAN γδ T CELL WAVES… A FIRST GLIMPSE?

The notion of a waved-based ontogeny of the γδ T cell compartment was first established in mouse.[31,32] Since its discovery, the layered development of these cells has been expanded until encompassing successive waves of γδ T cell subsets, which arise from the thymus during fetal, neonatal and adult life with distinct tissue-homing properties.[21,70] Each of these murine waves are linked to defined effector profiles (mainly associated with either IFN-γ or IL-17 production) and are associated with specific TCR features, that is: A specific Vγ segment is attributed per wave and the early fetal waves are associated with invariant CDR3 sequences (without junctional diversity, common in all mice).[70] Thus, mouse research set up the constitutive parts of γδ ontogenetic waves (see Box 1 "What constitutes a thymic γδ T cell wave?").

Since the first observations of layered development of γδ T cells in mouse were reported, several groups started seeking actively for parallels to these events in human.[38,39] Experiments using cloned T cells[38] or detection of rearrangements by RT-PCR/Southern blotting on whole thymic tissue *ex-vivo*[39] showed a TRDV2-based wave with minimal junctional diversity in the fetal thymus (8-22 weeks of gestation) that is paired with germline encoded TRG sequences, while diverse TRDV1-containing and TRGV-containing CDR3 sequences are found in the postnatal thymus (Figure 2A). A few years later, the newly identified phosphoantigen-reactive Vγ9Vδ2 subset[71–73] was reported to be detected at high prevalence in the fetal liver as early as 5–6 weeks of gestation,[74] becoming the *de facto* first human (prethymic) γδ T cell wave.

In more recent years, our group has focused on the assessment of the γδ TCR repertoire on sorted human fetal and postnatal γδ thymocytes *ex-vivo* by high-throughput sequencing, thus capturing thousands of CDR3γ and CDR3δ sequences. This allowed us to identify a distinct fetal thymic γδ TCR repertoire including the discovery

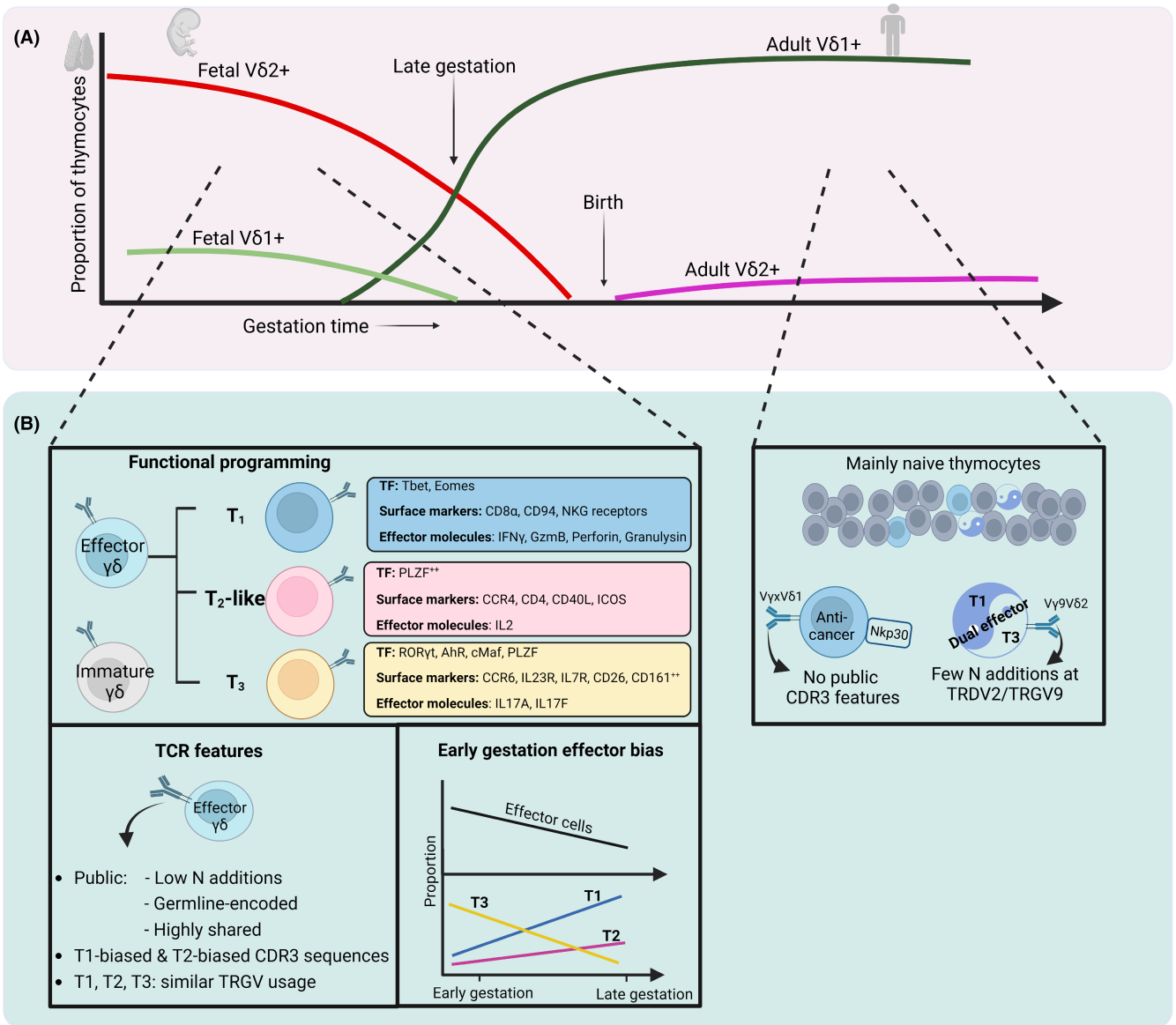


FIGURE 2 Human γδ T cell ontogeny: the old and the new waves. (A) Several techniques prior to the single-cell era (mainly TCR sequencing) indicate the presence of γδ T cell waves in the human thymus. See section in the text "Seeking for the human γδ T cell waves… a first glimpse?" for further explanation. (B) (sc) RNA gene expression (RNA-seq) combined with sc γδ TCR sequencing reveals new aspects from human γδ T cell ontogeny. Effector fetal γδ thymocytes are committed to either a type 1 (T1), a type 3 (T3) or a type 2-like (T2) effector fate, are enriched for public CDR3 features and T1 and T3 cells display enrichments for specific CDR3 sequences. These 3 effector clusters display a wave-like pattern depending on gestation age. By contrast, the pediatric thymus generates mainly γδ T cells with a naive phenotype. Two small effector populations can be detected: i) a TRDV1 (Vδ1+) subset with diverse CDR3 features that might be the origin of previously described anti-cancer adult γδ T cells[103,105,106]; ii) a small effector subset that is highly biased toward Vγ9Vδ2 TCR usage and shows a mixed type1/type3 effector profile.

of public fetal-specific CDR3γ and CDR3δ sequences,[1,75,76] which is paralleled by the presence of programmed γδ thymocyte effector functions in the fetus.[76] Of note, while Vδ1+ γδ T thymocytes are only a minority in the fetal thymus, the TRDV1-asscociated CDR3 sequences are clearly consisting of a fetal repertoire that is distinct from the later postnatal/adult Vδ1+ wave[76] (Figure 2A). Furthermore, we were able to show that the postnatal thymus is capable of producing a small percentage of Vγ9Vδ2 T cells that were developmentally unrelated to the Vγ9Vδ2 T cells from the fetal wave as their CDR3 sequences displayed profound differences such as increased level of N additions and distinct TRDJ segment usage[75] (Figure 2A). In addition, we found that fetal and adult stem cell progenitors produced *in vitro* the respective Vγ9Vδ2 progeny further supporting the notion that two distinct developmental waves of Vγ9Vδ2 T cell exist in humans.[75] This research brought into the spotlight a key difference between mouse and human γδ T cell ontogeny: while in mice, the development of γδ T cells is guided by exclusive TRGV gene rearrangements and thus the developmental-restricted appearance of specific Vγ-subsets, this appears not to be the case for humans. This discrepancy may be due to the highly different organization of the TRG locus between the two species: In the mouse, the TRGV gene segments are organized in various gene cassettes with associated TRGJ and TRGC segments, while in human, the TRGV gene segments are not interspersed with TRGJ or TRGC gene segments.[19,77] The appearance of a "second" postnatal/adult Vγ9Vδ2 T cells wave in humans might be related to their much longer life span (ca. 75 years) than mice (ca. 2y). The adult Vγ9Vδ2 T cells may then serve an important role until older age in humans since they appear to be resistant toward senescence upon aging.[78]

Apart from the phosphoantigen-reactive $V\gamma9^{+}V\delta2^{+}$ T cells, the fetal thymus generates plenty of $V\gamma9^{-}V\delta2^{+}$ γδ T cells, possessing invariant CDR3 sequences and programmed effector functions.[76] As with fetal Vγ9Vδ2 thymocytes, the innate effector programming and the public CDR3 features are linked to the nature of the fetal progenitor cells.[76] The intricacies of the effector thymic programming of both Vγ9Vδ2 and non-Vγ9Vδ2 γδ T cells were very recently revealed thanks to the application of the single-cell technologies,[79] as we discuss in the next section ("Breaking down the waves in the single cell era").

The dynamics of γδ T cell subsets in the fetal blood compartment throughout gestation further expands the insight into the fetal thymic/liver Vδ2-based wave and the postnatal Vδ1-enriched wave.[80] Effector Vγ9Vδ2 cells containing semi-invariant TCR sequences with low junctional diversities were identified as the dominant subset in blood during the late second trimester of gestation.[80] These cells were shown to be gradually replaced by $V\delta1^{+}$ γδ cells that increased until becoming the dominant subset in cord blood at term delivery.[80] In adult blood, TCR sequencing experiments defined the $V\delta1^{+}$ cells as adaptive-like, as preferential private expansion of specific clonotypes were observed across the life span[81] and under pathogenic challenges.[82–84] However, pathogenic challenge (cytomegalovirus, CMV) during fetal life results in a striking expansion of a public invariant Vγ8Vδ1 TCR in cord blood,[60] a TCR that is exclusively generated in the fetal thymic $V\delta1^{+}$ wave[76] (Figure 2A). A similar observation was found for $V\gamma9^{-}V\delta2^{+}$ γδ T cells: In adults, cells with this type of TRGV/TRDV usage constitute a very small subset and are described as adaptive-like and CMV-reactive,[85,86] while in the fetal thymus[76] and cord blood upon CMV-challenge,[60] they are highly prevalent and display public/invariant TCR features. We propose that during fetal development, γδ T cells with public/invariant γδ TCRs seed fetal peripheral tissues, such as lung or intestine, and that upon recognition of infected cells, they expand highly explaining their presence in the blood of CMV-infected fetuses.

4 | BREAKING DOWN THE WAVES IN THE SINGLE-CELL ERA

As we have shown in the previous section ("Seeking for the human γδ T cell waves… a first glimpse?"), the accumulation of distinct pieces of evidence in recent years has supported the notion of distinct waves in human γδ T cell ontogeny. However, a profound and deep characterization of these waves was lacking. Specifically, in the fetal mouse thymus, distinct waves of innate effector γδ T cells have been described, while this type of programming has been difficult to study in human due to the scarcity of fetal tissues. In a recent study, we took advantage of combining single-cell (sc) RNA gene expression (RNA-seq) with sc γδ TCR sequencing to investigate human γδ T cell development in fetal and pediatric thymus samples[79] (see also Box 2 "Study of γδ T Cells in the Single-Cell Omics era"). Our results significantly extend the nature of human γδ T cell ontogeny providing new insight into several levels (Figure 2B) that we believe to be part of the fundamental core of a γδ T cell wave (see also Box 1 "What constitutes a thymic γδ T cell wave?").

4.1 | Innate functional programming

Despite sorting for γδ TCR^{+} cells, fetal and pediatric thymocytes display a broad landscape of distinct maturing cell states, with the most immature cells being identified by the presence of *CD1* transcripts.[69,77] In the heterogeneity of the fetal thymus single-cell dataset, three clusters are identified whose transcriptional profile can be linked to the three major immune effector modules, that is, type 1, 2 and 3 immunity[79] (Figure 2B). The type 1 and 3 clusters show remarkable similarities with equivalent innate-like profiles described in mouse γδ ontogeny, γδIFN-cells and γδ17-cells, respectively.[21–23,87] While in humans, IL-17-producing γδ T cells have been described in certain pathological contexts,[88–93] the presence of a dedicated innate programming toward IL-17 production was not identified before in human thymus.[94] The fetal single-cell γδ thymocytes allowed the unambiguous identification of human γδ17 precommitted T cells, as *IL17A* and *IL17F* transcripts were only present in the type 3 cluster.[79] Tan and colleagues reported the presence of a type 3-associated cluster in cord (term delivery) and adult blood γδ T cells that show fetal-like TCR features, but no IL-17 transcripts

BOX 2 Study of γδ T Cells in the Single-Cell Omics era

In recent years, the assessment of gene expression at the single-cell level has revolutionized the study of the immune system, especially in humans where the scarcity of material drives research toward holistic study approaches. Single-cell omics techniques offer the possibility to capture the heterogeneity of samples almost entirely, thus generating "soups" of distinct cell types and cell states that would otherwise go unnoticed with traditional "bulk" omic approaches (i.e., RNA-seq). In parallel, new computational tools have been developed to maximize information extraction from the single-cell transcriptome, including lineage tracing methods that open the possibility of dissecting the molecular events that rule the pathways of the cell differentiation landscape. This "maximization approach" has been useful for scientists studying the ontogeny of the human immune system, overcoming the difficulties generated by the paucity of embryonic-derived or adult tissue samples, and has recently led to the generation of dense and profound datasets termed cell atlases.[42,43,205] However, the identification of γδ T cells has remained challenging in these complex cell mixtures due to the low cell capture rate (low abundance in the leucocyte fraction) and ambiguities generated by the absence of curated markers to identify these cells only by gene expression. For instance, in addition to the innate-like functional profile of γδ T cells that can be shared by other innate-like cell types, TRDC and TRGC gene segments (components of the γδ TCR) have been reported to be expressed in other immune cell types.[206–210] Despite the development of several tools to improve γδ T cell annotation in "noisy" transcriptomic datasets,[205,211] γδ T cell sorting (magnetic or fluorescence-associated) or cell hashing with specific γδ TCR antibodies should be kept as a gold standard in order to comprehensively study these cells at the single-cell level. Furthermore, immune repertoire profiling coupled with transcriptomics at the single-cell level using Single Cell VDJ 5' technology from 10x Genomics can provide information about the developmental trajectories and the TCR fingerprint of γδ T cells.

could be identified.[95] This indicates that in human, the expression of IL-17 (*IL17A* and *IL17F*) is highly regulated, and thus restricted to an early fetal period. Indeed, the earlier the gestation time, the higher the level of observed IL-17 transcription.[79] Besides the type 1 and 3 effector clusters, we were able to identify a cell cluster enriched for the expression of type 2 immunity genes such as *CCR4*, *CD4*, *IL4R*, or *CD40LG*[79] (Figure 2B) However, we termed the cluster as "type 2-like" because of two reasons: (i) the absence or poor expression of the type 2 signature cytokines *IL4*, *IL5*, and *IL13*; (ii) the high expression of *ZBTB16* (encoding the promyelocytic leukemia zinc finger (PLZF) transcription factor) and CD4, and the presence of IFNγ-producing cells reminiscent of the mouse iNKT-like γδ cells or γδNKT cells from fetal origin.[96–99]

The picture is highly different in pediatric γδ thymocytes, as here most cells do not show any signs of innate functional programming. Surprisingly, a small cluster has a blended type 1 and type 3 transcriptional profile and coincidentally, it is enriched with *TRDV2* and *TRGV9* transcripts (Figure 2B). These "type 1/3" postnatal Vγ9Vδ2 thymocytes may be the source of the small fraction of IL-17$^+$IFN-γ$^+$ cells present in *in vitro* stimulated adult Vγ9Vδ2 T cells[100] and might contribute to the IL-17 production in adult pathogenic conditions.[94] A similar IL-17 induction in the periphery under certain conditions (IL-23 exposure and tumor microenvironment) has also been reported in mouse Vγ4$^+$ γδ T cells from neonatal/adult origin.[101,102] Additionally, a pediatric γδ thymic cluster containing *TRDV1* transcripts is enriched for the natural killer (NK) receptor *NCR3* (Nkp30) and mediators of cytotoxicity (Figure 2B), suggesting that these cells can serve as the thymic origin of the anti-cancer Vδ1$^+$ NKp30$^+$ γδ T cells found in the blood of adult subjects.[103–106] Finally, assessment of the egress potential in the pediatric thymus data showed that this feature was more widely spread than in the fetal thymus dataset and includes TRDV1 clusters that did not show any innate preprogrammed features[79] (Figure 2B). These clusters are probably proving a source of more "naive-like" γδ T cells in the periphery that could be transformed to type 1 γδ T cells when receiving the right (cytokine) signal in the periphery.[107]

4.2 | TCR features

The advantage provided by the coupled sc RNA-seq and sc TCR-seq technique emerges from a precise characterization at the TCR level of the distinct cell subsets identified at transcriptomic level and the concomitant assessment of the origin and developmental pathways followed by these cells.

Flow cytometry results from second trimester fetal thymus showed that the proportion of Vδ1$^+$ cells display a steady increase with increasing gestation time, although Vδ2+ cells remain the main subset at the last gestation time measured (22wk).[79] Importantly, Vγ9$^-$Vδ2$^+$ γδ cells rather than Vγ9$^+$Vδ2$^+$ γδ T cells are the most abundant Vδ2$^+$ subset during this period in thymus,[79] while in the peripheral blood Vγ9$^+$Vδ2$^+$ T cells are much more abundant than the Vγ9$^-$Vδ2$^+$ subset.[80] This indicates that Vγ9$^-$Vδ2$^+$ γδ T cells leaving the thymus may seed distinct peripheral tissues than blood. In line with this, a recent atlas paper that combined sc RNA and TCR-seq to characterize the fetal immune system in peripheral tissues, reported that the γδ T cell fraction contains a large proportion of TRDV2 clones paired with TRGV8 chains.[108] This homing of Vγ9$^-$Vδ2$^+$ γδ T cells to solid tissues (for example to subcutaneous fat tissue[109]) could explain the specific association of the type-3 profile to blood Vγ9Vδ2 by Tan and colleagues.[95] TRDV2 cells are increased upon maturation toward the three effector fates in the fetal thymus.[79] Intriguingly, the TRDV2-containing CDR3

sequences of the three-effector clusters are enriched for public CDR3 features, including low amount of N additions, shorter CDR3 length, and increased proportions of TRDV2 sequences shared among individuals (high publicity), suggesting some sort of selection/restriction of the TRDV2 repertoire upon maturation toward effector profiles[79] (Figure 2B). Although this effect on TRDV2 repertoire was observed at comparable levels in the three effector clusters, we were able to identify enrichments of unique TRDV2-containing CDR3 sequences in the type 1- and type 2-like clusters, indicating that germline public TRDV2 sequences can influence the generation of specific effector profiles in human γδ T cell ontogeny[79] (Figure 2B). In contrast to the presence of these specific CDR3 sequences, we observed similar proportions of both $V\gamma9^{+}V\delta2^{+}$ and $V\gamma9^{-}V\delta2^{+}$ subsets in the three fetal effector clusters[79] (Figure 2B). This is different in the pediatric thymus, where a type 1/type 3 effector cluster was identified that was highly enriched for the $V\gamma9^{+}V\delta2+$ subset[79] (Figure 2B). Finally, this insight into the human thymic development of γδ T cells highlights the contrast of the TCR game between mouse and human: In mouse fetal waves, the TRGV (Vγ) segment is used as a proxy to identify distinct preprogrammed functions, while in human, the fetal thymic effector programming is rather controlled by more subtle TRD CDR3 features.

4.3 | Early gestation effector bias

The scRNA/TCR fetal thymus dataset was designed to include a series of fetal thymuses ranging from 14 to 22 weeks of gestation time in order to gain insight into potential "time-restricted" waves existing during human fetal γδ T cell ontogeny.[79] Thanks to this design, we found that the proportion of effector populations is highest at early gestation time points, indicating that the earlier the gestation the more biased are the γδ progenitors[76] toward the acquisition of effector fates (discussed in more detail in the next section "Mechanisms involved in the of generation of fetal γδ T cell thymocyte waves") (Figure 2B). One possible explanation is the germline TRDV2-CDR3 restrictive nature needed to generate these effector cell types, as TdT levels and thus N additions are higher at later gestation time points resulting in a reduced amount of the fetal public TRDV2-CDR3 sequence rearrangements. However, type 3-committed cells are more enriched at earlier gestation indicating that some evolution in the nature of the progenitors may also play a role in the generation of specific effector profiles during human fetal γδ T cell ontogeny. In line with this, the presence of a putative interspecies type 3 precursor among the immature fetal γδ thymocytes is detected, with a high abundance in early gestation thymi.[79,110] Whether this progenitor program is established prior to TCR expression in human remains to be established.

5 | MECHANISMS INVOLVED IN THE GENERATION OF FETAL γδ T CELL WAVES

Both intrinsic factors (stem/progenitor cells) and extrinsic factors (factors present in the fetal thymic environment) can contribute to the generation of fetal γδ thymocyte waves. Here, we discuss these possible factors and associated mechanisms, focusing on studies in mice where they have been mostly studied, and link them to recent observations in humans[76,79,111] (Figure 3).

5.1 | Stem cells and progenitors

In adulthood, newly generated cellular components in the blood, including white blood cells, are derived from hematopoietic stem and progenitor cells (HSPC) from the bone marrow (BM). T cells arise from immigrating precursor cells that lose their self-renewing properties while undergoing T cell lineage specification in the thymus.[16,112] Originally, it was thought that definitive (adult) BM-HSPC could give rise to all hematopoietic cell types including lymphocytes, but experiments based on adoptive transfer and fetal thymic organ culture systems showed that murine fetal liver (FL) HSPCs, unlike adult BM HSPCs, preferentially give rise to innate-like lymphocytes such as B-1a cells[113–115] and fetal-derived γδ T cell subsets[116–118] that persist as self-renewing tissue-resident cells in the adult. Other comparative studies further extended the differences between fetal- and adult-derived HSPCs, with early life HSPCs displaying different cell surface markers and proliferative and engrafting capabilities.[119–121] The transcription factor SRY-related HMG-box gene 17 (SOX17) is described as a key determinant involved in the establishment of the fetal HSPC identity, and its gradual downregulation is linked to the developmental switch toward adult HSPC phenotype.[122,123] The discovery of the post-transcriptional network formed by the RNA-binding protein Lin28b and the microRNA (miRNA) family lethal-7 (Let-7) provided a first clue to the fetal-HSPC constitutive (molecular) mechanism that is directly involved in the generation of fetal-restricted innate-like lymphocytes.[124] Lin28b is highly expressed in embryonic tissues and stem cells[125,126] where, among other functions, it represses the biogenesis of Let-7 family miRNAs. Yuan and colleagues showed that an ectopic expression of Lin28b in BM adult HSPCs was sufficient to generate fetal-HSPC dependent cells in mice.[124] In human, Lin28b action has been suggested to be responsible to drive the pro-tolerogenic bias in the fetal conventional αβ T cell compartment.[127] However, in the field of human innate lymphocyte ontogeny, we showed that Lin28b promotes the development of effector functions in γδ thymocytes: overexpression of Lin28b in HSPC leads to the production of γδ T cells with a fetal-like effector program[76] (Figure 3). Lin28b might promote the acquisition of this effector program by blocking the repressing action of Let-7 on the transcription factor PLZF,[128] as PLZF has been described to be important in the generation of different innate-like lymphocytes.[97] In the mouse, ectopic expression of Lin28b in adult HSPCs, leads to an increased production of the iNKTγδ T cell subset,[124] a γδ subset possessing an invariant germline encoded TCR (Vγ1Vδ6.3) restricted to the perinatal period and dependent on PLZF expression for its generation.[97] Of note, mouse invariant $V\gamma5^{+}$ γδ T cells, the first γδ T cell wave in

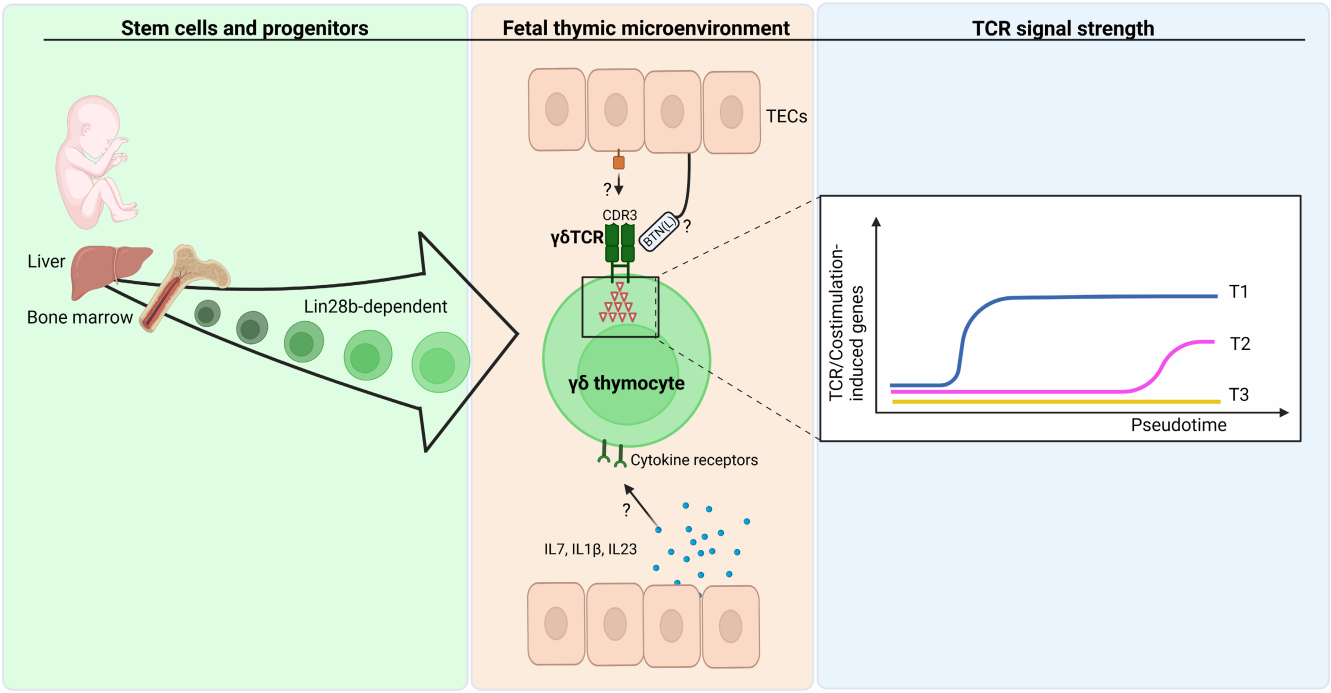


FIGURE 3 Possible factors involved in the generation of fetal γδ T cells in humans. Intrinsic factors such as the stem cell nature might be important regulators of fetal γδ T cell waves in the thymus. The RNA-binding protein Lin28b, of which the expression is highly restricted to fetal stem cells, has been shown to be a main regulator of the effector pre-commitment of fetal γδ thymocytes.[76] This influence on fetal γδ T cell development might be elicited at different stages of T cell development (depicted by the different cell sizes on the left side of the figure), with the highest level of Lin28b (dark green) present at the earliest stem/progenitor phase. Additional progenitor aspects, such as the anatomical niche of HSPCs may also influence this process. Fetal thymic microenvironment (TECs and other cell types) may act as an extrinsic factor through the expression of stage-specific ligands and/or cytokines as it has been shown before in mouse literature. Finally, and connected to this last point, our single-cell results indicate that TCR-agonist signaling events (identified by the expression of TCR signaling associated genes and genes involved in T cell co-stimulation) regulate the effector commitment of the fetal γδ T cell waves. For more details see section in the text "Mechanisms involved in the generation of fetal γδ T cell waves."

mice,[70] and the PLZF-dependent invariant second wave of Vγ6$^+$ γδ T cells[120] do not increase,[124] indicating that other intrinsic and/or extrinsic factors such as the fetal microenvironment play a role in the generation of these murine fetal γδ T cells (further discussed in the subsection "Fetal thymic environment").

We also reported an important role of Lin28b in driving the generation of a fetal γδ TCR repertoire in human γδ thymocytes.[1,76] It was previously shown in mouse models that a mechanism of short-homology repeat dictates the generation of invariant CDR3 sequences in fetal mouse γδ TCRs,[1,129,130] and we identified similar short-homology repeats in human that can drive invariant CDR3γ and CDR3δ sequences.[1,61,75,76] Overexpression of Lin28b in human HSPCs led to the generation of a more invariant γδ TCR repertoire, a mechanism that was linked to a reduced expression of TdT.[76] Based on this, we propose that Lin28b controls the generation of human fetal invariant γδ T cells in an active process by inhibiting TdT expression, which opens the possibility for the germline delta and gamma CDR3 rearrangements to occur by a short-homology repeat mechanism. This action on the TCR repertoire of Lin28b can then influence the effector commitment of fetal γδ T thymocytes, as effector clusters cells in the human fetal thymus γδ single-cell data are enriched for distinct TRD and TRG sequences that contain short-homology repeat sequences.[79] In addition to this TCR-mediated influence, Lin28b may modulate the effector program of fetal γδ thymocytes also by other non-mutual exclusive mechanisms.[1]

Besides the "Lin28b ecosystem" that characterizes fetal HSPCs, several lines of evidence point to additional intrinsic factors within the progenitors that might regulate the generation of innate-like waves of γδ T cells in mice. For instance, it has been shown that potential distinct HSPC waves with different T and B developmental potentials might co-exist during fetal and neonatal hematopoiesis in mouse[131] and humans.[132] In line with this, a layered progenitor colonization in the mouse thymus has been described, with early progenitors from embryonic day 13 showing increased potential to generate Vγ5$^+$ γδ T cells as opposed to a later wave of progenitors before birth that no longer produces fetal-restricted γδ T cells.[133] Additionally, the hematopoietic anatomical niche has been described to influence the generation of embryonic γδ T cell waves in mouse thymus.[134,135] The question that remains is: what molecules might regulate these distinct HSPC potentials? E protein family members, such as E2A or HEB, may be potential candidates for this role as their function appears to be involved in the temporal control of Vγ rearrangements.[136] In line with this, several proteins that are part of a transcriptional network that promotes the γδIL17 or type 3

fate in mouse early ontogeny, such as Sox4, Sox13, PLZF, and RAR Related Orphan Receptor A (Rora), are regulated by E protein activity.[22] Considering the high degree of conservation of Lin28b action on mouse and human HSPCs, it is tempting to speculate that other HSPC ontogenetic mechanisms are also conserved. For example, the high proportions of effector cell populations and the bias toward type 3 effector differentiation at earliest gestation time (Figure 2B) could be explained by a prewiring prior to TCR expression at the level of a precursor, and this could explain why we did not observe any type 3-associated CDR3 sequence in the fetal thymus single γδ T cell data.[79] This precursor could arise from fetal liver as it has been described that this organ dominates hematopoiesis until the mid-second trimester (around 16wk of gestation),[137–139] which it is coincidental with the reduction in type 3 proportion in the fetal thymus (at 17wk of gestation).[79] Later gestation γδ thymocytes might develop from an increased pool of BM-derived HSPCs, the dominant site of hematopoiesis during late second trimester (20 PCW),[139,140] which could be more limited in its commitment toward the type 3 lineage. After that, some additional signals from the BM niche may drive the transition/switch of the fetal-derived HSPC pool toward an adult HSPC phenotype (thus without Lin28b) during early life. The efficient sensing of the niche by HSPC can contribute to the change in their intrinsic potential.[141]

5.2 | Fetal thymic environment

The complex thymic microenvironment comprises epithelial and mesenchymal components that permit the normal development and the maturation of T cells. Little is known about the dynamics in the gland composition, especially in the epithelial compartment, during embryonic/neonatal life and the transition toward adulthood, and how this affects the developing immune system.[142–145] However, the study of γδ T cell ontogeny has provided indirect evidence that such dynamics exists in the organ, by indicating that developmentally restricted components of thymic stroma act as extrinsic factors that directly influence the development of γδ T cell waves. An example of this is observed in the development of DETC precursors (Vγ5Vδ1) that compose the first wave of γδ thymocytes in the mouse thymus.[31,118] FL-derived HSPCs are capable to generate Vγ5Vδ1 T cells only in fetal thymus organoid cultures (FTOC).[116,131] In line with an important role of the fetal microenvironment for the development of this murine γδ T cell subset is that ectopic expression of Lin28b in adult mice is not sufficient for their generation (see also the previous subsection 'Stem cells and progenitors').[124] One of these fetal exclusive thymic determinants is the butyrophilin-like (Btnl) protein Skint1, of which the expression by medullary thymic epithelial cells (mTECs) critically drives the selection and maturation of the Vγ5Vδ1 subset[146] (mechanism further discussed in "TCR strength signaling" subsection). Interestingly, Rankl$^+$ Vγ5Vδ1 cells influence on their turn the thymic organogenesis by promoting the maturation of mTECs via Rank signaling.[55] Of note, human fetal γδ thymocytes express as well high levels of *TNFSF11*, encoding RANKL protein.[79]

Fetal-restricted γδ17mouse waves have also been shown to be highly influenced by thymic stroma during their development. A homozygous missense mutation in the cortical TEC (cTEC)-specific proteasome subunit β5t leads to a deficiency of mature cTEC and substantially reduces mTEC numbers resulting in an increase in Vγ6 γδ T cell subset in adulthood, replacing the adult-like Vγ4 subset.[147] In line with this, mTECs have been recently shown to negatively influence the generation of Vγ6$^+$ cells through MHC class II,[148] a molecule of which the expression on the cell surface of mTEC is acquired upon their maturation.[149] Similarly, after a TEC-specific deletion of mechanistic target of rapamycin complex 1 (mTORC1), the controlled generation of γδ17 is lost and Vγ6 cells are continuously generated in the adult thymus.[150] Therefore, TEC maturation in the fetal thymus and normal mTORC1 functions may be responsible for timely switching from fetal (mainly Vγ6) to adult (mainly Vγ4) γδ17 cells.

Other additional extrinsic thymic determinants that influence γδ T cell differentiation are cytokines, with mTECs being an important source for their production.[151] IL-7 has been identified as an important driver of the expansion of mouse γδ17 cells during fetal thymic development.[152] A parallel with human may exist, as IL7R gene expression is enriched in the fetal thymus type 3 cluster, and it can be traced in immature cell states, suggesting that IL7 may be important for the generation of these cells in human fetal thymus[79] (Figure 3). Additional important cytokines involved in type 3 immunity such as IL-1β and IL-23, further support the differentiation of mouse γδ17 T cells in the OP9-DL4 co-culture system and in FTOC.[153]

Dynamics of thymic microenvironment during ontogeny is even less characterized in human. However, several lines of evidence indicate that changes in human thymic stroma might provide different windows of opportunity that support distinct T cell developmental programs. For instance, it seems that the fetal/neonatal thymus is more permissive toward or prone to the generation of specific αβ T cell subsets that undergo a stringent process of selection by thymic epithelium (agonist selection mechanism), such as CD4 αβ Tregs[154] and unconventional CD8 αβ thymocyte populations.[155] Some indications of the importance of fetal thymic microenvironment in the generation of fetal-restricted human γδ T cells can be found in experiments using the OP9-DL1 and OP9-DL4 in vitro culture systems. Culture of fetal-derived HSPCs (from blood or liver, 15-31 weeks of gestation) can generate γδ T cells with fetal-like properties at functional (innate-like) and TCR levels (germline-derived repertoire).[76] However, this system fails to mimic the complete fetal Vδ2$^+$ wave phenotype, as the Vδ2$^+$ output population is mainly composed of Vγ9$^-$Vδ2$^+$ cells, with the Vγ9$^+$Vδ2$^+$ subset accounting only for a small fraction of thymocytes.[75,76] A possible explanation is that the main fetal Vγ9Vδ2 progenitors are present at earlier gestation times than 15 weeks or are found in other anatomical origins than fetal liver or fetal blood. However, a similar diminished generation of the Vγ9Vδ2 subset has been observed recently in mouse-like human yolk sac hematopoietic development using pluripotent stem cell differentiation and the OP9DL4 system,[111] making the hypothesis of early embryonic timed progenitors as a main source of fetal Vγ9Vδ2 more unlikely. An alternative explanation could be found in the dependency

of Vγ9Vδ2 T cells on human-specific BTN for their phosphoantigen-reactivity.[17,18,156,157] While γδ T cells during their development in the OP9DL1 system may well interact with other human HSPC-derived cells,[52] including BTN(L)-expressing cells, interaction with (fetal) BTN-expressing TEC may be the driving force for a fetal wave of Vγ9Vδ2 T cells (Figure 3), signals that are absent in the mouse OP9 cells. It remains to be explored if a developmental time-restricted expression of distinct BTN(L) members exists in human thymus, and how this hypothetic event may regulate the ontogeny of human γδ T cell waves.

5.3 | TCR signal strength

Research in mice has shown that TCR signaling is required to determine the T cell lineage choice (αβ or γδ) followed by developing thymocytes,[158] with human research pointing toward a similar mechanism of commitment.[16] Furthermore, once the commitment step is surpassed, γδ TCR signaling is critically involved in the generation of cells poised toward IFN-γ production.[159–161] In particular, TCR-agonist selection plays an important role in the development of fetal Vγ5Vδ1 T cells and perinatal Vγ1Vδ6.3/6.4+ NKT-like γδ T.[162] As discussed above, Skint1 specifically expressed by TECs is required for the thymic maturation and effector differentiation of Vγ5Vδ1 γδ T cells. In the absence of this molecule DETCs are misdirected toward IL17 phenotype instead of the classical type 1 phenotype.[163] While evidence for direct Skint1-TCR binding is still missing, it has been suggested that a Skint1-based complex might provide TCR-mediated signals in a similar fashion to the described mode of action of other Btnl (mouse)/BTNL (human) molecules.[20] Trajectory analysis of human fetal-TRDV2 thymocytes revealed three distinct lineage pathways ending in the three effector clusters, with an early split between the type 1 lineage versus the type 2 and type 3 lineages.[79] Interestingly, human type 1-preprogrammed cells, akin to mouse γδIFN cells, may develop under conditions of TCR-agonist signaling as we detected an enrichment in the expression of genes described to be induced after TCR signaling along the type 1 lineage[79] (Figure 3). What the possible contribution of the type 1-enriched CDR3 sequence is (Figure 2B) to such strong TCR signal and/or whether BTN(L)-TCR interaction[17,18,20] contributes to this signaling remains to be explored (Figure 3). The common type 2/3 lineage is enriched for some naiveT cell markers that ends up generating type 2 and type 3 effector cells.[79] Although no TCR-signaling-associated signature can be found along the type 2/3 lineage, an increase in *CD5* expression is observed in the final type 2 effector cluster, which may suggest some late TCR signaling event in these effector cells (Figure 3), an observation that is in line with the suggested equivalent nature of these cells with the mouse perinatal NKTγδ T cell subset (Vγ1Vδ6.3/6.4).[164,165] Genes coding for proteins involved in T cell co-stimulation are also enriched in the human type 1 lineage, especially members from tumor necrosis factor receptor superfamily.[79] One of these genes is *CD27*, a surface marker that marks the commitment of γδIFN cells in mice and that plays an important role in the development of these cells, at least in part by inducing the expression of the lymphotoxin-β receptor and genes associated with trans-conditioning.[166,167] Whether a similar mechanism guides the generation of human type 1 fetal γδ thymocytes remains to be explored.

6 | WHY WAVES, YOU ASK?

Biological phenomena usually serve specific causes for them to persist in evolution. The rise of γδ T cells in waves is expected to fulfill missions contributing to the survival of the host. We have described the human γδ T cell waves based on their timing, TCR and programmed effector function and we will attempt here to explain their existence.

The first divergence of the waves concerns the effector versus naive γδ thymocytes observed in early versus late hematopoiesis. We hypothesize that the effector γδ T cells generated in early gestation follow specific assignments either linked to physiological roles or to infection control in a time where αβ T cells are not yet optimal.[168] The programmed effector function occurring in fetal development is further divided into the three types of immunity with a very first type 3 wave followed by a type 1/2 wave in mid-gestation.

The very first wave of effector γδ thymocytes is IL-17-biased (Figure 2B) which may serve non-immune roles after homing to non-barrier tissues. Indeed, mouse IL-17A-producing γδ T cells which reside in the adipose tissue promote thermogenesis after stimulating the IL-33 production by stromal cells, the proliferation of regulatory T cells and activating mitochondrial uncoupling protein 1 (UCP1) in adipocytes.[56] It has been also shown that mouse γδ T cells produce IL-17F, which stimulates the adipocytes to produce transforming growth factor-β (TGFβ), promoting tissue innervation by the sympathetic nervous system.[169] The early human type 3 precommitted γδ T cells express both IL-17A and IL-17F[79] which could help the fetus regulate its temperature or be prepared for birth, when the newborn faces a cold environment for the first time. Furthermore, meningeal mouse γδ T cells control short-term memory by producing IL-17A to boost brain-derived neurotrophic factor (BDNF) production by glial cells and enable glutamatergic (Glu) neuronal synaptic plasticity in the hippocampus.[57] The development of the human hippocampus is active at mid-gestation,[170] so this early human γδ17 wave might be crucial in the early stages of human brain formation. Interestingly, the fetal type 3-committed γδ T cells show transcriptomic similarities with Lymphoid tissue inducer cells (LTi).[171] These LTi cells are important in promoting the formation of secondary lymph nodes or Peyer's patches in the embryo, necessary structures to accommodate the coordinated function of the immune system.[172–175] So, the type 3 γδ T cells may provide helper functions in the formation of secondary lymphoid structures, during the 12-17 week gestational window where they both develop.[79,176]

Other than physiological functions, the type 3 γδ T cells may be involved in infection setups. There is evidence for protection by IL-17A-producing γδ T cells in children during *Clostridium difficile*

infection.[65] This is linked to intestinal γδ T cells, based on the fecal data of the children and parallel studies on mice.[65] So, the first wave of type 3 γδ thymocytes may home to the intestine in early life, to help fight bacterial infection. Finally, γδ T cells homing in the intestine in early development might fulfill roles in the crosstalk between immune cells and microbiota, notably in the selection of commensal communities.[177]

The second fetal wave is characterized by a rise of type 1 precommitted γδ T cells (Figure 2B). The type 1 bias is expected to help resolve infections and indeed there are studies in humans which evidence the *in-utero* response of γδ T cells to microbial attacks. First, the Vγ9Vδ2 T cells in the context of congenital toxoplasma infection, show increased cytotoxicity indicating their rapid response to infection and interaction with the phosphoantigen-producing parasite.[61] Second, the nonVγ9Vδ2 γδ T cells, including $Vγ9^{-}Vδ2^{+}$ and $Vγ9^{-}Vδ1^{+}$ (especially Vγ8Vδ1), expand in a unique fetal way (public clones) and react vividly during congenital CMV infection.[60] Certainly, these effector γδ T cells are also patrolling at birth, when the revolution of encountering a new world occurs, and the newborn body interacts with a multitude of organisms. The Vγ9Vδ2 T cells are the first cells to proliferate and widen their cytotoxic potential after birth, becoming a necessary weapon for the hurdles ahead.[62] Finally, the type 1 γδ T cells could also participate in physiological functions, via the high expression of granzyme A which can act on tissue remodeling of extracellular matrix[178] during fetal growth.

Finally, the fetal thymocyte programming includes a type-2-like effector cluster (Figure 2B). The type 2 precommitted γδ T cells could be of particular significance in helping in the production of antibodies during the first trimester when the maternal antibody transfer is relatively low.[179] One way that γδ T cells can interact with B cells to promote the development of B-cells into plasma cells is via costimulatory molecules such as CD40, Inducible T Cell Costimulator (ICOS), and CD28[180] which are expressed in the human γδ thymocytes[79] (Figure 2B). The contribution of γδ T cells to antibody production has been also investigated in the context of natural antibodies in early life, when the spontaneously activated γδ T cells might be in charge of shaping the developing repertoire of these antibodies.[181]

All these functions of the precommitted γδ thymocytes, theoretical/assumed or evidence-based, seem indispensable for the newborn baby to thrive. Why do they stop being generated by the thymus and a wave of naive, adaptive-like γδ T cells arise? Our hypothesis lies in the evolution of needs of the growing individual. The rapid growth of the developing infant slows down at around age 1 (after birth),[182] while the need to co-exist in a pathogen-loaded milieu brings new demands. It has been shown that mouse CD8 αβ T cells that are generated in a fetal wave specialize in rapid and innate-like functions, whereas those generated in an adult wave result in long-lived memory cells.[183,184] So, the shift from a vivid and fast response to a long-lasting one is preferred in adulthood, particularly to protect the host against reinfection. This may also occur to γδ T cells which while shifting from effector to naive thymocyte profile, they also shift from germline to diverse TCRs.[75,76] A restricted TCR might be more cross-reactive in order to maximize immune recognition by the small number of cells in early life.[185] In mouse CD8 αβ T cells, this has been linked to low TCR avidity.[186–188] In contrast, the post-natal/adult wave of thymocytes is affected by TdT, giving rise to a diverse TCR repertoire which in mouse CD8 αβ T cells is associated with increased avidity and specificity, a requirement for pathogen-specific memory responses.[185] Whether human fetal γδ T cells would also fall short in avidity because of their restricted TCR is not clear. This might depend on the type of TCR that is associated with a particular effector program, since fetal type 1-associated thymocytes show signs of strong TCR activation during their development.[79]

Since a similar pattern of adaptation seems to be applied in both the αβ and γδ T cell compartment, we wondered which are the fundamental advantages of possessing adaptive γδ T cells in postnatal life. Our hypothesis here is based on the differential mode of antigen recognition between αβ and γδ T cells. The latter recognize antigens in an MHC-unrestricted fashion which might be peptides, or not, processed or not (antibody-like fashion).[6] Additionally, γδ T cells are unique at sensing normality and playing a role in homeostasis.[189] Inspired by mouse data, constant interaction of the human γδ T cells with their BTN(L) ligands may be important in stress surveillance.[190–192]

It is noteworthy that γδ T cells have been considered innate, so their memory and adaptive features came as a surprise.[193,194] Similarly, other innate cells, such as NK cells, have been recently studied on their memory capabilities in infectious and cancer settings.[195–197] However, γδ T cells and NK cells cover distinct recognition needs. NK cells sense stress through missing self, whereas γδ T cells can establish a flow of constant sensing with their ligands (for example BTNL and intestinal γδ) and may regulate many components of tissue immunosurveillance, as it is described for mouse DETC.[192]

Finally, preferential localization of γδ T cells in barrier tissues, such as skin and intestine, promotes the rapid surveillance toward a plethora of tissue-disruptive challenges.[189] It is of increasing interest to investigate which γδ T cells home to which organs, at what time frames, and what mission, physiological, homeostatic or immune, they accomplish.

7 | CONCLUDING REMARKS AND PERSPECTIVES

Every year, we are getting closer at comprehending the distinct biology of γδ T cells, all their quirks and quarks, to be able to modulate them in our best interest. The ultimate research goals are usually centered around infection and cancer management, but they can be extended to regulation of physiological functions and control of autoimmune diseases.

We are just starting to color the puzzle pieces of human γδ T cell ontogeny. We know now that in early fetal life, three distinct functional waves arise. It is still unknown where these cells home to, and for how long they persist. Their maintenance in specific tissues could be linked to protection from cancer, a question that requires access to early life human tissues and tumor infiltrating γδ

T cells to be answered. Until now, studies on fetal γδ T cells in the human periphery have shown their prevalence in liver, intestine, and blood.[74,80,198,199] The human fetal skin appear to be a "special case," with a recent study showing the enrichment of αβγδ T cells (T cells co-expressing an αβ and a γδ TCR) in this fetal organ, while γδ T cells (that is $\gamma\delta^{+}\alpha\beta^{-}$ T cells) were only present at low frequency.[200] We would need to further investigate the TCR repertoire and function of these fetal tissue-associated γδ T cells as well as extend our knowledge of γδ T cells in other tissues, either in internal organs or at barrier tissues. Coupled sc-RNA-seq with sc-TCRseq (Box 2) seems to be the fittest way to address these questions, nowadays.

Besides the downstream of where γδ T cells reside and what they accomplish, it is important to know more about the upstream of the γδ T cell generation as well. It is well-known that the thymic environment affects the selection of αβ T cells but it remains an open field for the ontogeny of human γδ T cells. For example, expression of BTN(L), the anatomy of the thymus and the localization of the T cell progenitors might guide the development of human γδ T cells. More particularly, the distinction of the three fetal waves seems to be steered by TCR strength, which is probably at least partially dependent on the CDR3 sequences present in the γδ TCR.[79] It would be enlightening to investigate the potential interactions with γδ ligands such as BTN(L)s in the fetal thymus and their contribution to this TCR signaling strength. Moreover, the presence of type 3-biased precursor subset could contribute to the very first functional γδ thymocyte wave observed. This study would inquire examining the thymocytes before they express the γδ TCR on their surface.[16] Finally, it will be intriguing to study the mechanism of action of Lin28b which is regulating the γδ thymocytes in early and late stages of formation, including the TCR generation and selection and the effector function acquisition.[76]

The parallel study of γδ T cells in human and mouse models has provided insight into γδ T cells from early life until adulthood. The additional comparison with other species brings more insight into the uniqueness of γδ T cells in the immune landscape. γδ T cells constitute a small part of the big immune jigsaw puzzle, which is transforming during life, and might be able to solve compatibility problems in certain settings.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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