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Review

Thymic Determinants of γδ T Cell Differentiation

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γd T cells have emerged as major sources of the proinflammatory cytokines interleukin-17 (IL-17) and interferon-γ (IFNγ) in multiple models of infection, cancer and autoimmune disease. However, unlike their αβ T cell counterparts that require peripheral activation for effector cell differentiation, γδ T cells instead can be 'developmentally programmed' in the thymus to generate discrete γδ T cell effector subsets with distinctive molecular signatures. Nonetheless, recent studies have presented conflicting viewpoints on the signals involved in thymic γδ T cell development and differentiation, namely on the role of both T cell receptor (TCR)-dependent and TCR-independent factors. Here we review the current data and the ongoing controversies.

Thymic Commitment to a γδ T Cell Fate

Murine γδ T cells consist of various subsets characterized by distinct anatomical locations and functional properties (Table 1). Essentially all γδ T cells are generated in the thymus, where somatic rearrangement of TCR genes operates in different windows throughout ontogeny, leading to subset-characteristic Vγ usage (Table 1). A major finding of recent years was that, rather than leaving the thymus as functionally immature T cells to colonize secondary tissues (where terminal differentiation could occur, akin to αβ T cells), γδ T cells are 'developmentally preprogrammed', that is, they can acquire effector functions while still in the thymus.

αβ and γδ T cells differentiate in the thymus from a common $CD4^{-}CD8^{-}$ double negative (DN) progenitor in which TCRγ, TCRδ and TCRβ rearrangements initiate [1]. Initially, αβ and γδ lineages were defined solely on the basis of TCR expression. However, the presence of cells lacking CD4 and CD8 co-receptors ('γδ-like') in **TCR-transgenic mice** (see Glossary) in which premature TCRαβ expression occurs, or development of TCRγδ-dependent $CD4^{+}CD8^{+}$ double positive (DP) cells in preTCR-deficient mice, suggested that the expressed TCR did not always correlate with lineage fate [2–4].

Attempts to explain the mechanisms underpinning αβ/γδ lineage choice resulted in two models: the stochastic and the signal strength models. The stochastic model proposed that fate determination occurred prior to expression of the TCR; with subsequent TCR signaling simply endorsing continued development. In support of this, it was demonstrated that TCR^{-} DN cells with higher expression of IL-7Rα and SRY-box-containing gene 13 (Sox13), were biased towards entering the γδ lineage [5,6]. However, recent analysis of mice with a spontaneous mutation in *Sox13* revealed that this transcription factor is not absolutely required for commitment to the γδ lineage, but instead may be important for development of discrete γδ subsets with IL-17-secreting effector function (see later) [7].

By contrast, the signal strength model proposed that strength of signal delivered by any TCR dictates lineage choice; DN cells receiving a strong signal adopting a γδ fate, with those

Trends

Innate-like γδ T cell subsets share many phenotypic similarities with subsets of innate lymphoid cells, evoking common mechanisms of development and complementary functional roles.

During thymic γδ T cell development the effects of TCRγδ signaling can be temporally segregated: firstly in driving commitment to the γδ lineage, and secondly for thymic acquisition of specific cytokine-secreting effector fates.

TCR-independent precommitment, TCRγδ signal strength, and the presence or absence of thymic TCRγδ ligands influence thymic acquisition of γδ T cell effector function.

Peripheral γδ T cells are a heterogeneous mix of innate-like and adaptive-like subsets, with likely different contributions to immune responses in different anatomical locations, and to different immunological challenges.

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Table 1. Features of Mouse γδ T Cell Subsets. Nomenclature by Heilig and Tonegawa [72].

Subset	Most common VγVδ pairs	V(D)J diversity	Tissue distribution	Production of signature cytokines
Vγ1	Vγ1Vδ6.3	High	Lymphoid tissue, liver	• IFNγ and TNFα • Can produce IL-4 and IL-17
Vγ2			?	?
Vγ3			?	?
Vγ4		Variable (low/high)	Lymphoid tissue, lung, liver, dermis	• IL-17 • IFNγ
Vγ5	Vγ5Vδ1	Invariant	Epidermis	• IFNγ
Vγ6	Vγ6Vδ1	Invariant	Uterus, lung, tongue, liver, placenta, kidney	• IL-17 and IL-22 • Can produce IFNγ
Vγ7	Vγ7Vδ4 Vγ7Vδ5 Vγ7Vδ6	Intermediate	Intestinal mucosa	• IFNγ

receiving weaker signals committing to the αβ lineage. Evidence for this model was provided by manipulating TCRγδ signal strength in thymocytes expressing a transgenic TCRγδ, by altering either ligand availability or downstream signaling capacity [8,9]. This effectively equates to an instructional model, as generally the preTCR transduces a weak signal whereas TCRγδ transduces a strong signal [8,9]. Although this model is now widely accepted, the molecular pathways that define γδ commitment are only just being elucidated. For example, increased phosphorylation of extracellular signal-regulated kinase (ERK), the induction of early growth response (Egr) family transcription factors, and the upregulation of **inhibitor of DNA binding 3 (Id3)** (see later), were all identified as activated by strong signaling through TCRγδ [10].

The differential activation of ERK signaling that drives progenitors to a γδ fate has been recently attributed to the noncanonical interaction of its DEF-binding pocket (DBP) with DEF-domain containing targets, which in turn increases their stability and expression, and promotes a more efficient transactivation of downstream target genes [11]. This noncanonical function is a consequence of stronger and more prolonged signaling than that associated with commitment to the αβ fate. What generates these stronger and more prolonged ERK signals however, is still unclear. Thus, commitment to a γδ fate requires strong signaling transduced by, largely, TCRγδ complexes in DN cells, which may operate in the context of some degree of lineage precommitment. The signal strength model for αβ/γδ lineage choice is further discussed elsewhere [12].

The Role of TCRγδ in γδ T Cell Effector Differentiation

Beyond γδ lineage commitment, TCRγδ signaling also plays an important role in thymic differentiation of γδ subsets with distinct effector functions (Table 1). These two processes are likely temporally segregated, so that TCR signaling operates in sequential developmental windows with distinct outcomes. In support of this, histone methylation patterns in mature $CD27^+$ versus $CD27^-$ γδ T cell subsets, which contain $IFN\gamma^-$ and IL-17-producers, respectively [13], displayed identical positive histone marks (H3K4me2, associated with transcription) on critical determinants of γδ lineage development, whereas loci associated with IL-17 production (various cytokines, cytokine receptors and transcription factors) showed strikingly different H3K4me2 patterns in the two populations [14]. These results are consistent with γδ T cell effector subsets sharing a common early developmental program (focused on γδ lineage commitment) dependent on strong TCR signals; but diverging later into subpopulations with distinct TCR signaling requirements for functional differentiation. This idea is also supported by

Glossary

Butyrophilin-like 1 (Btnl1): a protein encoded by the *Btnl1* gene that belongs to a larger family of butyrophilin glycoproteins that are variously implicated in immune modulation.

Dendritic epidermal T cells (DETC): γδ T cells expressing a canonical Vγ5Vδ1 TCR, found in the epidermal compartment of the mouse skin.

Hypomorphic mutation: a mutation where the altered gene product has reduced activity compared to the wild-type gene product, but is not completely absent.

Inhibitor of DNA binding 3 (Id3): a protein that can heterodimerize with certain transcription factors and prevent their DNA binding and thus transcriptional activity.

Innate lymphoid cells (ILCs): a relatively recently described group of immune cells, enriched at mucosal sites, that mirror the functional diversity of T cells, but do not express antigen-specific receptors.

Intraepithelial lymphocytes (IELs): essentially comprise of T cells that reside in the epithelium of the intestine; a significant proportion of these cells are TCRγδ-expressing cells. In mice, these T cells frequently express the CD8αα homodimer.

Phycoerythrin (PE): a large protein from red algae that is commonly used in flow cytometry due to its ability to absorb and emit (red) fluorescence at specific wavelengths.

SKG mice: an inbred strain of mice that spontaneously develops chronic arthritis as a result of a point mutation in the gene encoding ZAP-70, an important signaling molecule in T cells.

T10/T22: nonclassical major histocompatibility complex (MHC) class I molecules that are recognized as TCR ligands by some mouse γδ T cells.

TCR-transgenic mice: the bulk of T cells in these mice express the same TCR as a result of a rearranged TCR gene inserted into the mouse genome.

studies using CD73 as a marker of TCRγδ signaling [15], and is further discussed elsewhere [16,17].

Based on expression, or not, of putative ligands for **T10/T22**-specific transgenic Vγ4$^+$ T cells, or thymic Vγ5$^+$ progenitors of **dendritic epidermal T cells (DETC)**, it was proposed that strong TCRγδ signals drive the adoption of an IFNγ-secreting fate (see later), whereas weaker TCR signals support the differentiation of IL-17-producing γδ T cells [18,19]. This was also consistent with a bias towards the adoption of an IL-17-secreting fate by γδ cells in the absence of ERK signaling [11]. However, the fact that IL-17-producing γδ T cells and their thymic progenitors display surface markers, such as CD44 and CD73, that are associated with TCR activation, is somewhat contradictory to a requirement for weak TCR signaling [20,21]. Indeed, **SKG mice** possessing a **hypomorphic mutation** in ZAP-70, and hence attenuated TCR signaling, displayed a pronounced deficiency of IL-17-producing γδ thymocytes [22]. Also, in double-heterozygous mice for CD3γ and CD3δ (CD3DH) [23], in which TCR signaling was decreased due to reduced surface TCRγδ expression, fetal (but not adult) IL-17-producing γδ T cells were significantly depleted, suggesting that different 'waves' of thymic γδ17 T cell progenitors (Vγ6$^+$ in the fetus, Vγ4$^+$ in the adult), may require different TCR signals. Clearly, further work is needed to understand the TCRγδ signaling criteria, and underlying signaling cascades, required for development of IL-17-secreting cells.

Consistent with distinct effector γδ T cell subsets having distinct TCR signal strength requirements during ontogeny (Figure 1), CD3DH mice also lack adult CD27$^+$CD122$^+$NK1.1$^+$CD45RB$^+$

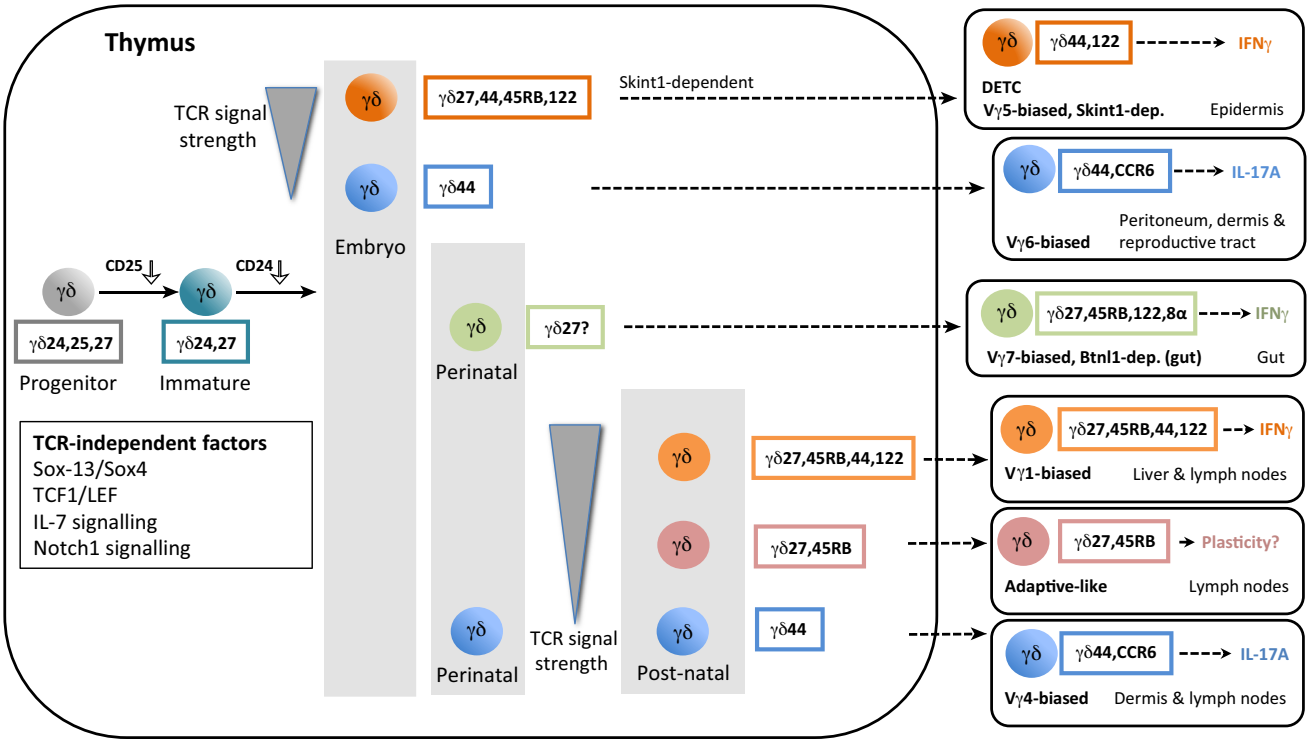

Figure 1. Development and Functional Differentiation of γδ T Cell Subsets. Phenotype of key γδ thymocyte subsets and their peripheral counterparts (present in indicated tissues) making interferon (IFN)γ or IL-17A. For simplicity, the prefix 'CD' was omitted from the superscript markers (CD24, CD27, etc.) expressed on the cell surface. Also depicted are TCR signal strength (represented as a gradient) and TCR-independent factors implicated in γδ T cell development. '?' denotes uncertainty; Abbreviations: Btnl1, Butyrophilin-like 1; dep., dependent.

γδ T cells expressing the highest levels of IFNγ [23]. These data support the multiple observations that suggest that NKT-like γδ T cells (that preferentially use a Vγ1Vδ6.3/6.4 TCR and express variously NK1.1, promyelocytic leukaemia zinc finger protein (PLZF), IFNγ and IL-4) require strong TCRγδ signals for development [24,25]. Notably, these cells also expand significantly in Id3$^{-/-}$, ITK$^{-/-}$, KLF2$^{-/-}$, SLP76-Y145F, and LAT-Y136F mice [1,10,26–30]. However, it is still unclear why these mutations, which largely affect the TCR-dependent PLCγ/Ca^{2+} signaling axis, specifically affect expansion of only NKT-like γδ T cells.

Also worth mentioning are the selective effects on some γδ T cell subsets (including DETC and Vγ4^{+} T cells) of a germline mutation in the extracellular domain of CD3ε, which prevents the outside-in transmission of conformational changes in the TCR [31]; and a recent study where nonsignaling CD3ζ chains were 'knocked-in' to replace wild-type CD3ζ, in which an impairment (~50% in numbers) in γδ T cell development was accompanied by a similar reduction in invariant αβ NKT cell numbers [32].

To what extent TCR 'signal strength' depends on ligand engagement or on intrinsic receptor properties such as surface expression levels, TCRγ/TCRδ pairing efficiencies, conformational changes after TCR stimulation, and involvement, or not, of differential downstream signaling cascades, remains to be established. Removal of the extracellular domain of TCRγδ, which is responsible for ligand binding, was not needed for *Tbx21* (T-bet) and *Ifng* expression in γδ thymocytes developing in fetal thymic organ culture [33]. Thus, an improved understanding of how TCR signal strength affects γδ T cell effector differentiation will likely require a qualitative appreciation of the intracellular signaling pathways triggered by different TCR complexes (with distinct Vγ-usage) in the presence or absence of TCR-ligand engagement. Of note, a detailed study on the TCRγδ-inducible molecule, CD73, suggested that most (~90%) γδ T cells receive TCR signals during effector cell differentiation [15]. Further clarification of this issue requires the identification of *bona fide* TCRγδ ligands and their role in thymic γδ T cell development.

TCR-Dependent versus TCR-Independent Transcriptional Networks in γδ Thymocytes

T-cell production of cytokines is tightly regulated at the transcriptional level [34–38]. Recent studies revealed that *Il17* versus *Ifng* gene expression in developing γδ T cells is controlled by shared (with their CD4^{+} T cell counterparts) and unique (lineage-specific) transcriptional networks [39], with variable dependence on TCR signaling for their establishment and maintenance. As in CD4^{+} T cells, the master transcriptional regulators of IL-17 and IFNγ expression in γδ T cells are *Rorc* (RORγt) and *Tbx21* (T-bet), respectively. Mice deficient for these transcription factors are severely depleted (in the case of T-bet and IFNγ), or even devoid (for RORγt and IL-17) of the corresponding cytokine-producing γδ T cells, both in the steady state and upon inflammatory or infectious challenge [40]. By contrast, other 'auxiliary' transcription factors (TFs) that contribute to Th1 or Th17 differentiation, such as Eomesodermin, RORα, BATF or IRF4, are dispensable in γδ T cells [40,41]. Interestingly, strong TCRγδ signaling is seemingly required to establish a TF network involving Egr2 and Egr3, which suppresses RORγt and the IL-17 pathway [19,23,42]. Egr2 and Egr3 were found to be upregulated during TCR-mediated thymic selection of fetal Vγ5^{+} thymocytes (which generate the DETC population of the murine skin), and also upon TCR stimulation of adult γδ thymocytes [19], that is also consistent with their TCR-mediated induction in NKT cells [43]. Importantly, Egr2 and Egr3 are transcriptional regulators of Id3, which inhibits the expression of E47, a key promoter of *Rorc* expression [42,44] and Id3 is critically required for the differentiation of IFNγ-producing effectors [10]. Agonist TCR signals seem to be especially required for the differentiation of a subset of CD27^{+} γδ thymocytes coexpressing the surface markers CD122 and NK1.1 and the highest levels of intracellular IFNγ [18,23,45]. The development of this subset was reported to also be

dependent on the TFs Th-inducing POZ-Kruppel factor (ThPOK) and PLZF, which are induced by TCR signaling via Id3 [46,47]. Thus, TCR-mediated selection seems to downregulate *Sox13* and *Rorc* expression and the 'default' IL-17 program to allow IFNγ expression by γδ thymocytes [19].

The TCR-independent mechanism of RORγt induction may depend on expression of two high-mobility group box TFs, Sox4 and Sox13, that are expressed before TCR signaling, and that have been shown to induce *Rorc* expression [26]. In support of this, an independent study demonstrated that a spontaneous mutation in *Sox13,* in a commonly used CD45.1$^+$ congenic C57BL/6 mouse substrain, led to a selective deficiency in IL-17$^+$ Vγ4$^+$ γδ T cells that was associated with reduced skin lesions in a model of psoriasis-like dermatitis [7].

The Sox4/Sox13/RORγt/IL-17 program appears to be counteracted, not only by the Egr2/Egr3/Id3 axis, but also by two other TFs, TCF1 and Lef1, which promote the development of IFNγ-producing γδ thymocytes [26]. Interestingly, TCF1 and Lef1 are downstream effectors of the canonical Wnt signaling pathway [48], potentially implicating another set of TCR-independent cues in the differentiation of effector (in this case IFNγ producers) γδ T cells. Conversely, Notch signaling, triggered by binding of Delta-like 4 (DLL4) to Notch1, and its downstream TF, Hes-1, were suggested to promote the development of IL-17-producing γδ thymocytes [49]. There is however a complex interplay between the Wnt and Notch pathways, as highlighted by evidence of direct activation of *Tcf7* (encoding TCF1) by early Notch signals in the thymus [50,51]. Of note, the TCF1 and Lef1 loci displayed extensive positive histone marks (H3K4me2) in lymph node and splenic CD27$^+$ γδ T cells, suggesting an epigenetic mechanism to maintain IFNγ production in the periphery [14].

In summary, although γδ T cells produce cytokines that are also made by CD4$^+$ T cells, and share RORγt and T-bet as core molecular determinants, effector function differentiation along the αβ and γδ T cell lineages seems to be regulated rather differently. Indeed, genome-wide analysis of histone H3 modifications showed that only one-third of the top 120 differentially marked loci in IFNγ- versus IL-17-producing γδ T cells were also differentially marked in CD4+ Th1 and Th17 cells [14]. TFs like Sox4 and Sox13; or Egr2, Egr3 and Id3, have unique roles in γδ T cell subsets (Figure 2). On the other hand, various important TFs in CD4$^+$ T helper cells, like STAT3 [49], IRF4 [41], RORα, BATF and Eomesodermin [40], are dispensable for effector γδ T cell differentiation. We postulate that such differences stem from the instructive cues constitutively available in the thymus versus the inflammatory stimuli that emerge in the periphery during T cell activation.

Innate versus Adaptive Features of γδ T Cells

The TCR-independent transcriptional events that control cytokine expression in γδ T cells evoke a relationship with **innate lymphoid cells (ILCs)**, since these cells differentiate into similar cytokine-producing subtypes in the absence of a TCR [23,52,53]. Interesting points of comparison are TCF-1 and PLZF, mentioned above as players in effector γδ T cell differentiation. TCF-1 expression seems to mark a precursor that can give rise to all ILC lineages [54]; and PLZF is highly expressed and required in ILC precursors [55]. Interestingly, PLZF seems to participate in the development of two distinct innate-like γδ T cell subsets: IFNγ-producing NK1.1$^+$ Vγ1$^+$ thymocytes [47] and IL-17-producing Vγ6$^+$ cells [56].

Also of note, Prinz and colleagues described a previously unrecognized population of IL-17$^+$ Thy1$^+$ thymocytes in Rag1-deficient mice, which must therefore have acquired their capacity to produce IL-17 independently of TCR rearrangement [57]. Whether these cells are ILC3-like thymocytes bearing the same transcriptional signatures as γδ17 thymocytes remains unknown. We believe dissecting the relationships between γδ T cell and ILC development

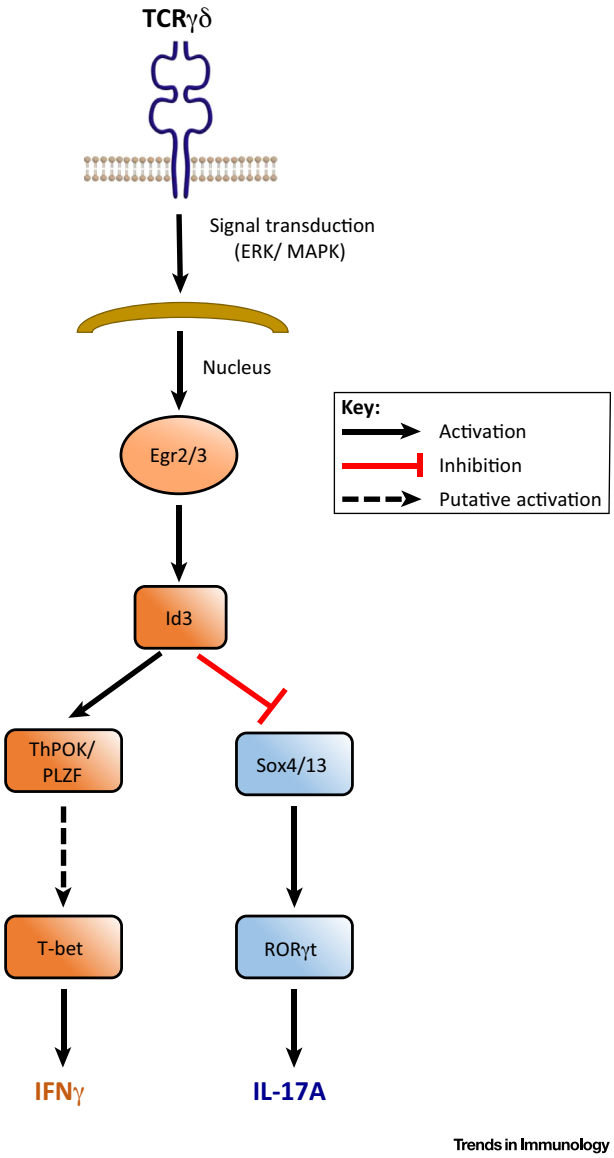

Figure 2. Simplified Model of TCR-Induced Transcriptional Events in Effector γδ Thymocyte Differentiation. Depicted are the transcription factors downstream of TCR signaling (transduced at least in part by extracellular-signal related kinases/mitogen-activated protein kinases, ERK/MAPK) – either activated (black arrows) or inhibited (red blockade). Abbreviations: Egr, early-growth response; PLZF, promyelocytic leukaemia zinc finger protein; ThPOK, Th-inducing POZ-Kruppel factor; Id3, inhibitor of DNA binding 3; Sox, Sry-related HMG box; ROR, RAR-related orphan receptor.

may contribute to understanding the evolutionary conservation of these distinct innate (-like) lymphocyte populations [58–61].

The 'developmental preprogramming' of γδ T cells in the thymus allows for rapid responsiveness to inflammatory cytokines in the periphery, without a requirement for TCR engagement; IL-17 by stimulation (of $CD27^{-}CCR6^{+}$ γδ T cells) by IL-1β, IL-7, and IL-23 [62,63]; IFNγ by stimulation (of $CD27^{+}$ $CD45RB^{hi}$ γδ T cells) by IL-18 plus IL-12 [22,40]. Interestingly, Hayday and colleagues suggested that the lack of peripheral responsiveness to TCR stimulation of these 'natural' effectors resulted from their previous TCR triggering during thymic

development [22]. This generates the thought-provoking concept that the TCR, a hallmark of adaptive immunity, can drive differentiation of innate-like lymphocytes.

This notwithstanding, a sizeable fraction of γδ T cells leaves the thymus as functionally immature cells with a naïve phenotype ($CD122^{lo}CD62L^{+}$ $CD44^{lo}$) and only acquires cytokine-producing capacity in the periphery. In physiological contexts, such 'induced' effector γδ T cells could be considered adaptive-like if their differentiation involved TCR engagement and clonal expansion. This scenario was described by Chien and coworkers for **phycoerythrin (PE)**-specific γδ T cells, which differentiated into IL-17-producers within 60 h after encountering antigen [64,65]. Peripheral differentiation was also recently associated with $V\gamma7^{+}$ intestinal **intraepithelial lymphocytes (IELs)** that require **butyrophilin-like 1 (Btnl1)** expression in intestinal (but not thymic) epithelial cells to become IFNγ producers [66]. Whether the same happens in other peripheral tissues, and the extent to which this applies to γδ T cell clones in general, remains to be investigated. Nonetheless, an adaptive-like mode of action for γδ T cells could underlie their contributions to infection, vaccination, and antigen challenge [22] where memory-like γδ T cell responses have been reported [67,68].

Finally, a further aspect to be considered in the periphery is the plasticity of γδ T cell subsets, including the differentiation of polyfunctional $IL\text{-}17^{+}$ $IFN\gamma^{+}$ double producers. $CD27^{-}$γδ T cells can be induced to produce both signature cytokines under highly inflammatory conditions characterized by abundant IL-1β and IL-23 [19,40]. This polyfunctional phenotype was also associated with memory-like responses and enhanced protection against oral *Listeria* infection [67–69].

Concluding Remarks

There are several outstanding questions in the field of γδ T cell development (see Outstanding Questions). The compelling similarities between ILCs and innate-like γδ T cells warrant further investigation into common developmental mechanisms and modes of function. That said, a defining difference between these cell types is the TCR, which also emphasises the need for an improved understanding of the role of thymic TCRγδ signaling in commitment to, and differentiation of, the γδ T cell lineage. Central to this is the involvement of thymic TCR ligands, and a better appreciation of how certain Vγ-specific TCRs induce the acquisition of particular effector phenotypes at particular anatomical locations. Finally, the balance of contributions between thymically preprogrammed innate-like γδ T cell subsets, and *de novo* expanded peripheral adaptive-like γδ T cell subsets, needs to be assessed in various settings. This will likely improve our understanding of the nonredundant roles played by γδ T cells in immune surveillance of infection, tissue damage, and cancer [70,71].

Outstanding Questions

What are the developmental relationships between innate-like subsets of γδ T cells and innate lymphoid cells? What clues will these provide for shared peripheral functions?

How is TCRγδ signaling that instructs γδ lineage commitment temporally and mechanistically distinguished from TCRγδ signaling that instructs acquisition of γδ T cell effector fate?

To what extent do TCR-independent factors influence thymic acquisition of γδ T cell effector fate?

How do TCRγδ signal strength and the presence or absence of thymic TCRγδ ligands instruct γδ T cells to adopt and maintain distinct cytokine-secreting fates? How do distinct Vγ-domains and specific TCRγδ specificities differentially impact on these selection events?

What are the downstream signaling cascades that link TCRγδ signaling to commitment to, and maintenance of, specific cytokine-secreting capacities?

To what extent do thymic-committed innate-like subsets of γδ T cells versus peripherally-committed adaptive-like subsets of γδ T cells contribute to the overall γδ T cell response in various immune settings?

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