T cell receptor signalling in γδ cell development: strength isn’t everything

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γδ cells have been conserved across ~450 million years of evolution, from which they share the distinction, alongside αβ T cells and B cells, of forming antigen receptors by somatic gene recombination. However, much about these cells remains unclear. Indeed, although γδ cells display ‘innate-like’ characteristics exemplified by rapid tissue-localised responses to stress-associated stimuli, their huge capacity for T cell receptor (TCR)γδ diversity also suggests ‘adaptive-like’ potential. Clarity requires a better understanding of TCRγδ itself, not only through identification of TCR ligands, but also by correlating thymic TCRγδ signalling with commitment to γδ effector fates. Here, we propose that thymic TCRγδ-ligand engagement versus ligand-independent signalling differentially imprints innate-like versus adaptive-like characteristics on developing γδ cells, which fundamentally dictate their peripheral effector properties.

Unresolved role for TCRγδ in γδ cell development

γδ T cells are predominantly tissue-resident lymphocytes that display diverse responses against pathogens and tumours [1]. Indeed, novel immunotherapies that target γδ cells are now being explored to combat chronic viral infections, atopic and autoimmune pathologies, and various cancers [2,3]. Stimulation through TCRγδ is crucial for γδ cell function [4]. However, by contrast to TCRαβ, through which ligand-dependent signalling is absolutely required for thymic αβ T cell development, the role of TCRγδ in γδ cell development remains controversial. TCRγδ signalling is clearly necessary for commitment to the γδ lineage, but the initiation, regulation and molecular nature of this commitment signal are still uncertain. Moreover, ligand-mediated positive and negative selection through TCRγδ remain poorly understood, as too does the correlation between thymic TCRγδ signalling and subsequent γδ effector fates. Here, we build on recent studies that have assessed the initiation and consequences of TCRγδ signalling in immature thymocytes [5–7], to propose that thymic TCRγδ-ligand engagement versus ligand-independent TCRγδ signalling may differentially impose innate-like versus adaptive-like features on developing γδ cells.

Heterogeneity of peripheral γδ subsets

Discussion of the role of TCRγδ in γδ cell development first requires appreciation of the heterogeneous nature of peripheral and thymic γδ subsets. Functionally distinct γδ subsets have been extensively characterised by surface phenotype. For example, dendritic epidermal T cells (DETCs), that reside in murine epidermis and predominantly express a TCRγδ that uses TCRγ variable-region-5 (Vγ5) and TCRδ variable-region-1 (Vδ1) (nomenclature from [8]), are CD44+CD62L+ express CD103 (αE integrin), and are CD122*, consistent with their dependence on interleukin (IL)-15 (Figure 1 and Table 1) [9,10]. DETCs also readily secrete interferon (IFN)-γ when activated. This CD44+CD62L CD122+ IFN-γ-secreting phenotype is also shared by a minor population of lymphoid γδ cells (~0.5%) whose TCRs bind to MHC class IB molecules T10b and T22b, but only in mouse strains expressing T10b and T22b [7]. Moreover, it also characterises a CD90lowCD27* ’NKT-like’ γδ subset that uses a restricted Vγ1Vy6.3* (or Vy6.4*) TCR and is known to secrete both IFNγ and IL-4 [11].

By contrast to DETCs, Vγ4-biased γδ cells (i.e. a γδ subset with over-representation of Vγ4-containing TCRγ chains) of the murine dermis secrete IL-17A, are CD44+CD122−, and express chemokine CC receptor (CCR6 and the scavenger receptor SCART2 [9,12,13] (Figure 1 and Table 1). These cells are probably CD27+, because they closely resemble IL-17A-secreting Vγ6-biased γδ cells from the peritoneal cavity and female reproductive tract that are CD27+CD44+CD122− and CD25+ [14,15]. A CD27−CD44+CD62L+CD122− phenotype accompanied by CCR6 and SCART2 expression is also shared by a minor population of IL-17A-producing γδ cells from the secondary lymphoid organs [13,16,17]. Nonetheless, the majority of lymphoid γδ cells from naive mice secrete large amounts of IFN-γ when activated, but no IL-17A [16]. These cells are CD27*, with a contrasting (for example, to DETCs) CD44+CD62L+CD122− phenotype (Figure 1 and Table 1). Finally, Vγ7-biased γδ intraepithelial lymphocytes (IELs) of the gut are CD27+ and express IFN-γ on activation [18]. These cells are often described as partially activated, and may be under constant stimulation from gut-associated antigens [19,20].

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Thymic γδ subsets

TCRγδ+ thymocytes are first evident from embryonic day-14 (E14), and are initially dominated by a population of Vγ5/Vδ1+ DETC progenitors [21]. Shortly after, a population of Vγ6/Vδ1+ progenitors emerge that are destined for the female reproductive tract, peritoneal cavity and tongue. Thymic terminal transferase is not expressed during these prenatal stages, resulting in simple V-D-J joins that characterise the canonical TCRs of foetal-derived γδ cells [21]. By contrast, postnatal thymic precursors of gut and lymphoid γδ cells possess diverse V-D-J joins in their Vγ1, 2, 4 and 7-containing TCRs. This sequential progression of γδ cell output is partly ordered by γV-region transcription and rearrangement. Nonetheless, other intrinsic differences between foetal and adult thymic progenitors [22,23], and requirement for age-specific thymic stromal factors [24], are also thought to influence subsequent γδ cell fate.

In the postnatal murine thymus, CD27, CD25, CD24 and CD44 identify five distinct γδ cell populations [16,25] (Figure 1 and Table 1). The most immature TCRγδ+ cells are CD27+CD25+CD24+CD44+, express low TCR levels but are highly proliferative [16,25]. These progenitors down-regulate CD25 to become CD27+CD25−CD24+CD44− cells, that can possibly already colonise the periphery [26]. They also probably represent precursors for three mature γδ thymocyte populations that lack surface expression of CD24. This includes a CD27+ subset that is CD44+CD62L+, largely CCR6+ [17], and is already committed to IL-17A secretion [16]. By contrast, mature CD27−CD24− γδ...
thymocytes, which have potential to secrete IFN-γ, can be further subdivided into CD44+CD62L+CD122+ and CD44+CD62L−CD122− subsets. The former lack CCR9 and are largely NK1.1+ [17,20]; being enriched for precursors of NKT-like γδ cells [11]. Conversely, CD27+CD24+CD44+CD62L−CD122− γδ thymocytes are probably progenitors to those of similar phenotype in peripheral lymphoid organs (see previous section). Thus, the thymus generates distinct γδ populations with clear phenotypic links to peripheral γδ subsets. Box 1

**Thymic commitment to a γδ cell fate**

γδ and αβ T cells share a common CD4+CD8− double negative (DN) thymic progenitor in which TCRγδ, TCRαβ, and TCRβ rearrangements initiate [21]. DN cells that express a preTCR (TCRβ paired with invariant preTCRα chain), traverse a ‘β-selection’ checkpoint to a CD4+CD8− double positive (DP) stage that marks commitment to the αβ lineage [27]. By contrast, TCRγδ expression appears to commit DN cells to a γδ fate. These observations initially suggested a qualitatively instructional role for preTCR and TCRγδ in αβ versus γδ fate determination [28]. However, this model failed to explain development of TCRγδ-dependent DP cells in preTCR-deficient mice [29,30], or that precocious expression of transgenic-TCRαβ induced the appearance of ‘γδ-like’ cells [31].

A competing pre-commitment model for αβ versus γδ lineage choice alternatively proposed that fate determination occurred before TCR expression. This initially correlated to heterogeneity in CD127 expression in CD44+CD25−DN (DN2) cells; CD127hi cells being biased toward the γδ lineage [32]. More recently, expression status of SRY-box containing gene 13 (Sox-13) has been similarly implicated [33], while commitment potential to the γδ lineage clearly varies with ontogeny [22] and the developmental stage at which TCRγδ is initially expressed [34]. Nonetheless, the extent to which subsequent TCRγδ signalling can override these pre-committed states remains unclear [35] Box 1.

**Box 1. Outstanding Questions**

- What are the cognate ligands for murine TCRγδ? Do these include self-ligands? Have canonical γδ TCRs been preserved through evolution for self-ligand recognition?
- To what extent are thymic progenitors pre-committed to innate-like γδ effector fates? Do innate-like γδ and αβ T cells share a common thymic progenitor?
- To what extent is thymic TCRγδ-agonist engagement required for γδ cell development? What contribution does thymic ligand-independent TCRγδ signalling make to the peripheral γδ pool? Do ligand-independent TCRγδ signalling initiate?
- How is the IL-17A-secreting effector programme initiated during thymic γδ cell development? Also, how do additional inputs, such as TGFβ3 or Notch signalling, affect this process?
- What are the signalling pathways, molecular mechanisms, and transcription factors that regulate thymic commitment to diverse γδ cell effector fates?

**Strong TCR signalling promotes a γδ cell fate**

Available data now best fit a model in which quantitative differences in TCR signal strength, irrespective of TCR identity, dictate αβ versus γδ fate determination; strong signalling promotes a γδ fate, and weaker signalling generates αβ-committed DP cells [36,37]. Operationally, this equates to an instructional model, because TCRγδ largely provides strong signals, whereas preTCR signalling is weaker. Although stronger signalling from TCRγδ appears to correlate with increased extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, induction of early growth response (Egr) family transcription factors, and upregulation of inhibitor of DNA binding 3 (Id3) [36],
the molecular pathways that define γδ commitment are only now being defined (as discussed later). Recent investigations using delta-like-1-expressing OP9 (OP9-DL1) stromal cell co-culture of TCRγδ+ DN3 thymocytes supports a ‘signal-strength’ model [35]. Thus, strong TCRγδ signalling combined with age and/or stage-specific pre-commitment factors promote a γδ cell fate (Figure 2).

**Generating strong TCR signals; engaging thymic TCRγδ ligands**

The paucity of known murine TCRγδ ligands has made investigation of ligand engagement during thymic γδ cell development problematic. Nonetheless, at least three γδ subsets are implicated in thymic ligand binding; thymus leukaemia (TL)-specific γδ cells, NKT-like γδ cells, and DETCs.

Interaction of TL-specific γδ cells (in either KN6 or G8 TCRγδ transgenic mice) with cognate T10b or T22b ligand (from the MHC TL region) during thymic development has been variously reported to cause tolerance, deletion, or trafficking of cells to the gut epithelium [38–40]. However, recent experiments with a T22-tetrameric FACS-staining reagent have instead suggested that thymic ligand-engaging TL-specific γδ cells develop to secrete IFN-γ, whereas thymic ligand-naïve TL-specific γδ cells secrete IL-17A [7]. This study further reported that T22-tetramer-negative γδ cells, which constitute ~99% of those observed in wild-type mice, share phenotypic features of ligand-naïve (i.e. IL-17A-secreting) TL-specific γδ cells, somewhat contradicting the perceived view of γδ cells as predominantly IFN-γ secreting. Importantly, the development of ligand-naïve γδ cells was suggested to result from TCR-oligomerisation-mediated ligand-independent TCRγδ signalling (see below) [7]. A subsequent report additionally suggested that TL-specific TCRγδ+ IELs also lack evidence of thymic ligand engagement [20].

TCR-ligand-mediated selection is also assumed for thymic development of NKT-like γδ cells, because their characteristic Vγ1*Vδ6.3* (or Vδ6.4*) TCR displays restricted Vδ-CDR3 length and amino acid composition [11,41]. Like TCRαβ+ NKT cells, NKT-like γδ cells are dependent on signalling lymphocytic activation molecule-associated protein (SAP) signalling [42] and the transcription factors T-helper-inducing POZ/Krüppel-like factor (ThPOK) [43] and promyelocytic leukaemia zinc finger (PLZF) [44]; the latter being necessary for IL-4 and IFN-γ secretion [44]. However, although PLZF is induced by TCRγδ cross-linking [44], disruption of the linker for activation of T cells (LAT)–IL2-inducible T-cell kinase (Itk)–Idd3 signalling pathway, which functions downstream of TCRγδ, paradoxically promotes expansion of Vγ1*Vδ6.3* cells [42,45–47]. Consistent with this, attenuation of TCR signalling
appears to expand the Vγ1Vδ6.3+ subset, and to elevate PLZF levels in those expanded cells [42]. Thus, very strong ligand-dependent TCRγδ signalling may not favour development of NKT-like γδ cells.

Finally, selection through TCRγδ is also implicated in DETC development, which correlates with thymic stromal expression of immunoglobulin superfamily gene Skin1 [48,49]. Although not necessarily a direct ligand for the TCR, Vγ5Vδ1+ foetal thymic progenitors that engage Skin1+ stromal cells upregulate Egr3 that, together with nuclear factor of activated T cells (NFAT) and nuclear factor (NFκB) signalling, promote the DETC phenotype that involves upregulation of Tbx21 and IFN-γ-secreting potential [5]. By contrast, Vγδ2Vδ1+ progenitors that develop in the absence of Skin1 fail to induce Egr3 and Tbx21, but express both Sox13 and Rorc that jointly promote what can be called a ‘non-selected’ phenotype that includes IL-17A-producing potential. Importantly, the reciprocal regulation of Egr3 versus Sox13 and Rorc could be demonstrated in adult γδ thymocytes by cross-linking with agonist anti-TCRδ antibody. Thus, this study begins to provide crucial insight into the molecular mechanisms that relate γδ cell functional specification to thymic ligand engagement.

**Ligand-independent TCRγδ signalling**

Despite the acknowledged presence of certain thymic TCRγδ-ligands, and that TCRγδ signalling is considered ligand-driven in peripheral immune responses, ligand engagement may not mediate all instances of TCRγδ signal initiation in DN thymocytes. Ligand-independent signal initiation has long been demonstrated for preTCR [50], being variously ascribed to pTα-mediated lipid-raft association [51], preTCR oligomerisation mediated by the extra-cellular Ig-loop of pTα [52,53], or to an intrinsically low signalling threshold in DN thymocytes [54]. Thymic ligand-independent signalling was similarly proposed for TCRγδ, possibly mediated by oligomerisation of the variable region of TCRδ [7]. However, a recent study has suggested that TCRγδ complexes that lack variable domains, or that lack both variable and constant Ig-like domains, can still initiate signals that drive Recombination activating gene 2 (RAG-2)-deficient thymocytes toward a γδ-like fate [6]. This implies that appropriate surface pairing of TCRγ and TCRδ chains that possibly bring CD3ε-containing signalling modules into close proximity of available lymphocyte-specific protein tyrosine kinase (Lck) is sufficient for TCRγδ signal initiation in DN thymocytes. Thus, strong TCRγδ signalling may not only be a consequence of ligand engagement; additionally, efficiently paired TCRγ/TCRδ chains that are expressed at the cell surface above a certain critical threshold will also commit DN progenitors to a γδ cell fate [6].

**Mapping thymic TCRγδ signalling to peripheral γδ effector fate**

Studies on DETC, NKT-like, and antigen-experienced T-cell-specific γδ cells clearly associate thymic ligand binding with a CD44+CD62L−CD122− phenotype and IFN-γ-secreting potential [5,7,44]. Nonetheless, the majority of CD27− lymphoid γδ cells also produce abundant IFN-γ when activated [16], despite displaying a contrasting CD44+CD62L−CD122+ phenotype that implies an absence of thymic TCR-ligand engagement. Thus, we suggest that thymic ligand-independent TCRγδ signalling may be sufficient to promote γδ cell commitment to subsequent IFN-γ production (and not predominantly to IL-17A production as suggested in previous reports [7]). Moreover, we also propose that a significant component of thymic TCR-ligand engagement may actually be interaction with ligand-presenting cells that provide crucial additional signals for subsequent γδ cell effector function; this may include provision of SAP-dependent signalling for NKT-like γδ cells, or access to Skin1 for developing DETCs.

By contrast to IFN-γ secretion, IL-17A production by γδ cells has been proposed as a default pathway in which TCRγδ thymocytes do not encounter TCR-ligand [7]. However, IL-17A-secreting CD27− γδ thymocytes display a uniformly activated CD44+CD62L− thymic phenotype similar to ligand-experienced γδ cells (although without CD122 or NK1.1 expression) [17,25]. Indeed, early studies suggested that Vγ6Vδ1+ thymic progenitors (that mature to secrete IL-17A) undergo ligand-driven TCR selection for canonical CDR3 sequences to a similar extent as Vγ5Vδ1+ DETC progenitors [55]. This notwithstanding, it seems unlikely that any such IL-17A-inducing thymic TCR ligand would behave as a full TCRγδ agonist [5,7].

Whatever the nature of the inductive event for IL-17A-secreting potential, the thymic progenitors of IL-17A-secreting γδ cells appear to enter a complex programme of development [5] that results from some significant degree of foetal thymus-associated pre-commitment [14]. It also appears to require signalling pathways that involve B lymphoid kinase [56], transforming growth factor (TGF)-β1 [57], and Hairy and enhancer of split 1 (Hes-1) [15]. Thus, it presently remains unclear whether an IL-17A-secreting γδ fate truly represents a ligand-independent γδ cell developmental pathway, or whether foetal/neonatal γδ progenitors of IL-17A-secreting γδ cells must also interact with thymic ligands that results in distinct but overlapping phenotypic changes to those observed for DETCs and NKT-like γδ cells.

**Implications for γδ cell function**

The common description of γδ cells as innate-like perhaps more accurately reflects tissue-associated γδ subsets with highly focused TCR specificities that represent prototypic stress-surveillance lymphocytes with rapid responses to autologous stress antigens [1]. As discussed above, precursors of these populations are probably selected on thymic ligands that endow them with specific effector functions, or reinforce homing to certain body locations [11,23]. These cells respond en masse to local tissue insults and make crucial contributions to both immune protection and tissue integrity [1]. IFN-γ-secreting DETCs represent a well-studied example of these stress-surveillance lymphocytes. However, it is tempting to also speculate whether IL-17A-producing γδ subsets, such as Vγδ6Vδ1+ cells from the female reproductive tract and peritoneal cavity, or Vγ4-biased dermal γδ cells, might also contribute to stress-surveillance. These subsets are predominantly tissue-located, and share the activated CD44+CD62L−...
phenotype of DETCs and NKT-like γδ cells, which could suggest some degree of thymic TCRγδ ligand engagement [16,25]. Certainly, their rapid responsiveness to cytokines such as IL-1 and IL-23 is consistent with an innate-like existence [58,59]. Investigations that determine whether IL-17A-producing γδ progenitors require thymic TCR-ligand interaction for development should clarify this issue. Moreover, they should test the hypothesis that innate-like γδ subsets require TCRγδ ligand engagement during their thymic development (Box 1).

Adaptive T cell responses are generally defined as clonal expansions of relatively few antigen-specific lymphocytes. Interestingly, this feature may well be shared by CD27⁺CD44⁺CD62L⁺CD122⁺ γδ cells that comprise the majority of γδ cells in secondary lymphoid organs [16]. This subset rapidly and extensively expands to secrete abundant IFN-γ on activation through TCRγδ (and CD27), and has been shown to include Murid herpesvirus-4-responsive cells [59]. Thus, γδ cells that lack evidence of thymic TCR-ligand engagement (i.e. with a CD27⁺CD44⁺CD62L⁺CD122⁺ phenotype), that probably develop as a consequence of ligand-independent TCRγδ signalling, may demonstrate peripheral adaptive-like responses, which includes abundant secretion of IFN-γ, on recognising non-thymic (possibly pathogen-associated) antigens. Nonetheless, there is little evidence of γδ memory cell generation or fixing of TCR specificities in the TCRγδ repertoire as a consequence of these expansions [7,26]; instead, expanded γδ cell clones appear relatively short-lived, being replaced by fresh naive γδ cells that presumably maintain a diverse TCRγδ repertoire.

Similarities with non-T lymphocyte development

Despite persistent temptation to align γδ cells with their αβ T cell cousins, comparison with non-T lymphocytes may instead reveal much about γδ cell biology. For example, developing B cells whose B cell receptors (BCRs) engage self-antigen often develop as B-1 B cells; a subset with innate-like features that include rapid functional responses and a restricted BCR repertoire [60]. Conversely, B cells expressing BCRs with no apparent self-reactivity primarily differentiate into conventional follicular B cells with classic adaptive-like qualities. Interestingly, BCR signalling of intermediate strength in response to limiting self-antigen drives marginal zone B cell generation in a B-cell-activating factor (BAFF)-dependent manner [61]. Here, BCR signalling induces expression of non-canonical NFκB pathway substrate p100, which suppresses survival and differentiation unless converted to active p52 by BAFF signalling [62]. This demonstrates that different B cell fates are generated by different qualities of BCR signalling that may or may not require ligand engagement and/or input from additional signalling pathways [60]. Thus, an alternative developmental perspective from a distantly related lymphocyte relative may provide fresh insight into the generation of different γδ effector fates that possibly result from similar differences in TCRγδ signalling and co-stimulation.

Concluding remarks

Recent studies have reinforced the importance of γδ cell responses in infections, cancer and autoimmunity [1]. Indeed, the administration of autologous activated human Vγ9Vδ2⁺ γδ cells now represents a promising approach for immunotherapy in diverse disease scenarios [2,3]. Clearly, an improved knowledge of γδ cell biology is essential, and great strides have been taken to characterise γδ subset phenotypes and functions throughout the body. A thorough understanding of thymic γδ cell development is equally important, at the forefront of which is elucidation of TCRγδ-mediated selection events. Here, we propose that thymic engagement of TCRγδ ligands generates innate-like γδ subsets with rapid cytokine responses to stress-associated stimuli. By contrast, γδ cells that do not recognise thymic ligands develop as adaptive-like γδ cells that expand clonally to secrete IFN-γ in response to non-thymic and potentially foreign TCRγδ ligands. Thus, we contend that thymic TCRγδ selection plays a crucial role in ascribing appropriate functional responses to self/non-self TCRγδ specificities.

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