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$\gamma\delta$ TCR ligands and lineage commitment

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Abstract

Two major T lymphocyte lineages - $\alpha\beta$ and $\gamma\delta$ T cells - develop in the thymus from common precursors. Differentiation of both lineages requires signals coming from TCRs. Development of $\alpha\beta$ T cells is driven at early stages by signaling from the pre-TCR, most likely in a ligand-independent fashion, and later – by signals delivered by $\alpha\beta$ TCRs binding to their ligands – classical or non-classical MHC molecules. $\gamma\delta$ lineage cells likewise require TCR signaling for their differentiation. Recent work from several groups suggests that TCR signaling not only ensures the developmental progression towards $\alpha\beta$ and $\gamma\delta$ lineages but that signal strength instructs lineage fate: weaker TCR signal results in $\alpha\beta$ and stronger – in $\gamma\delta$ lineage commitment. However, as most $\gamma\delta$ TCRs remain orphan receptors, it is still debated whether strong signals from $\gamma\delta$ TCRs in development are generated in a ligand-dependent manner (as in the case of $\alpha\beta$ TCRs), ligand-independent manner (as for pre-TCR) or both. Here we summarize evidence supporting a possible role for ligands in $\gamma\delta$ T cell lineage commitment and the generation of $\gamma\delta$ sublineages.

Introduction

Proliferation, cell death and lineage fate decisions in multicellular organisms are guided by a limited number of molecular pathways [1]. Most of them are initiated by an interaction of a receptor – either cell surface or intracellular – with its ligand, often produced by a different cell. Presence of these two constituents – the receptor and its ligand – adds an important level of control into the system. Deregulation of this interaction can lead to malignant transformation of a cell. Indeed, most examples of ligand-independent signaling were documented in tumor cells and are the result of mutations in components of the molecular pathways that are normally triggered by a ligand – as in the case of HER2/neu [2], c-Kit [3], or Notch [4,5].

One example of the receptors that, in collaboration with the other pathways, determine cell fate decisions in the adaptive immune system of jawed vertebrates are antigen receptors. They are distinct from most other receptor types as their diversity is generated by random recombination of gene segments. This allows for recognition of an almost infinite number of possible ligands (antigens) in the course of an immune response. Yet signals from the very same receptors control important developmental checkpoints and lineage fate decisions during early stages of immune cell differentiation.

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Three types of antigen receptors exist – B cell receptors (BCRs), $\alpha\beta$ T cell receptors ($\alpha\beta$ TCRs) and $\gamma\delta$ T cell receptors ($\gamma\delta$ TCRs) – and each type is expressed on a corresponding lymphocyte subset and regulates its development. B cells, which differentiate in the bone marrow, separate from the other two lineages early on, whereas $\alpha\beta$ and $\gamma\delta$ T cells differentiate in the thymus and share a large part of their developmental programs. CD4/CD8 double negative (DN) T cell precursors in the thymus initiate rearrangement of three out of four T cell receptor (TCR) loci – *Tcrb*, *Terg* and *Tcrd*. If the *Tcrb* rearrangement is productive, the cell expresses the TCR β chain in a complex with the germline encoded invariant pre-TCR α (pT α) chain. Expression of the TCR β /pT α complex – the pre-TCR – leads to a burst of proliferation and rapid progression to the CD4/CD8 double positive (DP) stage – a hallmark of $\alpha\beta$ lineage differentiation. The pre-TCR is a rare example of a receptor that is believed to signal in a ligand independent fashion under physiological conditions [6,7]. Importantly, this ligand-independent signaling event is very transient as pT α expression is dampened by the time the cell progresses to the DP stage [8]. At this stage cells rearrange *Tcra* loci and, if the rearrangement is productive, express TCR $\alpha\beta$ on the cell surface. Further TCR-driven fate decisions of $\alpha\beta$ T cells in the thymus are ligand dependent (reviewed in [9]). If a cell recognizes self MHC-peptide complexes with a high affinity it is eliminated by negative selection or is diverted to a lineage with regulatory properties such as Treg. If an $\alpha\beta$ TCR fails to recognize MHC at all, the cell attempts secondary rearrangements of *Tcra* [10,11] and if it still fails to generate an MHC-binding receptor the DP thymocyte deprived of TCR signal dies – so called death by neglect. Recognition of MHC-peptide complexes with moderate affinity leads to positive selection of the thymocyte and differentiation towards CD4 or CD8 sublineage – a choice also guided by TCR signal strength – with stronger (or longer) signals favoring CD4 and weaker – CD8 fate [12]. Survival of mature $\alpha\beta$ T cells in the periphery also requires TCR ‘tickling’ by self MHC-peptide complexes [13–16]. Thus most of the events in the development of $\alpha\beta$ T cells – with the notable exception of the pre-TCR guided step – depend on triggering of their TCRs by endogenous ligands – and lack of such triggering leads to cell death.

Most cells that successfully rearrange *Terg* and *Tcrd* loci in a wt mouse become $\gamma\delta$ T cells. As is the case for $\alpha\beta$ T cells, their development requires a signal coming from TCR and in the absence of such a signal – for instance in mice deficient for the adaptor molecule LAT – developmental progression of TCR $\gamma\delta$ expressing cells is completely blocked [17]. These cells in wt mice become functionally mature and egress to the periphery without progression through the DP stage [18]. Although the endogenous ligands for the majority of $\gamma\delta$ TCRs are unknown, it was long suspected that at least some of the $\gamma\delta$ T cells were self-reactive [19–21], because surface phenotype of many $\gamma\delta$ T cells resembles that of antigen-stimulated $\alpha\beta$ T cells, and they can produce cytokines shortly after stimulation or even without stimulation. The restricted TCR repertoire of peripheral $\gamma\delta$ T cells indicated that these cells may undergo selection – possibly ligand mediated – during their development in the thymus. Recent results from several experimental systems indicate that a strong TCR signal in fact instructs commitment to the $\gamma\delta$ lineage [22–25] – and at least in one system this strong TCR signal was the result of interaction of TCR with a cognate ligand [23]. However, formal evidence for the existence of endogenous ligands is missing for most $\gamma\delta$ TCRs. Moreover, certain data suggest that at least some $\gamma\delta$ TCRs may signal in a ligand-independent fashion [26] – much like the pre-TCR [6,7] – providing a potential alternative mechanism for the generation of the strong TCR signal. Thus, existence of endogenous $\gamma\delta$ TCR ligands and their possible role in $\gamma\delta$ T cell development is an area of hot debate. As another review in this issue [27] focuses on the arguments against the ‘ligand hypothesis’, we try to advocate it here. In our view neither hypothesis contradicts the bulk of the existing experimental data at the moment.

Strong TCR signals instruct commitment to the $\gamma\delta$ lineage

$\alpha\beta$ and $\gamma\delta$ lineages were initially defined on the basis of TCR expression. Progression along the $\alpha\beta$ lineage path is associated with the execution of a characteristic molecular program involving upregulation of CD4 and CD8 coreceptors, rearrangement of *Tcr* loci (which leads to the deletion of *Tcrd* encoded within *Tcr*) silencing of *Tcr* and induction of the transcription factor Ror γ t. As a common molecular program defines a lineage better than expression of a single receptor (TCR), the lineages are commonly defined on the basis of progression through the DP stage ($\alpha\beta$ lineage) or acquisition of functional maturity without this progression ($\gamma\delta$ lineage).

It is important to underline a fundamental difference in currently accepted definitions of $\alpha\beta$ and $\gamma\delta$ lineages. The $\alpha\beta$ lineage definition is ‘positive’ – cells that do execute the molecular program associated with progression through the DP stage. The $\gamma\delta$ definition, on the other hand, is ‘negative’ – cells that become functionally mature and egress the thymus without progression through the DP stage. This definition is based not on a common molecular program shared by all $\gamma\delta$ lineage cells – but rather on the lack of execution of the $\alpha\beta$ lineage program. This leaves the possibility that $\gamma\delta$ T cells are in fact a collection of several independent sublineages (equipollent to the $\alpha\beta$ lineage), that do not share a common $\gamma\delta$ lineage committed precursor (discussed in [28]). Nevertheless, as discussed below, many $\gamma\delta$ sublineages exhibit properties indicating that they might have received strong signals from their TCRs and are suspected to have endogenous TCR ligands.

Over time it became clear that $\alpha\beta$ and $\gamma\delta$ molecular programs do not always correlate with the TCR type. Studies in transgenic and knock-out systems demonstrated that both $\alpha\beta$ and $\gamma\delta$ TCRs are compatible with both lineage fates and only the pre-TCR exclusively drives $\alpha\beta$ lineage differentiation. Indeed, progression to DP was observed in animals that are not capable of expressing TCRs other than $\gamma\delta$ TCR such as TCR $\beta^{-/-}$ [29,30] and pTa $^{-/-}$ TCR $\alpha^{-/-}$ mice [31]. On the other hand, prematurely expressed $\alpha\beta$ TCRs in TCR transgenic mice divert some cells to the $\gamma\delta$ lineage as they became functionally mature while maintaining their endogenous *Tcr* loci in the germline configuration [32,33] and not progressing through the DP stage [34]. This lack of correspondence between lineage and type of TCR seems to hold true for minor populations in wt mice [29,35]. As the pTa can only be found in mammals, and thus the DP progression in other vertebrates has to be driven by $\alpha\beta$ and/or $\gamma\delta$ TCRs, these minor populations in wt mice may represent vestiges of once mainstream developmental pathways (for review see [28]).

Thus the TCR type *per se* does not determine the lineage fate. However, in wt mice there is a nearly perfect correlation between lineage fate and TCR. This discrepancy was resolved recently, at least in part, by studies demonstrating that TCR signal strength, rather than TCR type instructs lineage fate, with a stronger TCR signal leading to the commitment to $\gamma\delta$ and weaker – to $\alpha\beta$ lineage [22,23]. These studies took advantage of the fact that both $\gamma\delta$ and $\alpha\beta$ lineages develop in TCR $\gamma\delta$ transgenic mice. When TCR signal in these mice was attenuated – for instance by *lck* deficiency or CD3 ζ hemizygosity – this led to an emergence of higher number of DP cells at the expense of $\gamma\delta$ lineage cells [22]. On the contrary, an increase in TCR signal strength – for example on CD5 $^{-/-}$ or CD3 ζ transgenic backgrounds led to a decrease in $\alpha\beta$ and a corresponding increase in $\gamma\delta$ lineage cells [22].

Additional evidence for the role of TCR signal strength in $\alpha\beta$ versus $\gamma\delta$ lineage choice comes from a TCR transgenic system where an endogenous ligand for $\gamma\delta$ TCR is known and so its expression can be manipulated. KN6 TCR transgenic cells recognize non-classical MHC class Ib molecules T10 and T22 [36,37] – as do about 0.5% of total $\gamma\delta$ T cells in spleen and thymus of a wt mouse [26]. Expression of these molecules is dependent on β 2-

microglubulin ($\beta 2m$). In Rag deficient KN6 transgenic mice very few cells progress to DP stage and the majority stay DN, some acquiring a mature phenotype. When these mice were bred to $\beta 2m$ deficient background, large numbers of DP cells appeared in their thymi – at the expense of $\gamma\delta$ T cells with a mature phenotype [23].

These results have at least two potential explanations. In the first scenario, TCR signal strength directly instructs lineage fate: a cell which receives a strong TCR signal becomes a $\gamma\delta$ T cell, whereas a weaker signal (as in the absence of $\beta 2m$) leads to adoption of $\alpha\beta$ fate and to DP progression. In the second scenario strong TCR signal merely deletes cells, already committed to the $\alpha\beta$ lineage, and attenuation of the signal results in the rescue of DP cells that are otherwise eliminated. The latter scenario implies that commitment occurs in a TCR independent manner. Indeed, several studies suggested that commitment to $\alpha\beta$ and $\gamma\delta$ lineages may occur prior to TCR expression (so-called precommitment) [38,39]. To discriminate between these two possibilities, the lineage fate of single TCR $\gamma\delta$ -expressing precursors in OP9-DL1 cultures (which allow for both $\alpha\beta$ and $\gamma\delta$ lineage differentiation [40]) was followed – as in bulk cultures and *in vivo* it is impossible to exclude selection. TCR $\gamma\delta$ -positive cells from the same clones that gave rise to DP cells on OP9-DL1 monolayers were irreversibly diverted to $\gamma\delta$ lineage when their TCRs were crosslinked with antibody [24]. Thus lineage commitment can happen after TCR expression, and TCR signal strength plays an instructive role in this process. The role of TCR signal strength in $\alpha\beta$ versus $\gamma\delta$ lineage choice is discussed in depth in another review in this issue [41].

As strong TCR signal is required for $\gamma\delta$ lineage commitment, and at least in one case (KN6 TCR) generation of such strong TCR signal is a result of ligand-mediated TCR triggering, it is probable that other $\gamma\delta$ lineage cells likewise require endogenous ligands for their differentiation.

Transcription regulators induced by strong TCR signals

Strong TCR signaling that instructs the $\gamma\delta$ lineage fate should lead to an execution of a certain molecular program. Indeed, several transcription regulators that can be induced by strong TCR signals are expressed by $\gamma\delta$ T cells.

It was reported that Egr family members and their target Id3 - a negative regulator of E protein function [42] - are induced by strong TCR signal in $\gamma\delta$ lineage cells [23]. Although expression was not analyzed at the single cell level, numbers of V γ 4 cells in the spleen and V γ 5 DETCs in the skin (V γ nomenclature after [43]) were drastically decreased in Id3 deficient animals [25] suggesting that these subsets expressed Id3, possibly in response to strong TCR signal, and their development required its expression. Moreover, KN6 transgenic cells diverted to the $\alpha\beta$ lineage in the absence of Id3 even when the ligands were expressed, suggesting that Id3 is an important player in TCR-instructed lineage choice [25]. However, overall numbers of $\gamma\delta$ lineage cells were increased in Id3 knockout mice – due to an increase in V γ 1V δ 6.3 cells [25,44–46]. This suggests that $\gamma\delta$ T cells can be subdivided into Id3-dependent and independent subsets. Whether the increase in V γ 1V δ 6.3 cells in Id3^{-/-} mice reflects a cell-intrinsic role of Id3 or is a secondary event is unclear.

Others [44,46] and we [47] recently demonstrated that the V γ 1V δ 6.3 subset expressed the BTB-ZF family transcription factor PLZF and required its expression for proper development. PLZF had been previously reported to be expressed by invariant NKT cells [48,49] – cells that recognize lipid antigens in context of CD1d molecules, require CD1d expression for their thymic development, and are thought to be self-reactive [50]. Thus we tested whether PLZF could be induced by a strong TCR signal. Indeed, TCR crosslinking turned on PLZF expression in polyclonal $\gamma\delta$ thymocytes [47] suggesting that its induction *in vivo* likewise may be ligand dependent.

Another transcription factor of the BTB-ZF family – ThPOK – was recently demonstrated to be expressed by about half of $\gamma\delta$ splenocytes [44]. ThPOK is crucial for CD4 $\alpha\beta$ T cell development and is induced by relatively strong TCR signals that can be mimicked by TCR crosslinking with an antibody [51]. Interestingly, all PLZF-positive $\gamma\delta$ T cells coexpress ThPOK [44].

The role of these transcription regulators in $\gamma\delta$ T cell development is addressed in detail elsewhere in this issue [52]. It seems likely that – by analogy with CD4 T cells for ThPOK, KN6 transgenic cells for Id3 and cultured polyclonal $\gamma\delta$ thymocytes for PLZF – induction of these transcription regulators in developing $\gamma\delta$ T cells *in vivo* may likewise occur upon ligand mediated TCR crosslinking. Taking in account a possibility that $\gamma\delta$ sublineages may not share a common molecular program [28], it is important to mention that both Id3-dependent and Id3-independent subsets of $\gamma\delta$ T cells show evidence for strong TCR signaling – the former because Id3 itself is induced by a strong TCR signal, and the latter because they require TCR-inducible PLZF for functional maturation.

Restricted $\gamma\delta$ TCR repertoires may suggest ligand-mediated selection

Despite the low number of V segments, $\gamma\delta$ TCRs are potentially the most diverse antigen receptors due to their ability to utilize more than one D segment to build CDR3 loops [53–55]. Yet the peripheral repertoire of $\gamma\delta$ T cells – especially in some anatomical locations – has a strong bias towards relatively small numbers of particular $\gamma\delta$ TCRs – often with limited junctional diversity. At a first glance this may indicate selection, possibly driven by the ligands. However, other factors can impose the limited diversity of $\gamma\delta$ TCRs. For instance, several $\gamma\delta$ sublineages develop during fetal/neonatal periods when terminal deoxynucleotidyl transferase (TdT) is not expressed and the addition of N-nucleotides is impossible. This includes V γ 5V δ 1 T cells which home to the epidermis, V γ 6V δ 1 cells found in the lungs, tongue and epithelium of the female reproductive organs and V γ 1V δ 6.3 cells found in spleen, liver and some other locations [56] – although the latter can also develop in adult thymus [47]. Another limitation of $\gamma\delta$ TCR diversity is imposed by the inability of certain TCR δ /TCR γ chain combinations to produce a functional TCR [57,58].

Without known ligands, in most cases it is arguable whether the limited TCR diversity of $\gamma\delta$ T cells found in certain anatomical sites is solely driven by the mechanisms described above – or whether the $\gamma\delta$ TCR repertoire is in addition shaped by positive selection. Convincing evidence for positive selection exists for murine skin $\gamma\delta$ T cells. This subset develops exclusively in the embryonic thymus. The cells home to the epidermis where they perform at least in part regulatory functions. Due to their characteristic morphology these cells are called dendritic epidermal T cells (DETCs). In the absence of V γ 5 or V δ 1 other $\gamma\delta$ T cells take over the epidermal niche, adopt the characteristic morphology but exhibit different functional properties and on certain genetic backgrounds TCR δ ^{–/–} mice develop spontaneous skin inflammation that can be rescued by a transfer of fetal V γ 5⁺ but not V γ 5[–] thymocytes [59]. Moreover, in TCR δ ^{–/–} mice the niche is overtaken by $\alpha\beta$ T cells [60]. No TCR $\gamma\delta$ -positive cells could be detected in the skin of knock-out mice lacking several V γ segments, including V γ 5 [61]. Expression of a V γ 4 transgene restored the DETC compartment. However, unlike V γ 5V δ 1 DETCs in wt mice, V γ 4 transgenic DETCs in these mice were highly enriched for V δ 2 positive cells – indicating a possible selection event. In the fetal thymus of these mice V γ 4V δ 4 and V γ 4V δ 5 were present, so chain pairing restrictions or preferential rearrangement of V δ 2 genes could not explain this result. Importantly, V γ 4V δ 4/V δ 5 fetal thymocytes did not acquire a mature phenotype suggesting that selection of V γ 4V δ 2 DETCs at least in part takes place in the thymus [61].

Identified or suspected endogenous $\gamma\delta$ TCR ligands: MHC and MHC-like molecules

The observation that a strong TCR signal is required for $\gamma\delta$ lineage commitment and evidence for positive selection of some $\gamma\delta$ T cell suggest that $\gamma\delta$ TCRs may have endogenous ligands (indirect evidence for that is summarized in Table 1). However, so far direct evidence for this is scarce (Table 2). A few MHC or MHC like molecules were suggested as possible endogenous ligands for $\gamma\delta$ TCRs (reviewed in [55,62]). In addition to T10/T22 discussed above, another non-classical MHC class Ib molecule – Qa-1 - was identified as a potential $\gamma\delta$ TCR ligand [63]. This early study demonstrated that a $\gamma\delta$ T cell hybridoma was reactive to a B-cell lymphoma cell line pulsed with poly-Glu-Tyr synthetic peptide and this reactivity could be blocked by anti Qa-1 antibodies – suggesting that this response was Qa-1 restricted [63]. However, Qa-1 is also a ligand for the CD94/NKG2C and CD94/NKG2E activating receptors [64] and thus it is possible that the $\gamma\delta$ T cell hybridoma was activated via these receptors rather than the TCR.

A similar problem exists for other putative $\gamma\delta$ TCR ligands. Human stress-induced MHC-like molecules MICA and MICB, which were shown to activate human V δ 1 T cell lines [65], are recognized by another NK cell receptor - CD94/NKG2D - which is expressed on many $\gamma\delta$ T cells. Indeed, anti-NKG2D antibodies inhibited the responses of MICA/MICB-reactive $\gamma\delta$ T cells [66]. However, TCR transfer from MICA-responsive but not MICA unresponsive $\gamma\delta$ T cells into a NKG2D-negative cell line conferred MICA tetramer binding to these cells – formally demonstrating that MICA is a TCR ligand [67]. Thus MICA plays a dual role as a TCR ligand and a costimulatory molecule.

Another MHC-like molecule – CD1c – was also suggested to serve as a TCR ligand for some human $\gamma\delta$ T cells [68]. Importantly, the authors were able to transfer the CD1c reactivity to Jurkat cells by transferring the TCR chains from responsive $\gamma\delta$ T cell line and thus proving that TCR was specific for CD1c.

Finally, MHC class II molecules were suggested as $\gamma\delta$ TCR ligands in mice [69,70]. In one study the response was allogeneic [69] and thus did not represent a case of self-reactivity. In another study the frequency of V δ 4 intestinal intraepithelial $\gamma\delta$ T cells in mice with various H-2 haplotypes was compared and it was suggested that I-E^k was involved in selection of these cells in mice [70]. However, a later report utilizing MHC class II knock-out mice failed to confirm this hypothesis [71].

Identified or suspected endogenous $\gamma\delta$ TCR ligands unrelated to MHC

Human V γ 9V δ 2 T cells can be activated by so called phosphoantigens – small phosphorylated compounds (reviewed in [72]). The most potent phosphoantigens identified so far - such as hydroxy-methyl-butyl-pyrophosphate - are bacterial metabolites. Nevertheless, isopentenyl pyrophosphate (IPP) – a compound produced by mammalian cells – can stimulate V γ 9V δ 2 T cells but at a 10 000 fold higher concentration when compared to hydroxy-methyl-butyl-pyrophosphate [73]. However, no evidence of direct binding of phosphoantigens to TCR exist so far [72]. V γ 9V δ 2 cells can be also activated by F1-ATPase that can be detected on the surface of tumor cells [74] and some data suggest that it may directly interact with the V γ 9V δ 2 TCR [74] – but the evidence for this is still incomplete.

A recent report suggests that an autologous peptide antigen may be recognized by a $\gamma\delta$ TCR [75]. In this study the authors, in an attempt to characterize insulin-responsive $\alpha\beta$ T cells from a TCR α transgenic NOD mouse, accidentally generated a $\gamma\delta$ hybridoma responsive to the insulin B:9–23 peptide. Although the response required very high peptide concentrations

(100 µg/ml) – it was sequence-specific. The response did not require APC, as a single cell could be activated by the peptide, as judged by NFAT-LacZ reporter expression. It is unclear whether the response was dependent on MHC molecules expressed by the hybridoma. Peptide responsiveness could be transferred into a TCR-negative hybridoma by TCR transfection. Moreover, the authors were able to generate several independent B:9–23-responsive hybridomas expressing various TCR δ /TCR γ chain combinations, from NOD but not B6 mice.

Indirect, yet extremely intriguing, evidence for a $\gamma\delta$ TCR ligand exists for murine skin $\gamma\delta$ T cells. As discussed above this population in wt mice is dominated by V γ 5V δ 1 cells. Development of the canonical V γ 5V δ 1 DETCs is blocked in one, but not the other, FvB substrain [76] – a phenotype controlled by a single gene. TCR crosslinking in culture could rescue this developmental block (as judged by a change in surface phenotype) suggesting that the phenotype might be due to a mutation in a TCR ligand. The mutation was later mapped to the novel gene *Skint1* which is expressed in the thymus and skin and encodes a transmembrane protein [77] – consistent with the idea that it may be a V γ 5V δ 1 TCR ligand or a part of it. However it is possible that *Skint1* performs other functions – such as costimulation or ligand processing. Physical demonstration of a *Skint1*-TCR interaction would be required to resolve this uncertainty. It is likewise unclear whether *Skint1* is required for commitment to the $\gamma\delta$ lineage and whether in its absence some V γ 5V δ 1 cells are diverted to the $\alpha\beta$ lineage.

Thus, although several endogenous $\gamma\delta$ TCR ligands have been postulated, many of them were not formally proven to interact with $\gamma\delta$ TCRs, and none of the ligands or ligand candidates with the exception of T10/T22 and *Skint1* were shown to play a role in $\gamma\delta$ T cell differentiation.

Functional evidence for endogenous $\gamma\delta$ TCR ligands

In addition to the cases where molecular identities of a $\gamma\delta$ TCR ligands are known or at least suspected, in some cases functional evidence for the presence of a ligand exist - but does not point to a particular molecule.

First evidence of $\gamma\delta$ T cell autoreactivity came from the fact that some $\gamma\delta$ T cell hybridomas could secrete IL-2 and IL-4 without stimulation [19,21]. The fact that the cytokine production could be blocked by anti-TCR antibodies [19,21] and, more importantly, that the spontaneous cytokine secretion could be initiated in non-secreting TCR-negative hybridoma by transfection of TCR chains [78,79] indicates that this property may be a result of recognition of autoantigen expressed by the hybridoma cells themselves. Interestingly, these putatively autoreactive hybridomas were V γ 1-positive and often - V δ 6-positive [80–83] and were able to secrete IL-4 [21] - properties characteristic for a subset of NKT cell-like $\gamma\delta$ T cells [84] that express the transcription factor PLZF [44,47]. PLZF can be induced in polyclonal immature $\gamma\delta$ thymocytes by TCR crosslinking [47] (see above). Altogether, these observations suggest that V γ 1V δ 6 TCRs may indeed recognize a self antigen. Cytokine secretion by the hybridomas was enhanced by cardiolipin (a lipid from inner mitochondrial membrane) providing one candidate antigen [85]. However, a number of other treatments, such as mycobacterial hsp60 [19,80], synthetic peptides including poly-Glu-Tyr [86] and even culture under reduced serum concentrations [85] resulted in enhanced cytokine production. It is unclear whether some of these manipulations trigger the TCR directly, induce a ligand or activate cells in a ligand-independent way.

Similar results were obtained with primary V γ 5V δ 1 epidermal T cells and cell lines derived from them. In this case spontaneous responses were not observed - however cocultures with primary keratinocytes or keratinocyte cell lines but not fibroblasts led to the secretion of

IL-2 which could be inhibited by anti-TCR antibodies [20]. Keratinocytes were irradiated, raising a possibility that a putative ligand was stress-induced. However, TCR transfer experiments were not performed, leaving a possibility that keratinocytes activate DETCs in a TCR-independent fashion. It would be interesting to see whether DETC-activating properties of keratinocytes rely on the expression of Skint1.

Recombinant $\gamma\delta$ TCRs

A promising approach to identification of $\gamma\delta$ TCR ligands is the utilization of recombinant TCRs in combination with biochemical and/or expression cloning methods. Such tools were developed by several groups [62,87,88], but so far their use has been limited to cell staining. Recombinant V γ 4V δ 5 TCR specific for T10/T22 was shown to bind only to T22-expressing cells, providing some confirmation for the specificity of the approach [88]. V γ 1V δ 6.3, V γ 5V δ 1 and V γ 6V δ 1 TCR stained a variety of cell lines, including those derived from keratinocytes, fibroblasts and thymic epithelium [88]. The staining pattern for these TCR was overlapping but somewhat different, especially when V γ 1V δ 6.3 was compared to the other two TCRs, suggesting that if the binding indeed reflected the recognition of the cognate ligands, the ligands for these TCRs may be different [62,88]. TCRs stained T and B cell-derived cell lines weakly or did not stain at all [88] but at least two of them - V γ 1V δ 6.3 and V γ 6V δ 1 - did bind to the surface of peritoneal macrophages [89]. Although several controls were made to confirm the specificity of the staining, including inhibition of the binding by pretreatment of multimerized recombinant TCRs with antibodies against variable regions of the TCR chains [62,89], ultimate specificity tests are only possible when the actual ligands are identified. Nevertheless, it would be interesting to see what stromal and/or thymocyte populations can bind the recombinant TCRs and thus represent potential selecting cells for the developing $\gamma\delta$ T cells.

Evidence against a role for ligands in $\gamma\delta$ T cell lineage commitment

Although another review in this issue focuses on the arguments against the role of the ligands in $\gamma\delta$ lineage commitment [27] we will briefly discuss some of them here.

An important observation questioning the role the ligands in $\gamma\delta$ lineage come from the work of Jensen and colleagues [26]. They notice that unlike in the KN6 TCR transgenic system, [23] non-transgenic mice on wt or β 2m deficient backgrounds have a comparable frequency of T10/T22 restricted $\gamma\delta$ T cells. This may indicate that the cells non-transgenic mice are not diverted to the $\alpha\beta$ lineage by an attenuation of TCR signaling. However, other explanations are possible.

β 2m $^{-/-}$ mice lack CD8 $\alpha\beta$ T cells – and $\gamma\delta$ T cells are shown to compete with them for some niche [90]. Indeed, β 2m $^{-/-}$ mice exhibited an increase in frequency of total $\gamma\delta$ T cell [91]. Although $\gamma\delta$ T cell subset composition was not addressed, it is possible that it would differ from that in wt mice, since different $\gamma\delta$ T cell subsets have different abilities for homeostatic expansion upon transfer into a lymphopenic host [92]. It is thus possible that similar frequencies of T10/T22 restricted cells in wt and β 2m $^{-/-}$ mice reflect secondary accumulation of these cells in the thymi of the latter mice due to a vacant niche possibly compensating for the diversion of some cells to the $\alpha\beta$ lineage in the absence of β 2m.

Some T10/T22-restricted TCRs may also crossreact with other non β 2m-dependent ligand(s). The absence of β 2-microglobulin could shift the window between putative positive and negative selection in such a way that the repertoire but not the total number of T10/T22 specific cells is affected. In this regard, it is interesting that two T10/T22 specific TCRs – G8 and KN6 – have a 15-fold difference in their affinities to T22 [93] providing a sufficient range for such a shift. In such a scenario T10/T22-specific cells in β 2-

microglobulin $\beta 2$ mice would represent at least in part a population that is deleted when $\beta 2$ -microglobulin is present. These putative rescued cells then may receive a sufficiently strong TCR signal to commit to the $\gamma\delta$ lineage – either from a ligand other than T10/T22, or in a ligand independent fashion.

The authors also provide some evidence for ligand-independent $\gamma\delta$ TCR signaling. To this end they utilize the BaF3 cell line which depends on IL-3 signaling. When these cells are transfected by chimeric molecules containing extracellular domain of protein of interest and intracellular part of human erythropoietin receptor (EPOR), which like IL-3 receptor activates the Jak-STAT pathway, only those extracellular domains that are able to aggregate can provide BaF3 survival in the absence of IL-3. Some but not all combinations of TCR γ /TCR δ chains rescued BaF3 cells cultured in the absence of IL-3. Surprisingly, however, the rescue was also observed in the case of pT α -EPOR construct – in the absence of TCR β chain (an observation that confirms previously published results [7]). As pT α does not signal in the absence of TCR β *in vivo* the rescue may be an overexpression artifact. The extent of the rescue by $\gamma\delta$ TCRs was not higher than by pT α -EPOR – so the interpretation of this result is difficult. It is also cannot be excluded that BaF3 cells themselves express $\gamma\delta$ TCR ligands.

Thus, although observations made by Jensen and colleagues provide an important piece of evidence supporting ligand-independent $\gamma\delta$ lineage commitment, these results are still compatible with ligand-driven $\gamma\delta$ lineage commitment.

Although selfreactivity of $\gamma\delta$ T cells has been long suspected and recent data indicate that strong TCR signaling is required for the commitment to $\gamma\delta$ lineage, most $\gamma\delta$ TCRs remain orphan receptors. Without identification of their ligands or generation of compelling evidence for ligand-independent $\gamma\delta$ TCR signaling the discussion on role for ligands in $\gamma\delta$ T cell lineage commitment remains speculative.

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Table 1

Indirect evidence for endogenous $\gamma\delta$ TCR ligands and their possible role in $\gamma\delta$ lineage development.

Arguments	Counterarguments
<u>Teleological:</u> Initiation of most signaling events depends on a ligand Ligand-independent signaling can result in malignant transformation	Some signaling is believed to happen in a ligand-independent fashion (pre-TCR)
<u>Developmental – commitment to $\gamma\delta$ lineage:</u> Requirement for strong TCR signal to commit to $\gamma\delta$ lineage In one system strong TCR signal-instructed commitment depends on ligand	Not necessarily true for all $\gamma\delta$ T cells under physiological conditions Strong TCR signal could be generated without a ligand
<u>Developmental – repertoire:</u> Although $\gamma\delta$ TCRs are potentially the most diverse antigen receptors, TCR diversity is limited – which may indicate ligand-driven selection	In part this is due to limited junctional diversity of fetal $\gamma\delta$ T cells Chain-pairing restrictions further limit the repertoire $\gamma\delta$ TCRs may exhibit different capabilities for ligand-independent signaling
<u>Activated signature:</u> Many $\gamma\delta$ T cells exhibit a surface phenotype and an ability for rapid effector responses characteristic of activated conventional $\alpha\beta$ T cells $\gamma\delta$ T cells express transcription factors that can be induced by strong TCR signal	activated signature could be acquired by a different mechanism than in $\alpha\beta$ T cells (e.g. ligand-independent TCR signaling or independently of TCR)
<u>Spontaneous or self-induced cytokine production:</u> some V γ 1 hybridomas produce cytokines spontaneously (autoreactivity?) V γ 5V δ 1 DETC lines produce cytokines in cocultures with keratinocytes	Properties of primary cells are different from lines and hybridomas Spontaneous production may be ligand-independent keratinocytes may stimulate cells via pathways other than TCR
<u>Physical interaction:</u> Recombinant $\gamma\delta$ TCRs can bind to surfaces of various cell lines and some primary cells	Formal proof of binding specificity can only be obtained when putative ligands are identified and their expression is manipulated

Table 2

Known or suspected endogenous $\gamma\delta$ TCR ligands

Ligand	Species	TCR	Evidence	Notes	Formally proved TCR recognition
MHC or MHC-like molecules					
T10/T22	Mouse	Various TCRs with a particular motif in TCR δ CDR3 loop [94,95]	Found as a ligand for KN6 TCR transgene [36,37] Was co-crystallized with TCR [94]	About 0.5% peripheral $\gamma\delta$ T cells bind T22 tetramer [26,95]	yes
CD1c	Human	V δ 1	Several $\gamma\delta$ T cell lines can be activated by CD1c-transfected cells Reactivity can be transferred to Jurkat cells by TCR transfection [68]	Frequency of responding cells <i>in vivo</i> is unknown	yes
MICA, MICB	Human	V γ 9V δ 1	Activate human V δ 1 T cell lines [65] TCR transfer into a NKG2D negative cell line conferred MICA tetramer binding [67]	Is also recognized by CD94/NKG2D receptor [66]	yes (MICA)
I-E ^k	Mouse	V δ 4	Frequencies of V δ 4 IELs (intraepithelial lymphocytes) correlated with I-E haplotype [70]	Analysis of MHC II $-/-$ mice failed to confirm the hypothesis [71]	no
Qa-1	Mouse		Anti-Qa-1 antibodies block activation of a $\gamma\delta$ hybridoma [63]	No direct evidence for TCR interaction Could act via NK cell receptors Frequency of responding cells <i>in vivo</i> is unknown	no
Unrelated to MHC					
Skint1	Mouse	V γ 5V δ 1	Expression on thymic stroma is required for the development of V γ 5V δ 1 DETCs [76,77] A block observed in the absence of Skint1 can be rescued (at least on the level of surface phenotype) by TCR crosslinking [76]	No direct evidence for TCR interaction $\Rightarrow$ could be required for processing of the ligand or for activation of a receptor other than TCR	no
Isopentenyl pyrophosphate	Human	V γ 9V δ 2	Can stimulate V γ 9V δ 2 T cells [73]	No direct evidence for TCR interaction [72] Works at a 10 000 fold higher concentration than some microbial phosphoantigens [72]	no
F1-ATPase	Human	V γ 9V δ 2	V γ 9V δ 2 cells can be activated by F1-ATPase on the surface of tumor cells [74]	Evidence for interaction with TCR is insufficient	no
insulin B-9-23	Mouse	various	Hybridomas from NOD mice responded to the peptide. No requirement for APC (single cell assays). Transfer of reactivity by TCR transfection [75].	Very high peptide concentrations (100 μ g/ml) were required [75].	no
Cardiolipin	Mouse	V γ 1	Can enhance cytokine production by V γ 1 hybridomas [85] Reactivity can be transferred by TCR transfection [85]	V γ 1 hybridomas produce cytokines spontaneously [19,21] Various treatments [19,80,86] including low serum culture	no

<u>Ligand</u>	<u>Species</u>	<u>TCR</u>	<u>Evidence</u>	<u>Notes</u>	<u>Formally proved TCR recognition</u>
				conditions [85] enhance cytokine production by V γ 1 hybridomas No direct evidence for TCR interaction	