

Authors' address

Na Xiong\*, David H. Raulet  
Department of Molecular and Cell Biology and  
Cancer Research Laboratory, University of  
California, Berkeley, CA, USA.

\*Present address: Center of Molecular Immunology and  
Infectious Diseases, Department of Veterinary and Biomedical  
Sciences, The Pennsylvania State University, 115 Henning  
Building, University Park, PA 16802, USA.

Correspondence to:

David H. Raulet  
Department of Molecular and Cell Biology and Cancer  
Research Laboratory  
485 LSA  
University of California, Berkeley  
Berkeley, CA 94720-3200, USA  
Tel.: +1 510 642 9521  
Fax: +1 510 642 1443  
E-mail: raulet@berkeley.edu

**Summary:** Two main lineages of T cells develop in the thymus: those that express the  $\alpha\beta$  T-cell receptor (TCR) and those that express the  $\gamma\delta$  TCR. Whereas the development, selection, and peripheral localization of newly differentiated  $\alpha\beta$  T cells are understood in some detail, these processes are less well characterized in  $\gamma\delta$  T cells. This review describes research carried out in this laboratory and others, which addresses several key aspects of  $\gamma\delta$  T-cell development, including the decision of precursor cells to differentiate into the  $\gamma\delta$  versus  $\alpha\beta$  lineage, the ordered differentiation over the course of ontogeny of functional  $\gamma\delta$  T-cell subsets expressing distinct TCR structures, programming of ordered V $\gamma$  gene rearrangement in the thymus, including a molecular switch that ensures appropriate V $\gamma$  rearrangements at the appropriate stage of development, positive selection in the thymus of  $\gamma\delta$  T cells destined for the epidermis, and the acquisition by developing  $\gamma\delta$  T cells of cues that determine their correct localization in the periphery. This research suggests a coordination of molecularly programmed events and cellular selection, which enables specialization of the thymus for production of distinct T-cell subsets at different stages of development.

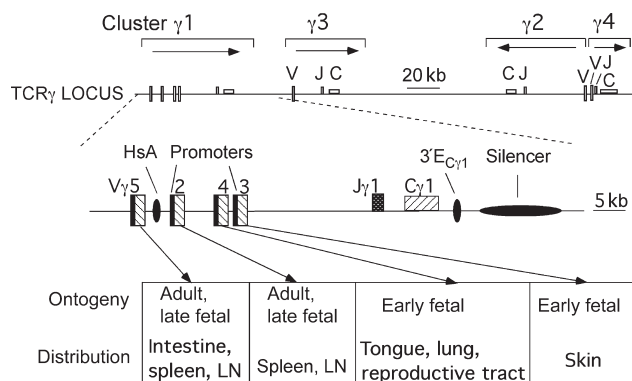
**Keywords:**  $\gamma\delta$ T cells, T-cell development, T-cell receptors, T-cell receptor gene rearrangement, tissue localization

## Introduction

Unlike conventional  $\alpha\beta$  T cells, which reside primarily in secondary lymphoid organs and play a central role in adaptive immune responses, many  $\gamma\delta$  T cells reside in epithelial layers of tissues underlying internal and external surfaces of the body, such as the skin, intestinal epithelium, lung, and tongue, where they function as a first line of defense (1–4). In these locations, the diversity of the repertoire of T-cell receptor (TCR) genes is much more limited than that observed in  $\alpha\beta$  T cells or  $\gamma\delta$  T cells that reside in secondary lymphoid organs (3). Furthermore, the repertoire of expressed variable region genes differs strikingly in the various anatomical locations. It is believed that the highly restricted TCRs expressed by different subsets of  $\gamma\delta$  T cells enable them to recognize ligands that are specifically expressed in infected, diseased, or stressed cells in those anatomical sites (3, 4). Early T-cell development in the thymus is characterized

by progressive waves of differentiation of distinct  $\gamma\delta$  subsets characterized by expression of specific V $\gamma$  and V $\delta$  gene segments. The underlying processes presumably evolved to produce functionally distinct sets of  $\gamma\delta$  T cells in an organized fashion.

A major focus of our research on  $\gamma\delta$  T cells over the years has been the molecular and cellular processes that dictate the highly organized differentiation of  $\gamma\delta$  cells and  $\gamma\delta$  subsets in mice. Therefore, we have addressed the regulation of V $\gamma$  gene rearrangements in one of the clusters of murine TCR $\gamma$  genes, the C $\gamma$ 1 cluster (Fig. 1). All the V genes in this cluster rearrange to a single J $\gamma$  gene segment (J $\gamma$ 1), yet occur in a strikingly ordered fashion over the course of ontogeny (Fig. 1). Thus, this cluster includes V $\gamma$  genes expressed by cells that arise at the fetal stage (V $\gamma$ 3 and V $\gamma$ 4) and at the adult stage (V $\gamma$ 2 and V $\gamma$ 5), using the nomenclature described by Garman *et al.* (5). We chose this cluster because its complex developmental regulation was accompanied by a compact size (approximately 50 kb) and relatively simple composition (four V $\gamma$  genes and one J $\gamma$  and C $\gamma$  gene), suggesting that it would be amenable to molecular analysis. In this review article, we describe findings from our laboratory and others, which have led to a greater understanding of many of these underlying processes. In addition, another focus of the laboratory has been the decision made by precursor cells at the adult stage of T-cell development to differentiate along the  $\alpha\beta$  versus  $\gamma\delta$  lineage. Overall, the findings show that programmed differentiation events and cellular selection processes work together in a systematic fashion to produce functional  $\gamma\delta$  (and  $\alpha\beta$ ) T-cell sets.



**Fig. 1. Organization of mouse TCR $\gamma$  genes and cis-acting regulatory elements, the developmental pattern of V $\gamma$  gene rearrangements, and the peripheral localization of specific subsets of  $\gamma\delta$  T cells defined by V $\gamma$  expression.** The locus consists of three functional clusters ( $\gamma$ 1,  $\gamma$ 2, and  $\gamma$ 4) and a non-functional one ( $\gamma$ 3). The  $\gamma$ 1 cluster, explored in this review in detail, is expanded to show specific gene segments and regulatory elements (2, 4, 16).

## Overview of $\gamma\delta$ T-cell development in mice

Role of TCR expression in commitment of thymocytes to differentiate into  $\alpha\beta$  T cells versus  $\gamma\delta$  T cells

$\gamma\delta$  T cells and  $\alpha\beta$  T cells arise from a common progenitor cell in the thymus. The molecular events leading to the lineage decision of developing CD4<sup>+</sup>CD8<sup>+</sup> thymocytes to differentiate into  $\gamma\delta$  T cells versus  $\alpha\beta$  cells have not been fully resolved. It is clear that the decision does not reflect a specialization in TCR gene rearrangements, such that one subset of cells rearranges TCR  $\gamma$  and  $\delta$  genes while another rearranges TCR $\beta$  genes, the latter event being necessary for expression of the pre-TCR that is important for early stages of  $\alpha\beta$  T-cell development. Indeed, studies show that complete rearrangements of TCR  $\gamma$ ,  $\delta$ , and  $\beta$  genes are present in both mature  $\alpha\beta$  and  $\gamma\delta$  T cells. Furthermore, complete rearrangements (V–D–J or V–J) occur at about the same time in T-cell development (6, 7).

Most TCR gene rearrangements result in joining of the V and the J segments in the incorrect translational reading frame and are therefore non-productive, so the fact that a cell makes a particular type of rearrangement is not an indication that it can ever express a corresponding TCR chain. Approximately 33% of complete rearrangements are productive. The randomness underlying the creation of a productive TCR gene rearrangement has been envisaged as one plausible determinant of lineage in this instance. Indeed, we and others reported evidence that developing T cells that express a functional  $\gamma\delta$  TCR are generally excluded from the  $\alpha\beta$  lineage (8–10). Whereas these data are at least partially consistent with the notion that the expressed TCR dictates the alternative lineage fate decision of the cell ( $\alpha\beta$  versus  $\gamma\delta$ ), they do not prove this hypothesis. The data are also consistent with the possibility that the expressed TCR simply enables the survival and further differentiation of cells that already made a lineage decision consistent with the identity of the expressed TCR. For example, progenitor cells may differentiate into  $\alpha\beta$ - or  $\gamma\delta$ -committed populations before TCR gene rearrangement occurs in development. Further successful differentiation may only occur in the subset of each population that subsequently succeeds in productively rearranging and expressing a TCR that matches its predetermined lineage.

A study from our group provided evidence for the notion that lineage determination to some extent is independent of the type of TCR expressed (11). At the double-negative 2 (DN2) stage of thymocyte development (phenotype CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup>CD44<sup>hi</sup>), at which stage complete TCR gene rearrangements are not yet made in the great majority of cells, cells that express high or low levels of the interleukin-7

receptor (IL-7R) on the cell surface can be distinguished. Analysis of the developmental potential of these cells, based on intrathymic injection experiments, showed that the IL-7R<sup>hi</sup> DN2 cells were more likely to differentiate into  $\gamma\delta$  T cells, whereas IL-7R<sup>low</sup> cells were more likely to differentiate into  $\alpha\beta$  T cells (11). These data suggested that some DN2 cells exhibit a bias in their lineage potential at a stage before TCRs are ever expressed on the cell surface. Recent clonal analysis of the potential of DN2 cells in a Notch ligand-dependent cell culture system arrived at a similar conclusion (12).

Other recent studies have focused on whether lineage determination might result from differences in the quality or strength of the signal propagated by the pre-TCR versus the  $\gamma\delta$  TCR (13–15). These studies provided strong evidence that the strength of signal delivered by the TCR in developing T cells has a major impact on the outcome of the process. The  $\gamma\delta$  TCR appears to provide stronger signals that promote  $\gamma\delta$  TCR development, whereas weaker signals associated with pre-TCR signaling promote the development of  $\alpha\beta$  T cells (14, 15). The effects of TCR signal strength are not necessarily incompatible with the aforementioned TCR-independent bias in lineage potential. Effective rescue of  $\gamma\delta$ -committed precursors may simply require a stronger TCR signal, whereas rescue of  $\alpha\beta$ -committed precursors may require a weaker TCR signal. Alternatively, it was suggested that the bias in lineage potential, which arises early in DN2 cell differentiation may be reversible in the case of a sufficiently strong instructive signal at a later stage in the process (11). Success in identifying the molecular signals responsible for lineage determination should ultimately resolve this issue in a definitive fashion.

#### Differentiation of subsets of $\gamma\delta$ T cells

Many tissue-specific  $\gamma\delta$  T cells express different specific subsets of TCRs with little or no diversity (2, 4, 16, 17). At one extreme, nearly all  $\gamma\delta$  T cells in the epidermal epithelium express an identical ('canonical')  $\gamma\delta$  TCR composed of V $\gamma$ 3–J $\gamma$ 1C $\gamma$ 1 and V $\delta$ 1–D $\delta$ 2–J $\delta$ 2C $\delta$  chains with identical junctional sequences (18). These skin  $\gamma\delta$  T cells are usually called dendritic epidermal T cells (DETCs). Similarly,  $\gamma\delta$  T cells in the tongue, lung, and reproductive tract epithelium express a canonical V $\gamma$ 4–J $\gamma$ 1C $\gamma$ 1/V $\delta$ 1–D $\delta$ 2–J $\delta$ 2C $\delta$  TCR (19). In a less exclusive situation, the epithelium of the small intestine contains both  $\gamma\delta$  and  $\alpha\beta$  T cells (20, 21) and the  $\gamma\delta$  T cells predominantly express V $\gamma$ 5<sup>+</sup> and V $\gamma$ 1.1<sup>+</sup> V $\gamma$  chains, but with diverse junctional sequences (22). Finally, in secondary lymphoid organs, where  $\gamma\delta$  T cells represent only a small fraction of total T cells,  $\gamma\delta$  TCRs predominantly include V $\gamma$ 2, V $\gamma$ 1.1, and V $\gamma$ 1.2 V regions, although with extensive, even dramatic, junctional diversity (22, 23).

The different subsets of  $\gamma\delta$  T cells arise in the thymus at different stages of ontogeny. V $\gamma$ 3<sup>+</sup> T cells are exclusively generated in the early fetal thymus, where they are the first T cells detected in ontogeny, around day 13 of gestation. From the fetal thymus, these  $\gamma\delta$  T cells migrate to the epidermal epithelium, where they expand locally to reach adult numbers and are normally maintained there for the life of the animal (18, 24). V $\gamma$ 4<sup>+</sup> T cells are also produced in the early fetal thymus and they migrate to their epithelial destinations, such as the reproductive tract, tongue, and lung. V $\gamma$ 3 and V $\gamma$ 4 TCRs display a high frequency of invariant canonical junctional sequences in the  $\gamma$  chains due, in large part, to the absence in fetal thymocytes of terminal deoxynucleotidyl transferase (TdT), which is necessary for the addition of N-nucleotides at V–J junctions, coupled with a preference for rearrangement at the sites of microhomologies at the ends of the V $\gamma$  and J $\gamma$  gene segments (25, 26). Similar mechanisms have been invoked to account for canonical junctional sequences in the V $\delta$ 1 rearrangements that encode the dominant TCR $\delta$  chain expressed by both V $\gamma$ 3<sup>+</sup> and V $\gamma$ 4<sup>+</sup> T cells (27). Positive selection during development in the thymus may also play a role in allowing only those cells with the canonical junctional sequences to mature.

V $\gamma$ 3<sup>+</sup> and V $\gamma$ 4<sup>+</sup> T cells are not generated in the adult thymus. Instead, the adult thymus switches to the production of  $\gamma\delta$  T cells expressing V $\gamma$ 2, V $\gamma$ 1.1, and V $\gamma$ 1.2 gene segments with highly diverse junctional sequences, which localize to the secondary lymphoid organs on exiting the thymus (1, 28–30). Adult V $\gamma$ 5<sup>+</sup> cells localize to the intestinal epithelium as well as secondary lymphoid organs (22). There are also reports suggesting that some intestinal epithelial V $\gamma$ 5<sup>+</sup>  $\gamma\delta$  T cells develop extrathymically (31, 32).

The sequential generation of specific subsets of  $\gamma\delta$  T cells at different stages of ontogeny is a fixed developmental program that cannot be easily reordered. For example, disruption of the generation of  $\gamma\delta$  T cells in the early fetal thymus by injecting anti- $\gamma\delta$ -TCR antibody into pregnant mothers results in the absence of DETCs in adult mice (3). Furthermore, DETCs cannot be replaced with bone marrow stem cells in adult mice, following full-body irradiation and reconstitution (33).

#### Programmed rearrangement of TCR V $\gamma$ gene segments

Evidence that ordered V $\gamma$  gene rearrangement is a programmed process

The appearance of cells with different V $\gamma$  gene rearrangements at different stages of development could reflect a developmental program that controls the rearrangement machinery or could be

because of a cellular selection process that promotes the survival of different  $\gamma\delta$  subsets at different stages of development, or both. Several lines of evidence showed that developmentally regulated V $\gamma$  gene segment recombination is a developmentally programmed process. Initially, scrutiny of V $\gamma$  gene rearrangements showed that non-productive V $\gamma$  gene rearrangements in cell lines were usually of the same type as the productive rearrangements (16). Because non-productive rearrangements cannot be subject to cellular selection processes, this finding suggested that developing  $\gamma\delta$  T cells are programmed to choose specific V $\gamma$  gene segments for rearrangement. Consistent with an intrinsic program of V $\gamma$  gene rearrangement, we later found that germline transcription of V $\gamma$  genes correlates with their rearrangement pattern. Germline transcription is usually correlated with greater accessibility of genes in chromatin to recombinases, suggesting a possible link between gene accessibility and rearrangement in this system (34).

As a more direct test of whether the ordered pattern of TCR $\gamma$  gene rearrangement occurs in the absence of cellular selection, we generated several lines of transgenic mice that harbored a 39-kb genomic transgene (called  $\gamma$ B) that spans most of the unrearranged TCR $\gamma$  C $\gamma$ 1 cluster. Each of the three V $\gamma$  genes in the transgene (V $\gamma$ 2, V $\gamma$ 4, and V $\gamma$ 3) contained a frameshift mutation that prevented functional expression of the corresponding V region (35). Despite the fact that the transgene could not encode intact TCR $\gamma$  chains that might influence the selection of cells in which they occurred, rearrangements of the V $\gamma$  genes in the reporter transgene, like rearrangements of the endogenous V $\gamma$  genes, appeared at the appropriate stage in thymic development. In a different but related approach, it was shown that a targeted deletion of the TCR $\delta$  gene, which abrogates the formation of functional  $\gamma\delta$  TCRs (although not protein expression of TCR $\gamma$  genes), did not disrupt the normal developmental pattern of V $\gamma$  or V $\delta$  gene rearrangements (27). These data provided compelling evidence that programmed rearrangement of V $\gamma$  genes is a major determinant of the ordered generation of different subsets of  $\gamma\delta$  T cells at different stages of ontogeny.

#### Regulation of programmed V $\gamma$ gene rearrangement

The finding that V $\gamma$  and V $\delta$  rearrangement patterns are programmed independent of cellular selection led us to investigate the underlying molecular mechanisms. As possible determining factors of the rearrangement patterns, we assessed both the role of cis-acting regulatory elements in the genes and the location of the V genes within the cluster. Both these factors have been proposed or implicated as determinants of rearrangement patterns in studies of other rearranging genes.

Over the years, we and others have identified and characterized several cis-acting elements in the TCR $\gamma$  C $\gamma$ 1 cluster, which play unique and overlapping roles in regulating the V $\gamma$  gene rearrangement process as well as transcription and expression of TCR $\gamma$  genes (Fig. 1). The roles and properties of some of these elements are described in more detail and in different contexts later in this review, but their role in ordered rearrangement is addressed first. The elements include the promoter regions of each V $\gamma$  gene, two enhancer-like elements (3'-EC $\gamma$ 1 and HsA) that cooperate to regulate transcription of the locus, and a putative silencer element that prevents TCR $\gamma$  expression in  $\alpha\beta$  T cells.

#### V $\gamma$ gene promoter regions determine the rearrangement pattern in the adult thymus

Numerous studies have shown that the accessibility of immunoglobulin (Ig) and TCR genes is a critical determinant of rearrangement patterns during development. Accessibility refers to whether chromatin is in a sufficiently open configuration for recombinase to access the recombination signal sequences (RSSs) that flank the gene. Accessibility usually correlates with transcription of the unrearranged genes (germline transcription). As already mentioned, early studies found a correlation between germline transcription and rearrangement of individual V $\gamma$  genes in the adult thymus. Specifically, germline transcription from the unrearranged V $\gamma$ 3 gene was strongly suppressed at this stage, while germline transcription from the unrearranged V $\gamma$ 2 gene remained high, suggesting greater accessibility of the V $\gamma$ 2 gene. Subsequent studies of adult thymocytes substantiated the conclusion that the V $\gamma$ 2 gene is more accessible than the V $\gamma$ 3 gene at this stage, including a report that showed a much greater degree of histone acetylation in the vicinity of the V $\gamma$ 2 gene than in the vicinity of the V $\gamma$ 3 gene (36). Furthermore, it was shown that an inhibitor of histone deacetylation, which is expected to increase the acetylation of the V $\gamma$ 3 gene and therefore its accessibility, stimulated increased levels of V $\gamma$ 3 rearrangements in the adult thymus (36).

Differences in accessibility of the different V $\gamma$  genes suggested that cis-acting regulatory elements associated with the V $\gamma$  genes might be responsible for controlling V $\gamma$  gene rearrangement at the adult stage. Therefore, we designed experiments to test whether the promoter segments of these genes play a role in imposing the adult pattern of rearrangement. For this purpose, we generated a new transgene based on the  $\gamma$ B transgene discussed above. The frameshift mutations in the V $\gamma$  gene segments of this construct should prevent interference in the outcome of an experiment by cellular

selection events. The new transgene, called  $\gamma$ B-Pr-Sw, was identical to  $\gamma$ B except that the promoter segments of the  $V\gamma 3$  and  $V\gamma 2$  genes were reciprocally swapped (35) (Fig. 2). Compared with the  $\gamma$ B transgene, the rearrangement patterns of  $V\gamma 2$  versus  $V\gamma 3$  were reversed in the adult thymus of the  $\gamma$ B-Pr-Sw transgenic mice. Consistent with the reversed pattern of rearrangement, the pattern of germline transcription of the  $V\gamma 2$  and  $V\gamma 3$  genes was also reversed in adult thymocytes of  $\gamma$ B-Pr-Sw mice, as compared with  $\gamma$ B mice. These data showed that the promoter regions of the individual  $V\gamma$  genes determine the preference for  $V\gamma 2$  rearrangements in the adult thymus, probably by controlling the accessibility of the corresponding  $V\gamma$  genes to the recombinase enzymes.

A distinct mechanism for establishing the rearrangement pattern in the fetal thymus

Our initial expectations were that the rearrangement patterns in the fetal and adult thymus would reflect the same basic mechanism and that the switch between these two patterns would consist of altered regulation of this basic mechanism. However, the notion that promoter activity is responsible for the fetal rearrangement pattern was rapidly refuted by analysis of  $\gamma$ B-Pr-Sw mice. Despite the reversed rearrangement pattern in the adult thymus of these mice, the exchanged promoter segments had no significant effect on transgene rearrangements in the fetal thymus, where  $V\gamma 3$  rearrangements predominated to a similar extent as in the  $\gamma$ B transgenic mice. These results indicated that differential promoter activity cannot account for

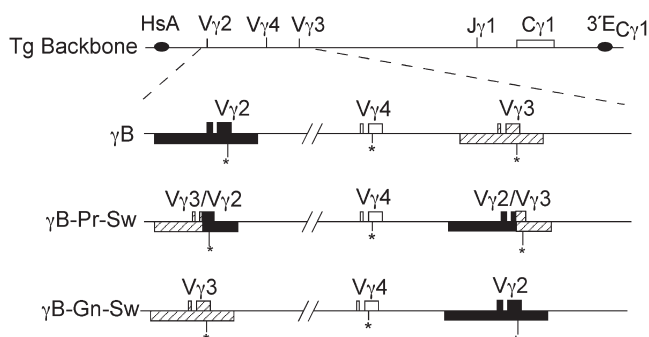
the predominance of  $V\gamma 3$  (and  $V\gamma 4$ ) rearrangements in the fetal thymus. We therefore considered other explanations.

The location of  $V\gamma$  genes in the TCR $\gamma$  locus is a major determinant of the rearrangement pattern in the fetal thymus

An obvious candidate as a determinant of the fetal rearrangement pattern is the location of  $V\gamma$  genes within the TCR $\gamma$ 1 locus. Previous studies have documented preferential rearrangement at the fetal stage of 3' - (D- or J-proximal) V genes in the IgH and TCR $\delta$  loci, in addition to the comparable findings in the TCR $\gamma$  locus already discussed above (5, 29, 37–39). In the IgH locus, an IL-7-dependent shift in the adult stage to rearrangement of 5' -VH genes has been documented (40, 41). However, other studies indicate that even in the adult stage, newly generated B cells show a bias for rearrangement of 3' -VH gene segments (42, 43). Based on transfections of cell lines with plasmids containing various recombination substrates, it was proposed that the preferential rearrangement of 3' -VH genes in the IgH locus was because of the potency of the RSS associated with these genes (44). In contrast, another study showed that the rate of recombination observed *in vivo* in the natural locus did not correlate with the rate of recombination of corresponding substrates in transfected cell lines, but did correlate with the position of the gene in the locus (45). The consistency of these latter findings with ours suggested that location of a V gene within a V gene array might be a major determinant of rearrangement at the fetal stage.

To directly test the role of  $V\gamma$  gene location in rearrangement, we generated a new transgene in which the entire  $V\gamma 2$  and  $V\gamma 3$  gene segments were swapped within the  $\gamma$ B transgene (Fig. 2). The exchanged fragments were approximately 1.6–2.3 kb in length and included the promoter regions, coding regions, and some downstream sequence including the RSS. This transgene, called  $\gamma$ B-Gn-Sw, showed a reversed pattern of rearrangement in the fetal thymus compared with  $\gamma$ B, such that  $V\gamma 2$  rearrangements were several fold more frequent than  $V\gamma 3$  rearrangements (46). These data clearly showed that the relative location of  $V\gamma$  genes is a dominant factor in determining their rearrangement levels in the fetal thymus. In recent unpublished experiments, we generated gene-targeted mice that have corroborated this conclusion (our unpublished data).

How does the position of  $V\gamma$  genes in the locus determine rearrangement levels? One model to consider is that the  $V\gamma$  gene position somehow determines whether the  $V\gamma$  gene is in an open chromatin configuration and is therefore accessible to the recombinase machinery and transcription apparatus. This model is reminiscent of a study showing that proximity of



**Fig. 2. Configuration of  $\gamma$ B,  $\gamma$ B-Pr-Sw, and  $\gamma$ B-Gn-Sw transgenes.** The transgenes include all contiguous sequence as present in the endogenous locus. The configuration of  $\gamma$ B is identical to that of the endogenous locus. In  $\gamma$ B-Pr-Sw, the promoter regions and leader exon (which encodes the signal peptide) of the  $V\gamma 2$  and  $V\gamma 3$  gene segments were exchanged. In  $\gamma$ B-Gn-Sw, 1.6- to 2.3-kb fragments containing the entire  $V\gamma 3$  and  $V\gamma 2$  gene segments were exchanged, including the promoters and RSS. The asterisks indicate the sites where frameshift mutations were introduced into each  $V\gamma$  gene segment. [Reprinted with permission from Proceedings of the National Academy of Sciences, USA (48).]

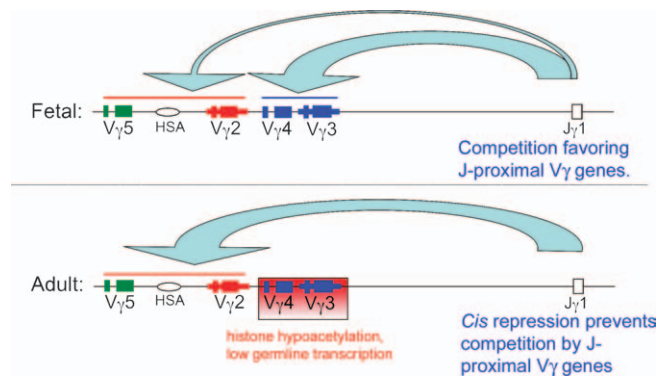


globin genes to the  $\beta$  globin locus-control region (LCR) determines the extent of globin gene transcription in the early yolk-sac stage of erythropoiesis (47). We have shown, however, that combined deletion of both known enhancers in the C $\gamma$ 1 locus, 3'-EC $\gamma$ 1 and HsA did not substantially perturb the fetal or adult patterns of V $\gamma$  gene rearrangement, nor did it cause a major reduction in the extent of V $\gamma$  gene rearrangement (48). Therefore, proximity of the V genes to these elements cannot be a necessary determinant of the patterns of rearrangement. While it might be proposed that differential accessibility is controlled by proximity to a distinct unidentified regulatory element, direct tests showed that gene position did not alter the accessibility of the V $\gamma$  genes at the fetal stage, even as it did alter the tendency of the V $\gamma$  genes to rearrange. Thus, comparing fetal thymocytes from  $\gamma$ B-Gn-Sw transgenic mice with those from  $\gamma$ B transgenic mice, we found no significant difference in germline transcription or restriction enzyme accessibility of the V $\gamma$ 2 or V $\gamma$ 3 genes (46). Consistent with the transgenic studies, in the endogenous TCR $\gamma$  locus, both genes showed high levels of germline transcription and histone acetylation in fetal thymocytes (36, our unpublished observations). All these data are in agreement that both V $\gamma$ 2 and V $\gamma$ 3 genes are 'open' and accessible in the natural locus at the fetal stage and suggest that the regulatory mechanism that dictates differential V $\gamma$  gene rearrangement at the fetal stage differs from that in the adult stage, where regulated accessibility of the V $\gamma$  genes appears to play a dominant role.

#### Models of regulated V $\gamma$ gene rearrangement

Two specific mechanisms are under consideration to account for preferential rearrangement of the 3'-V $\gamma$  gene at the fetal stage. One possibility, reminiscent of the one first proposed to account for preferential rearrangement of 3'-VH genes (37, 38), is that rearrangement at the C $\gamma$ 1 cluster involves some form of one-dimensional 'tracking' mechanism along the DNA by the recombinase, in which downstream V $\gamma$  genes are encountered before upstream genes because of their greater proximity to the J $\gamma$  gene segment. A second possibility is that the 3'-V $\gamma$  gene RSS, being more closely tethered to J $\gamma$ 1 in genomic DNA, is more likely to collide with the J $\gamma$ 1 gene RSS than a 5' V $\gamma$  gene RSS during three-dimensional diffusion, initiating rearrangement.

Whatever the specific molecular mechanism to account for the fetal pattern of rearrangement is, the available data support the general model depicted in Fig. 3 to account for the 'developmental switch' in V $\gamma$  gene rearrangement that occurs in ontogeny. The model proposes that in the fetal thymus, the whole TCR $\gamma$  locus is open for rearrangement, but the downstream V $\gamma$ 3 (and V $\gamma$ 4) genes have a competitive advantage



**Fig. 3. A model of programmed V $\gamma$  gene rearrangement.** At the fetal stage, both the 5'- and the 3'-V $\gamma$  genes are in an open 'accessible' chromatin configuration. Rearrangement of the 3'-V $\gamma$  genes (V $\gamma$ 3 and V $\gamma$ 4) is preferred at the fetal stage because of their location in the cluster, possibly related to their proximity to J $\gamma$ 1. This location preference may reflect competition of the V $\gamma$  gene segments for the rearrangement apparatus. At the adult stage, events involving the specific V $\gamma$  promoters result in suppressed accessibility of V $\gamma$ 3 and V $\gamma$ 4. Rearrangement of 5'-V $\gamma$  genes is strongly increased at this stage, possibly because the inaccessible V $\gamma$  genes can no longer compete with the 5'-gene segments.

because of their proximity to the J $\gamma$ 1 gene and, therefore, are preferentially rearranged over other upstream V $\gamma$  genes at the fetal stage. In the adult thymus, as a result of the activity of the associated promoter elements, the upstream V $\gamma$ 2 (and V $\gamma$ 5) genes are maintained in an open and accessible state, while the accessibility of the downstream V $\gamma$ 3 (and 4) genes is suppressed. The altered accessibility shifts the balance in favor of rearrangement of the upstream V $\gamma$ 2 (and V $\gamma$ 5) genes that are therefore preferentially rearranged in the adult thymus.

#### The potential role of transcription factor E2A in regulating V $\gamma$ gene rearrangement

The role of specific transcription factors in regulating cis-acting elements in the TCR $\gamma$  locus has not been comprehensively studied. However, studies have implicated the E2A transcription factor in the process, specifically in imparting the adult pattern of V $\gamma$  gene rearrangement. E2A is a member of the basic helix-loop-helix transcription factor family and has been found to play various important roles in regulating lymphocyte development (49). In mice lacking E2A, V $\gamma$ 2 gene rearrangements were decreased in the adult thymus, while V $\gamma$ 3 gene rearrangements were increased (50). The switch in V $\gamma$ 2 versus V $\gamma$ 3 rearrangement in E2A knockout mice was associated with a corresponding change in the germline transcription of these V $\gamma$  genes (50), suggesting that E2A contributes to the programmed V $\gamma$  gene rearrangement pattern at the adult stage by differentially regulating the accessibility of the V $\gamma$ 2 versus V $\gamma$ 3 genes. Whereas little effect of E2A deficiency on V $\gamma$  gene

rearrangement was noted in the fetal thymus at a very late stage in fetal ontogeny (day 19 of gestation) (50), we observed a substantial defect in rearrangement of both the  $V\gamma 3$  and the  $V\gamma 2$  genes in the early fetal thymus (day 15), at a time when  $V\gamma 3$  rearrangements normally predominate (N. Xiong, C. Murre, D. H. Raulet, unpublished data). These findings suggest that E2A plays distinct roles in regulating the development of  $\gamma\delta$  T cells at different stages in ontogeny.

E2A may interact directly with cis-acting elements in the TCR $\gamma$  locus to regulate rearrangement of  $V\gamma$  genes differentially. It was reported, for example, that E2A sites are present in the RSS of most  $V\gamma$  and  $V\delta$  gene segments (50), and candidate E2A sites have been identified in the promoter regions of  $V\gamma 2$ ,  $V\gamma 3$ , and  $V\gamma 4$  genes. The sites in the promoter regions might be more important, judging from the aforementioned evidence that the adult pattern of rearrangement is dependent on the promoter sequences and not on the  $V\gamma$  gene segments or RSS. However, evidence that E2A acts directly on  $V\gamma$  genes *in vivo* is lacking.

To help define cis-acting elements through which E2A may act in regulating  $V\gamma$  gene rearrangement *in vivo*, we crossed E2A knockout mice with  $\gamma B$  transgenic mice. In adult thymocytes from  $\gamma B$  transgenic mice, E2A deficiency resulted in an enhancement in transgene  $V\gamma 3$  rearrangements and a reduction in transgene  $V\gamma 2$  rearrangements, much as it does for the endogenous  $V\gamma$  genes (Table 1). These data are consistent with the possibility that E2A interacts with cis-acting elements within the span of the  $\gamma B$  transgene to regulate  $V\gamma$  gene rearrangement.

To address whether E2A regulates rearrangement through interaction with the promoter regions of  $V\gamma$  genes, we crossed the E2A knockout mice with  $\gamma B$ -Pr-Sw and  $\gamma B$ -Gn-Sw transgenic mice. The rationale of this analysis was that if the promoter regions of  $V\gamma$  genes are the dominant interaction sites for E2A to regulate rearrangement of the corresponding  $V\gamma$  genes, E2A deficiency should alter rearrangement of  $V\gamma$  genes in these transgenes according to the origin of the promoter regions ( $V\gamma 2$  or  $V\gamma 3$ ) that flank the  $V\gamma$  gene, not the origin of the  $V\gamma$  gene itself. This prediction was borne out in the case of the  $V\gamma 2$  promoter, in which we observed decreased rearrangements of

those  $V\gamma$  genes in each construct that was flanked by the  $V\gamma 2$  promoter fragment ( $V\gamma 2$  in the  $\gamma B$ -Gn-Sw transgene and  $V\gamma 3$  in the  $\gamma B$ -Pr-Sw transgene) (Table 1, N. Xiong, C. Murre, D. H. Raulet, unpublished data). Hence, it is plausible that E2A acts directly on the  $V\gamma 2$  promoter to enhance rearrangement at the adult stage, perhaps by maintaining accessibility of this region of the locus. In contrast, the prediction was not borne out in the case of the  $V\gamma 3$  promoter because in both the  $\gamma B$ -Gn-Sw and the  $\gamma B$ -Pr-Sw transgenes, E2A deficiency caused a decrease rather than an increase in rearrangement of those  $V\gamma$  genes that were flanked by the  $V\gamma 3$  promoter (Table 1, N. Xiong, Murre, D. H. Raulet, unpublished data). These data suggest that the  $V\gamma 3$  promoter region is not a dominant interaction site for E2A regulation of  $V\gamma$  gene rearrangement. In both these transgenes, the  $V\gamma 3$  promoter was relocalized to the 5' position normally occupied by the  $V\gamma 2$  promoter. A possible explanation for the puzzling results with these transgenes is that E2A acts on another site near this upstream region to enhance rearrangement in wildtype cells. A deeper understanding of the role of E2A in regulating this locus will require detailed analysis of the roles of the various E2A sites in the context of endogenous genomic DNA.

### Role of enhancer elements in promoting rearrangement and transcription of TCR $\gamma$ genes

Likely redundancy of enhancer elements in regulating TCR $\gamma$  gene rearrangement

Although the local  $V\gamma$  promoters influence the accessibility of the corresponding  $V\gamma$  genes, there is likely to be a higher order regulation of the chromatin condensation of the TCR $\gamma$  locus by additional cis-acting elements, including the aforementioned enhancer elements HsA and 3'-EC $\gamma 1$ . Indeed, deletion of small DNA segments containing HsA and 3'-EC $\gamma 1$  from a genomic transgene abrogated rearrangement of the transgene *in vivo* (51), suggesting the roles of HsA and 3'-EC $\gamma 1$  in maintaining the chromatin in an open configuration for rearrangement. Surprisingly, however, simultaneous deletion of both elements in the endogenous locus by gene targeting only modestly

**Table 1. Effect of E2A deficiency on endogenous and transgenic  $V\gamma 2$  and  $V\gamma 3$  gene rearrangements**

	Endogenous		$\gamma B$		$\gamma B$ -Pr-Sw		$\gamma B$ -Gn-Sw	
	$V\gamma 2$	$V\gamma 3$	$V\gamma 2$	$V\gamma 3$	$V\gamma 2$	$V\gamma 3$	$V\gamma 2$	$V\gamma 3$
Change in E2A <sup>-/-</sup> mice*	↓ 2–10†	↑ >3	↓ 3–5	↑ 3–5	↓ 3–5	↓ 2–3	↓ 3	↓ 4–5

\*Change in E2A<sup>-/-</sup> mice indicates increased (↑ fold change) or decreased (↓ fold change)  $V\gamma$  gene rearrangements in E2A<sup>-/-</sup> mice compared with that in E2A<sup>+/+</sup> littermates.

†The data for endogenous  $V\gamma 2$  rearrangements in E2A<sup>-/-</sup> mice are from Bain *et al.* (50).

inhibited V $\gamma$  gene rearrangement, and knocking out either element in isolation had no effect on rearrangement or accessibility (48). Considering that rearrangement of other Ig/TCR genes requires enhancer elements (52), it appears likely that an unidentified cis-acting element(s) necessary for recombination exists in the endogenous C $\gamma$ 1 locus, separate from the segment that comprised our transgene. However, the putative additional regulatory element(s) cannot, by itself, support transcription of most of these rearranged V $\gamma$  genes because transcription of rearranged V $\gamma$ 2, V $\gamma$ 3, and V $\gamma$ 4 genes was nearly abolished in the HsA/3'-EC $\gamma$ 1 double knockout mice. Thus, whereas HsA and 3'-EC $\gamma$ 1 together possess properties of an LCR and are capable of maintaining the genomic accessibility of an ectopically integrated TCR $\gamma$  transgene, they probably function redundantly with other cis-acting regulatory elements in the endogenous locus to promote genomic accessibility for gene rearrangement.

There is an indication that the putative additional regulatory element(s) that promotes recombination in the C $\gamma$ 1 cluster may reside upstream of HsA. In gene-targeted mice in which HsA was replaced by a PGK-*neo* gene cassette, development of V $\gamma$ 2<sup>+</sup> cells was severely reduced (data not shown), whereas deletion of PGK-*neo* resulted in restored numbers of V $\gamma$ 2<sup>+</sup> cells. In contrast, replacement of 3'-EC $\gamma$ 1 with PGK-*neo* did not significantly affect  $\gamma\delta$  T-cell development (data not shown). Because insertion of a PGK-*neo* gene between a cis-acting element and the gene it regulates often inhibits the function of the element (53), these data may imply the existence of an unidentified regulatory element(s) upstream of HsA, which plays a role in V $\gamma$  gene recombination and/or transcription. These results together with the results described earlier in this review suggest that V-J rearrangement in the C $\gamma$ 1 cluster is regulated by multiple cis-acting regulatory elements that function in a hierarchical complex fashion, in which some elements regulate larger segments of the cluster while others play a fine tuning role by regulating accessibility of individual V $\gamma$  genes.

Redundant roles of HsA and 3'-EC $\gamma$ 1 in regulating TCR $\gamma$  gene transcription and  $\gamma\delta$  T-cell development in the thymus As expected, in addition to their roles in regulating V $\gamma$  gene rearrangement, cis-acting elements in the C $\gamma$ 1 cluster are also important in controlling expression of the rearranged TCR $\gamma$  genes and development of corresponding  $\gamma\delta$  T cells. The various elements contribute differentially to rearrangement versus transcription. In particular, although the HsA and 3'-EC $\gamma$ 1 enhancer elements are not required for rearrangement in the endogenous locus, they play critical roles in transcription.

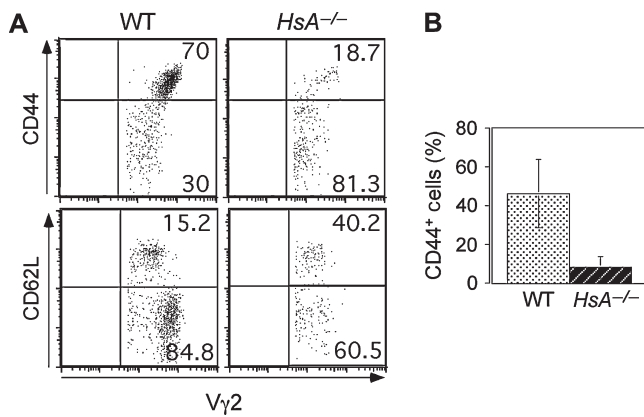
The roles of HsA and 3'-EC $\gamma$ 1 in regulating TCR $\gamma$  gene expression and  $\gamma\delta$  T-cell development were probed with gene-targeted mice lacking one or both of these elements (48). Although germline deletion of HsA and 3'-EC $\gamma$ 1 only impaired V $\gamma$  gene rearrangement modestly (by twofold), the numbers of V $\gamma$ 2<sup>+</sup> and V $\gamma$ 3<sup>+</sup> (and presumably V $\gamma$ 4<sup>+</sup>)  $\gamma\delta$  T cells in the thymus were dramatically decreased by more than 20-fold. Impaired development of these subsets was correlated with a dramatic reduction in the transcription of the TCR $\gamma$  genes located between HsA and 3'-EC $\gamma$ 1, including V $\gamma$ 2, V $\gamma$ 4, and V $\gamma$ 3 genes, whereas the upstream V $\gamma$ 5 and V $\gamma$  genes in other clusters were not substantially affected. Interestingly, separate deletions of HsA or 3'-EC $\gamma$ 1 had little or no effect on transcription of TCR $\gamma$  genes and development of  $\gamma\delta$  T cells in the thymus. This study, along with a study of enhancer elements in the Ig $\kappa$  locus, provided the first direct evidence of redundantly functioning enhancer elements in regulating transcription of an antigen-receptor gene (48). It is noteworthy that although expression of rearranged V $\gamma$ 2–4 genes was highly impaired in the double knockout mice, a few T cells expressing V $\gamma$ 2 and V $\gamma$ 3 at normal levels were detectable in these mice, suggesting that while initiation of gene expression is greatly reduced in the absence of these elements, a few cells that succeed in initiating gene expression can express adequate levels of the corresponding mRNAs.

#### A unique function of HsA in upregulating TCR $\gamma$ gene expression in peripheral V $\gamma$ 2<sup>+</sup> $\gamma\delta$ T cells

Although separate deletions of HsA or 3'-EC $\gamma$ 1 did not impair expression of TCR $\gamma$  genes in the thymus or thymic development of  $\gamma\delta$  T cells, deleting HsA resulted in a significant defect in the periphery. The number of V $\gamma$ 2<sup>+</sup>  $\gamma\delta$  T cells in the spleen or lymph nodes of HsA knockout mice was reduced by a factor of 4 when compared with wildtype mice (48). No such defect was evident in 3'-EC $\gamma$ 1 knockout mice, suggesting a unique role for HsA at the post-thymic stage of  $\gamma\delta$  T-cell development.

Analysis of the cell surface phenotype of  $\gamma\delta$  T cells provided a possible clue as to the cause of the depressed V $\gamma$ 2<sup>+</sup>  $\gamma\delta$  T-cell compartment in the periphery of HsA knockout mice. Most peripheral V $\gamma$ 2<sup>+</sup>  $\gamma\delta$  T cells in wildtype mice have probably been previously exposed to antigen, as they exhibit a memory phenotype characterized by expression of CD44 but not CD62L (54) (Fig. 4). In contrast, the majority of residual V $\gamma$ 2<sup>+</sup> T cells in HsA knockout mice were CD44<sup>low</sup> cells, suggesting that memory V $\gamma$ 2<sup>+</sup> T cells either fail to form or fail to survive in the absence of HsA. As a possible explanation for this defect, it may be significant that in wildtype mice, the TCR levels were elevated by approximately threefold on CD44<sup>+</sup>V $\gamma$ 2<sup>+</sup> T cells as





**Fig. 4. Role of HsA in peripheral development of V $\gamma$ 2<sup>+</sup> cells.** (A) Reduced percentage of CD44<sup>+</sup> cells or CD62L<sup>+</sup> cells among V $\gamma$ 2<sup>+</sup> lymph node cells in HsA<sup>-/-</sup> mice. Gated V $\gamma$ 2<sup>+</sup> cells are shown. (B) Mean percentages ( $\pm$  standard deviation) of CD44<sup>+</sup> cells among V $\gamma$ 2<sup>+</sup> cells in lymph nodes of wildtype versus HsA<sup>-/-</sup> mice ( $n > 5$ ). The total number of lymph node V $\gamma$ 2<sup>+</sup> cells was also reduced (by 4- to 5-fold) in the HsA<sup>-/-</sup> mice.

compared with CD44<sup>-</sup>V $\gamma$ 2<sup>+</sup> T cells, suggesting that formation of V $\gamma$ 2<sup>+</sup> memory cells is associated with increased levels of V $\gamma$ 2<sup>+</sup> TCRs. Memory  $\alpha\beta$  T cells usually show depressed TCR levels. In HsA knockout mice,  $\gamma\delta$  TCR levels (and transcripts) were relatively low on residual V $\gamma$ 2<sup>+</sup> T cells, consistent with the possibility that upregulation of the V $\gamma$ 2<sup>+</sup> TCR on memory cells requires the action of HsA (48). We propose that TCR upregulation and consequent increased TCR signaling may be necessary for differentiation and/or maintenance of V $\gamma$ 2<sup>+</sup> memory cells.

#### Thymic selection and development of tissue-specific $\gamma\delta$ T cells

The findings discussed above have documented the role of programmed V $\gamma$  gene rearrangement in the sequential development of  $\gamma\delta$  subtypes during ontogeny. In principle, programmed V $\gamma$  (and V $\delta$ ) rearrangement might be a sufficient explanation for the sequential appearance of  $\gamma\delta$  T-cell subsets, but it has long been proposed that cellular selection also plays a role in the process. Findings have been somewhat contradictory on this point. A related issue concerns how the appearance of  $\gamma\delta$  T-cell subsets in the thymus is coordinated with the localization of these cells to distinct peripheral sites and, potentially, to the acquisition of different functional activities. Recent studies provide compelling evidence that thymic selection plays a key role in the development of at least some  $\gamma\delta$  T-cell subsets in the periphery, especially the invariant  $\gamma\delta$  T-cell subsets that arise in the fetal thymus and in localization of these  $\gamma\delta$  T cells to their preferred home in the periphery. Studies examining selection in the adult versus fetal thymus are reviewed separately.

#### Selection of $\gamma\delta$ T cells in the adult thymus

The necessity for class I and class II major histocompatibility complex (MHC) molecules in the positive selection of  $\alpha\beta$  T cells in the thymus was clearly shown by the absence of these cells in mice lacking MHC class I and/or class II molecules (55, 56). In contrast, the absence of MHC class I and class II molecules had no observable effect on the development of  $\gamma\delta$  T cells in the thymus (56–58). These data suggested that most  $\gamma\delta$  T cells are not selected by classical MHC molecules or by non-classical class I molecules that depend on  $\beta$ 2-microglobulin ( $\beta$ 2m) for expression on the cell surface. However, it remained possible that MHC does play a role in selection of a subset of  $\gamma\delta$  T cells. Furthermore, the natural ligands for  $\gamma\delta$  T cells are in most cases not known and it remains possible that many or all the cells are positively selected by interactions with unidentified ligands in the thymus.

Because of limited knowledge of the natural ligands for  $\gamma\delta$  TCRs, many studies designed to examine whether developing  $\gamma\delta$  T cells undergo selection have used the G8 and KN6 transgenic mouse strains. The two transgenic strains harbor different  $\gamma\delta$  TCRs, although both include a V $\gamma$ 2 chain. The two TCRs are specific for the same ligands, the T10 and T22 non-classical MHC class I molecules (59–62). T10 and T22 are encoded in the TL region of the MHC and are expressed highly in B6 mice (H-2<sup>b</sup> haplotype) but only weakly in BALB/c mice (H-2<sup>d</sup> haplotype) because of defective T22 gene expression in the latter strain (59). Studies with T22 tetramers show that a small subset of naturally arising mature peripheral  $\gamma\delta$  T cells in normal mice is specific for these same ligands (63).

Early studies with G8 and KN6 transgenic mice reported that the transgenic  $\gamma\delta$  T cells underwent negative selection in mice that express strong ligands for the receptors, such as the B6 strain, but not in mice that express a weak ligand, such as the BALB/c strain (64, 65). It has been much more controversial whether interactions of these transgenic  $\gamma\delta$  T cells with ‘weak’ ligands are necessary for positive selection of the cells. Several studies suggested that positive selection was necessary, based on the finding that transgenic  $\gamma\delta$  T cells developed much less efficiently in  $\beta$ 2m-deficient mice, which lack surface expression of T10 and T22, than in BALB/c mice, which express a weak ligand (14, 66, 67). Another study attributed much of this difference to negative selection, because of variations in the genetic backgrounds of the mice under study (68). On top of this contradiction, a study that used T22 tetramers to directly assay the development of natural T10/T22-specific  $\gamma\delta$  T cells in non-transgenic mice concluded that development of these cells is not impaired in  $\beta$ 2m-deficient mice (K. Jensen & Y. Chien, personal communication). In lieu of positive selection, these

authors attribute the relatively high frequency of T10/T22-reactive  $\gamma\delta$  T cells in normal mice to programmed rearrangement mechanisms that assemble a reactive TCR in a small but significant population of developing  $\gamma\delta$  T cells. Given the variability in experimental outcomes and the possibility that these TCRs may be positively selected by an unknown  $\beta 2m$ -independent ligand, it remains uncertain whether positive selection is required for the development of T10/T22-reactive  $\gamma\delta$  T cells in the adult thymus.

#### Selection of $\gamma\delta$ T cells in the fetal thymus

As an approach to investigate the requirement for thymic selection of  $\gamma\delta$  T cells in the early fetal thymus, especially the  $V\gamma 3^+$  DETC population, several studies have investigated mice equipped with various  $\gamma\delta$  TCR transgenes non-native to the skin. The underlying assumption of most of these studies was that the transgenic TCR would exclude expression of TCRs normally found in the fetal thymus, especially the canonical  $V\gamma 3V\delta 1$  TCR found on most DETCs. Positive selection of DETCs, if necessary, should be aborted if the transgenic TCR had the inappropriate specificity. It was shown that  $\gamma\delta$  T cells expressing 'inappropriate' transgenic TCRs were able to develop normally into DETCs in the skin, suggesting that DETC development is not dependent on expression of  $V\gamma 3$  or  $V\delta 1$  and therefore is not dependent on positive thymic selection by ligands that are specifically recognized only by the  $V\gamma 3V\delta 1$  DETC TCR (69, 70). The assumption that the transgenic TCR necessarily excludes expression of endogenous TCRs was later proven incorrect, however, raising doubts about the earlier conclusions. For example, whereas plentiful transgene-expressing skin-resident  $\gamma\delta$  T cells developed in  $V\delta 6.3$  transgenic mice, none developed when the transgene was crossed onto a  $TCR\delta^{-/-}$  background in which endogenous  $TCR\delta$  chains cannot be expressed (71). These data suggested that development of the skin-resident  $\gamma\delta$  T cells depends on endogenously encoded  $TCR\delta$  chains in the transgenic mice, such as the  $V\delta 1$  chain. This finding resurrected the possibility that DETC development requires positive selection on specific ligands in the thymus.

As a related approach to this question, the requirement for the  $V\gamma 3V\delta 1$  TCR in development of DETCs was assessed by knocking out the  $V\gamma 3$  or  $V\delta 1$  genes. Disabling either of these genes did not prevent development of DETCs, proving that TCRs other than  $V\gamma 3V\delta 1$  are compatible with DETC development (72, 73). Analysis of  $\gamma\delta$  TCR<sup>+</sup> DETCs in the  $V\gamma 3^{-/-}$  mice detected  $V\gamma 1.1$ ,  $V\gamma 2$ , and  $V\gamma 5$  paired  $V\delta 1$  and other  $V\delta$  chains. The possibility that the DETCs in  $V\gamma 3$  knockout mice had undergone specific intrathymic positive selection was inferred from the finding that the TCRs on these DETCs had a specific

'idiotypic' marker detected with the 17D1 monoclonal antibody, a marker that is also found on  $V\gamma 3V\delta 1$  TCRs (72). However, this marker was absent on the DETCs that arose in  $V\delta 1$  knockout mice, suggesting that 17D1 reactivity might be associated with  $V\delta 1$ -containing TCRs rather than with specificity of the TCR (73). A more compelling suggestion that the DETCs in  $V\gamma 3$  knockout mice had undergone selection was the finding that these cells produced IL-2 when stimulated with keratinocyte cell lines, a specificity previously shown to be associated with DETCs, but not other subsets of  $\gamma\delta$  T cells (72).

Evidence for thymic positive selection of DETC precursors The previous studies were consistent with the selection of DETC precursors, but did not distinguish whether the selection occurred in the skin itself or was the result of selection in the thymus. We recently used gene targeting to reinvestigate the requirement for specific  $V\gamma$  chains in the development of DETCs. The results provided compelling evidence for selection of DETCs and clear evidence that it occurs in the thymus, and at the same time these studies showed a possible mechanism to couple thymic selection with emigration of the T cells to the epidermis (74).

We generated mice with a large deletion of the  $C\gamma 1$  cluster that encompassed  $HsA$ ,  $V\gamma 2$ ,  $V\gamma 4$ ,  $V\gamma 3$ ,  $J\gamma 1$ ,  $C\gamma 1$ , and 3'- $EC\gamma 1$  (234J $C\gamma 1^{-/-}$  mice). The remaining  $C\gamma$  clusters remained intact, and  $V\gamma 1.1^+$  and probably  $V\gamma 1.2^+$  T cells developed normally in the thymus of the knockout mice, where they were first detected at days 15 and 16 of gestation. Normal numbers of  $\gamma\delta$  T cells, most of them  $V\gamma 1.1^+$  cells, were detected in the spleen and intestines of these mice, but  $V\gamma 2^+$ ,  $V\gamma 3^+$ , and  $V\gamma 5^+$  T cells were absent, as expected. Remarkably, despite the abundance of  $\gamma\delta$  T cells in other peripheral sites and in the fetal thymus, there were virtually no  $TCR\gamma\delta^+$  DETCs in the epidermis of these mice (74). Therefore, DETC development was dependent on expression of specific  $\gamma$  chains, consistent with a requirement for positive selection of the cells. These data were in contrast to the findings using  $V\gamma 3$  knockout mice reported above, where  $V\gamma 1.1$  and other  $\gamma\delta$  T cells were detected in the epidermis in lieu of  $V\gamma 3^+$  T cells. The basis for this discrepancy in results is currently under investigation.

Additional evidence that the DETC precursors undergo specific positive selection and that selection occurs in the fetal thymus came from analysis of marker expression on fetal thymocytes. Consistent with earlier studies (75, 76), we found that approximately 70% of  $V\gamma 3^+$   $\gamma\delta$  T cells in the fetal thymus express various activation markers, including CD122, which is the  $\beta$  chain of the receptors for IL-2 and IL-5. We showed that CD122 expression on  $V\gamma 3^+$  fetal thymocytes was correlated

with expression of V $\delta$ 1, the chain characteristic of DETCs (74). V $\gamma$ 3<sup>+</sup> fetal thymates lacking CD122 showed a much more diverse expression of TCR V $\delta$  chains, indicating that CD122 expression occurred specifically on V $\gamma$ 3<sup>+</sup>V $\delta$ 1<sup>+</sup> fetal thymocytes and not on thymocytes with V $\gamma$ 3 paired to other TCR $\delta$  chains. Remarkably, CD122 was completely absent on the  $\gamma\delta$  T cells in the fetal thymus of 234JCY1<sup>-/-</sup> mice, which lack DETCs, showing a correlation between the expression of activation markers in the thymus and development of DETCs. Thus, expression of DETC-specific V $\delta$ 1 was associated with CD122 expression in the thymus, which was in turn correlated with DETC development. These data suggested that among fetal thymic precursors expressing various  $\gamma\delta$  TCRs, those expressing V $\gamma$ 3 and V $\delta$ 1 engaged a thymic ligand, leading to expression of activation markers and ultimately development of DETCs.

While highly suggestive of selection, the correlations described above could theoretically arise by other mechanisms. However, as a result of an unexpected observation, we were able to provide strong additional evidence that these events reflected positive selection. As one approach to test whether the absence of DETC development in 234JCY1<sup>-/-</sup> mice was specifically because of the absence of V $\gamma$ 3, we attempted to complement the 234JCY1<sup>-/-</sup> mice with a transgene encoding a rearranged functional V $\gamma$ 2–J $\gamma$ 1CY1 chain. Surprisingly, the transgene restored DETC development and all the resulting DETCs expressed the transgenic V $\gamma$ 2 chain (74). While this finding could have been seen to resurrect the notion that DETC development does not depend on specific TCR expression, analysis of the DETCs showed instead a very specific TCR configuration: all the cells expressed V $\gamma$ 2, as expected, and showed great enrichment for V $\delta$ 7 expression and little or no expression of other TCR $\delta$  chains, including V $\delta$ 1. Furthermore, the V $\gamma$ 2<sup>+</sup>V $\delta$ 7<sup>+</sup> DETCs, but not peripheral  $\gamma\delta$  T cells, produced IL-2 when stimulated with keratinocyte cell lines, suggesting that the V $\gamma$ 2V $\delta$ 7 receptor combination, like the V $\gamma$ 3V $\delta$ 1 combination, confers specificity for a keratinocyte ligand. The story was brought full circle when the analysis of the V $\gamma$ 2 transgenic/knockout mice showed that the V $\gamma$ 2 transgene resulted in restored expression of CD122 on a subset of V $\gamma$ 2<sup>+</sup> fetal thymocytes. Furthermore, the V $\gamma$ 2<sup>+</sup> fetal thymocytes that expressed CD122 predominantly expressed V $\delta$ 7, whereas V $\gamma$ 2<sup>+</sup> fetal thymocytes that did not express CD122 expressed a diversity of V $\delta$  genes. These data strongly supported the conclusion that only specific subsets of developing fetal thymic  $\gamma\delta$  T cells, including cells expressing the V $\gamma$ 3V $\delta$ 1 pair and the V $\gamma$ 2V $\delta$ 7 pair, exhibit reactivity with an unknown ligand that is expressed by fetal thymic stromal cells and probably shared with keratinocytes. Moreover, such reactivity leads to expression of CD122 on developing fetal thymocytes and is necessary for development of DETCs (74).

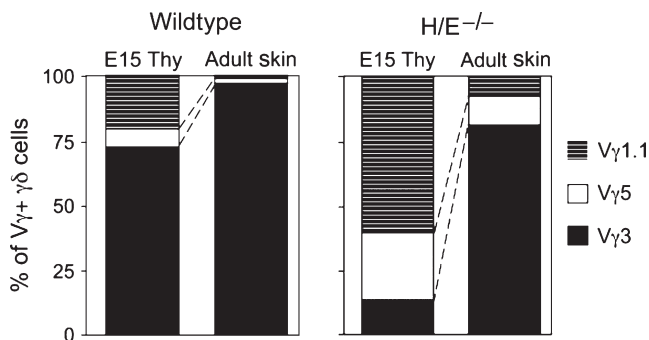
A recent report provides independent support for positive selection of DETC precursors in the thymus. A comparison of mouse strains showed normal development of V $\gamma$ 3<sup>+</sup> DETCs in FVB-Jax strain mice but none in the related FVB/N-Taconic strain, and chimera experiments showed that the defect in FVB/N-Taconic was consistent with the absence of a selecting ligand for DETCs in the fetal thymus (77). The identity of the putative selecting ligand has not yet been reported.

#### Preference for the V $\gamma$ 3V $\delta$ 1 pair among DETCs in wildtype mice

If different V $\gamma$ V $\delta$  pairs are compatible with DETC development, why does the V $\gamma$ 3V $\delta$ 1 pair predominate in wildtype mice? A likely possibility, already discussed above, is that the V $\gamma$ 3V $\delta$ 1 pair is more likely to arise in the fetal thymus than other V $\gamma$ V $\delta$  pairs, as a result of favored rearrangement of these genes in the fetal period. However, this preference cannot fully account for the dominance of the V $\gamma$ 3V $\delta$ 1 pair. In wildtype mice, a small but significant percentage of DETCs in the epidermis are V $\gamma$ 3<sup>-</sup> in the neonatal period, whereas such cells are nearly undetectable in the adult stage (73, 78). Thymic production of V $\gamma$ 3<sup>+</sup> cells ceases by the time of birth, so the change cannot reflect greater thymic production of V $\gamma$ 3<sup>+</sup> cells at the adult stage.

Other evidence suggests that the V $\gamma$ 3V $\delta$ 1 TCR is favored among DETCs, even when it is not the most commonly expressed  $\gamma\delta$  TCR in the early fetal thymus. In the previously discussed gene-targeted mice in which the 3'–EC $\gamma$ 1 and HsA enhancer elements were simultaneously deleted in the CY1 cluster, the number of V $\gamma$ 3<sup>+</sup> T cells in the fetal thymus was reduced by fivefold to 20-fold at all time points, but V $\gamma$ 1.1<sup>+</sup> and V $\gamma$ 5<sup>+</sup>  $\gamma\delta$  T cells developed normally. Therefore, V $\gamma$ 3<sup>+</sup> T cells represented only a small percentage of  $\gamma\delta$  T cells in the fetal thymus of these mice (47) (Fig. 5). Nevertheless, approximately 75% of DETCs in the skin of adult knockout mice expressed V $\gamma$ 3, suggesting selection for the canonical V $\gamma$ 3V $\delta$ 1 TCR among DETCs (47) (Fig. 5).

Between the neonatal and adult period, DETCs are believed to expand greatly in the epidermis, and it is likely that the local environment in the skin is important for selective expansion of the V $\gamma$ 3<sup>+</sup>  $\gamma\delta$  T cells. The expansion could result from local engagement of the DETC TCR with epidermal ligands, although it is believed that the putative ligand is only expressed well in damaged or stressed epidermis (79). Alternatively, one could speculate that the V $\gamma$ 3V $\delta$ 1<sup>+</sup> DETCs may receive more potent positive selection signals in the fetal thymus, perhaps because of a higher affinity of the V $\gamma$ 3V $\delta$ 1 TCR for the selecting ligand, which may enhance the ability of the cells to survive and expand later in the epidermis.



**Fig. 5. The composition of different  $\gamma\delta$  subsets in the fetal thymus (day 15 of gestation) and adult skin (DETC), comparing wildtype littermates with  $H/E^{-/-}$  mice lacking both known  $C\gamma 1$  enhancer elements (HsA and 3'-EC $\gamma 1$ ). Most DETCs express  $V\gamma 3$  in mice of both genotypes, but  $V\gamma 3^{+}$  cells are quite rare in the fetal thymus of mice. These data suggest a strong preference for  $V\gamma 3^{+}$  DETCs, even when these cells are produced in limiting numbers. Data are based on analysis of cells from three or more mice of each type on a mixed (C57BL/6/129Sv) genetic background.**

Positive versus negative selection in the thymus may vary at different developmental stages

Differences have been noted in comparing negative selection in the thymus of adult and fetal (or neonatal) mice, which may be instructive in understanding the different roles of the  $\gamma\delta$  T-cell subsets that arise at these stages. In the adult thymus, T cells expressing the G8  $\gamma\delta$  TCR underwent negative selection in mice that express the strong TL<sup>b</sup> ligand, but little or no negative selection occurred in neonatal mice of the same genotype, despite indications that the G8 TCR was engaged with its ligand at that stage (80). Another report indicated that transgenic  $V\gamma 3V\delta 1^{+}$   $\gamma\delta$  T cells underwent negative selection in adult but not fetal thymi in certain genetic backgrounds (81). Finally, treatment of fetal thymic organ cultures with anti- $\gamma\delta$ -TCR antibodies did not delete  $\gamma\delta$  T cells, whereas the same treatment causes deletion of adult thymic  $\gamma\delta$  T cells (82). These indications suggest that developing fetal thymic  $\gamma\delta$  T cells are highly resistant to negative selection. Perhaps this resistance reflects the specialization of the fetal thymus to select cells with highly defined, invariant specificities. The highly constrained repertoire of rearrangements and junctional diversification that occur in the fetal thymus is predicted to produce a large number of cells with receptors of the optimal affinity for the correct ligand. If the selecting ligands in the thymus are identical to or have the same affinity for the receptor as the ligands recognized by the cells in the periphery, negative selection, if it occurred, would presumably result in deletion of the cells. Because  $\gamma\delta$  T cells that arise at the adult stage are highly diverse, effective negative selection is probably necessary to reduce the risk of generating pathogenic autoreactive  $\gamma\delta$  T cells. These consid-

erations may aid in understanding why and how the thymus specializes in producing different types of T cells at different stages of development.

How does positive selection in the fetal thymus enable development of  $\gamma\delta$  T cells in the epidermis?

Having provided evidence for positive selection of DETCs, we sought to understand how selection in the thymus is coordinated with T-cell development in the skin. In theory, thymic selection could influence migration from the thymus, migration to the epidermis, and survival and/or expansion in the epidermal environment.

Consistent with a role in lymphocyte migration, we found that positive selection in the fetal thymus resulted in a coordinated switch in expression of several chemokine and homing receptors in the selected cells (74). Chemokine receptor CCR10 and sphingosine 1-phosphate receptor-1 (S1P1) were upregulated, while CCR6 was downregulated in the positively selected fetal thymic  $\gamma\delta$  T cells. Together, these changes are expected to enhance emigration from the thymus and may direct cells specifically to the epidermis. Recent studies show, for example, that S1P1 is essential for the exiting of mature  $\alpha\beta$  T cells from the thymus in adult mice (83). Also, the ligand for CCR6, CCL20, is highly expressed in the thymus (84–87). Upregulation of S1P1 and loss of CCR6 are therefore predicted to promote thymic export. Furthermore, the ligand for CCR10, the chemokine CCL27, is constitutively expressed in the skin (88–90), including the fetal skin (74). Hence, CCR10 upregulation may promote skin homing of positively selected  $\gamma\delta$  T cells after they exit the fetal thymus. These predictions remain to be tested experimentally.

Positive selection in the thymus may also influence propagation of DETCs in the epidermis in other ways. A likely possibility is that the survival and expansion of DETCs in the skin are enabled by the upregulation of CD122 that occurs as a result of positive selection of DETC precursors in the fetal thymus. CD122 is the  $\beta$  chain of the IL-2/IL-15 receptor, and IL-15 is known to be essential for DETC development (91), whereas IL-2 is not essential (92). In both CD122 and IL-15 knockout mice,  $V\gamma 3^{+}$   $\gamma\delta$  T cells were generated in the fetal thymus nearly as efficiently as in wildtype mice, but DETCs did not develop (91, 93). It has been proposed that IL-15 expressed by keratinocytes maintains survival/expansion of DETCs in the skin, apparently through interaction with IL-15 receptors expressed on skin-specific  $\gamma\delta$  T cells (91). Therefore, the survival and expansion of DETCs in the skin are probably promoted by the upregulation of cytokine receptors, which occurs during positive selection in the thymus. It remains

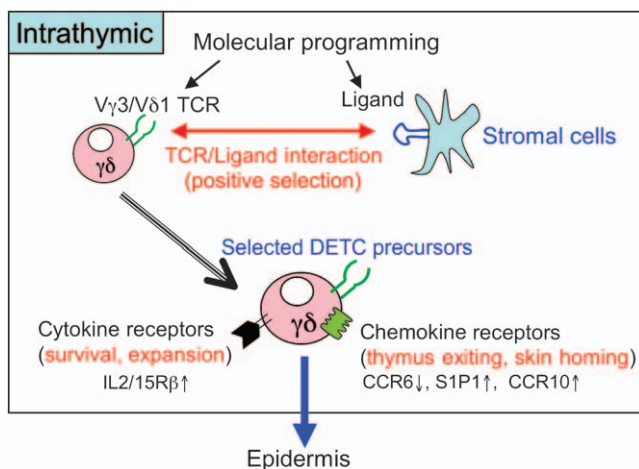


possible that additional stimulation of the cells in the epidermis is also important in maintaining expression of these cytokine receptors.

#### A proposed model for development of tissue-localized $\gamma\delta$ T-cell subsets

Programmed V $\gamma$  gene rearrangement and cellular selection cooperate to regulate development of different tissue-specific  $\gamma\delta$  T cells (Fig. 6). In the early fetal thymus, a programmed rearrangement process based on proximity of the gene to J $\gamma$ 1 favors the generation of V $\gamma$ 3V $\delta$ 1<sup>+</sup>  $\gamma\delta$  T cells, the natural skin-specific  $\gamma\delta$  T-cell precursors, and V $\gamma$ 4V $\delta$ 1<sup>+</sup> T cells, destined for other epithelial locations. Subsequently, the V $\gamma$ 3V $\delta$ 1<sup>+</sup>  $\gamma\delta$  T cells are positively selected by engagement of the V $\gamma$ 3V $\delta$ 1 TCR with a ligand expressed in the fetal thymus, resulting in a coordinate switch in expression of chemokine receptors and cytokine receptors, which in turn directs the specific migration of these cells to the skin and enhances their subsequent expansion and survival there.

In the adult thymus, V $\gamma$ 3 and V $\gamma$ 4 (and V $\delta$ 1) rearrangements are suppressed by the programmed rearrangement process, favoring production of V $\gamma$ 2<sup>+</sup> and other adult-type  $\gamma\delta$  T cells. Although it remains somewhat murky whether positive selection acts on adult-type  $\gamma\delta$  T cells, it is likely that a maturation process, whether selection or another process, confers these cells with their specific homing properties,



**Fig. 6. Proposed overall scheme of developmental events leading to development of  $\gamma\delta$  T cells in the skin (DETCs).** In the first phase, programmed rearrangement events lead to a striking bias in TCR gene rearrangements, favoring production of the V $\gamma$ 3V $\delta$ 1 canonical TCR, and may direct expression on thymic stromal cells of ligands necessary for positive selection of DETCs. Positive selection ensures that only cells with the correct specificity differentiate further and bestow the selected cells with chemokine receptors, which enable the cells to exit the thymus and migrate to the epidermis, and with cytokine receptors, which aid in subsequent survival and expansion of the cells.

enabling them to exit the thymus and home to secondary lymphoid organs.

Presumably, the directed differential homing of specific subsets of  $\gamma\delta$  T cells is ultimately important for positioning these different T cells appropriately for their specialized roles. In the case of invariant  $\gamma\delta$  subsets, such as the V $\gamma$ 3V $\delta$ 1<sup>+</sup> DETCs, the cells are localized to the site where they can recognize their specialized ligands, which are induced in the skin by the pathophysiological changes (79). The much more variable adult  $\gamma\delta$  T-cell subsets, such as V $\gamma$ 2<sup>+</sup> T cells, are believed to be involved in surveillance for more diverse sets of ligands, appropriate to their localization to the secondary lymphoid organs.

#### Concluding remarks

Our research has addressed all stages of the development of  $\gamma\delta$  T cells, including the decision to differentiate along the  $\alpha\beta$  versus  $\gamma\delta$  lineage, ordered production of V $\gamma$ -defined subsets of  $\gamma\delta$  T cells over the course of ontogeny and the acquisition during development of guidance information that directs the cell to its appropriate destination. Our findings together with those of others show that programmed events, such as V $\gamma$  gene rearrangement and junctional diversification mechanisms, cooperate with subsequent cellular selection processes, ultimately generating distinct sets of specific tissue-localized  $\gamma\delta$  T cells. For example, in the absence of selection, most of the productive V $\gamma$  rearrangements in the early fetal period are canonical V $\gamma$ 3 rearrangements (9). Subsequent development of DETCs nevertheless requires a cellular selection process. Hence, with respect to  $\gamma\delta$  T-cell specificity, the molecular program greatly restricts the possible repertoire at the fetal stage, and selection reinforces this restriction. In the normal course of events, many of the cells subjected to selection will already express the canonical V $\gamma$ 3V $\delta$ 1 receptor, so it might be surmised that shaping the repertoire is not the sole or even main role of positive selection. But under exceptional circumstances, the selection process is capable of selecting alternative TCRs, including the V $\gamma$ 2V $\delta$ 7 receptor, which apparently provides specificity similar to that of the canonical receptor.

The role of selection extends beyond shaping the repertoire, however, by inducing expression of specific chemokine receptors and cytokine receptors that are likely to determine the subsequent localization, survival, and proliferation of the cells. This outcome of positive selection at the fetal stage may be 'hardwired' in the sense that all  $\gamma\delta$  lineage cells at this stage will make this response to TCR ligation. We found, for example, that



fetal thymocytes stimulated with anti- $\gamma\delta$ -TCR antibodies upregulated the same chemokine and cytokine receptors as DETC precursors, even if the thymocytes lacked a known DETC-compatible receptor (74). These considerations suggest that the specialization of the fetal thymus to produce DETCs is imposed at numerous levels of development and prompt the question of why such stage-specific specialization evolved. We suggest that it is necessary because the repertoires, diversity, and functions of the cells produced at the fetal versus adult stages differ so greatly. Specialization of the thymus for production of different subsets at different stages is one way to vary developmental processes at different stages to impart different repertoires and functionality on the corresponding cells. As examples, the absence of TdT at the fetal stage is critical to producing a high frequency of canonical V $\gamma$ 3 rearrangements appropriate for DETC production (24, 25), and we have proposed in this review that the reduced susceptibility of fetal thymocytes to negative selection may be important for producing cells like DETCs, with a fixed specificity for a self-ligand.

Despite its role in the production of DETCs and V $\gamma$ 4V $\delta$ 1 invariant T cells at the fetal stage, ontogenic specialization is clearly not required to produce other specialized T-cell subsets. Aside from the obvious CD4/CD8 subsets, some of the T-cell subsets that arise in the adult thymus have properties similar to those of DETCs. Examples are natural killer (NK) T cells, CD8 $\alpha\alpha^+$  T cells, mucosal-associated invariant T cells (MALT), which exhibit restricted TCR repertoires, are localized to specific peripheral tissues, and exhibit distinctive immune functions. The development of these cells is not separated in ontogeny from the production of conventional  $\alpha\beta$  T cells and adult-type  $\gamma\delta$  T cells, but the differentiation process they undergo may share one or more of the features of DETC development outlined in this review. Developmentally, many of these cells undergo selection processes that are unlike those of conventional  $\alpha\beta$  T cells but similar in some respects to those of  $\gamma\delta$  T cells. For example, NK T cells are positively selected by a self-glycolipid antigen associated with CD1 (94, 95), while MALT cells are positively selected by the self-antigen MR1 (96, 97). Strong TCR/antigen interactions, which usually result in

negative selection of conventional T cells, were found to promote development of CD8 $\alpha\alpha^+$  T cells (98). It remains to be seen whether positive selection of these and other T-cell subsets also induces chemokine and cytokine receptors that aid in peripheral localization and survival of the cells.

$\gamma\delta$  T cells in the human also display unique tissue distribution patterns. For example, V $\delta$ 1 $^+$   $\gamma\delta$  T cells are predominant in intestines and skin (99, 100), where they are believed to play an important role in tumor surveillance and maintenance of tissue integrity, while V $\delta$ 2 $^+$   $\gamma\delta$  T cells are the dominant population of secondary lymphoid organs and blood (101). The findings in the mouse system will ultimately provide a guide for understanding the development and localization of  $\gamma\delta$  T cells in humans.

Detailed molecular events that dictate programmed V $\gamma$  gene rearrangement and generation of corresponding  $\gamma\delta$  T cells remain to be defined. In understanding the adult pattern, identification of transcription factors that interact with the cis-acting elements in the TCR $\gamma$  locus, especially the promoters, is one of the most prominent issues to be resolved. Understanding the role of E2A in this process will be an important step. However, E2A cannot be the sole determinant of the adult developmental pattern because E2A-deficient mice still have plentiful V $\gamma$ 2  $\gamma\delta$  T cells, but no V $\gamma$ 3 $^+$   $\gamma\delta$  T cells in the adult thymus (N. Xiong, C. Murre, D. H. Raulet, unpublished data). In understanding the fetal pattern, it will be necessary to dissect how the rearrangement pattern is controlled by gene location in the cluster, independent of differences in V $\gamma$  gene accessibility. With respect to  $\gamma\delta$  T-cell selection, it is critical to identify the ligands that mediate positive selection of DETCs and potentially other  $\gamma\delta$  T-cell subsets and to determine if and how their expression is restricted to specific developmental stages or cell types in the thymus. To understand tissue localization, it will be essential to test directly the roles of specific chemokine receptors and other molecules that influence cell localization. Finally, an essential issue to explore is whether and how positively selected  $\gamma\delta$  T cells at different stages of ontogeny (and therefore selecting environments) are bestowed with different homing properties.

## References

1. Raulet DH. The structure, function, and molecular genetics of the  $\gamma/\delta$  T cell receptor. *Annu Rev Immunol* 1989;7:175–207.
2. Haas W, Pereira P, Tonegawa S. Gamma/delta cells. *Annu Rev Immunol* 1993;11:637–685.
3. Allison JP, Havran WL. The immunobiology of T cells with invariant  $\gamma\delta$  antigen receptors. *Annu Rev Immunol* 1991;9:679–705.
4. Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol* 2000;18:975–1026.
5. Garman RD, Doherty PJ, Raulet DH. Diversity, rearrangement and expression of murine T cell gamma genes. *Cell* 1986;45:733–742.
6. Capone M, Hockett RD, Zlotnik A. Kinetics of T cell receptor  $\beta$ ,  $\gamma$ , and  $\delta$  rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44+CD25+ Pro-T thymocytes. *Proc Natl Acad Sci U S A* 1998;95:12522–12527.

7. Livák F, Tourigny M, Schatz DG, Petrie HT. Characterization of TCR gene rearrangements during adult murine T cell development. *J Immunol* 1999;**162**:2575–2580.
8. Livak F, Petrie HT, Crispe IN, Schatz DG. In-frame TCR  $\delta$  rearrangements play a critical role in the  $\alpha\beta/\gamma\delta$  lineage decision. *Immunity* 1995;**2**:617–627.
9. Dudley EC, Girardi M, Owen MJ, Hayday AC.  $\alpha\beta$  and  $\gamma\delta$  T cells can share a late common precursor. *Curr Biol* 1995;**5**:659–669.
10. Kang J, Baker J, Raulet D. Evidence that productive rearrangements of TCR $\gamma$  genes influence the fate of developing T cells. *Eur J Immunol* 1995;**25**:2706–2709.
11. Kang J, Volkmann A, Raulet DH. Evidence that gammadelta versus alphabeta T cell fate determination is initiated independently of T cell receptor signaling. *J Exp Med* 2001;**193**:689–698.
12. Ciofani M, Knowles GC, Wiest DL, von Boehmer H, Zuniga-Pflucker JC. Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. *Immunity* 2006;**25**:105–116.
13. Saint-Ruf C, Panigada M, Azogui O, Debey P, von Boehmer H, Grassi F. Different initiation of pre-TCR and gammadeltaTCR signalling. *Nature* 2000;**406**:524–527.
14. Haks MC, et al. Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity* 2005;**22**:595–606.
15. Hayes SM, Li L, Love PE. TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity* 2005;**22**:583–593.
16. Raulet D, et al. Control of  $\gamma\delta$  T cell development. *Immunol Rev* 1991;**120**:185–204.
17. Allison JP. Gamma delta T-cell development. *Curr Opin Immunol* 1993;**5**:241–246.
18. Havran WL, Allison JP. Thy-1<sup>+</sup> dendritic epidermal cells of adult mice arise from fetal thymic precursors. *Nature* 1990;**344**:68–70.
19. Itoharu S, et al. Homing of a  $\gamma\delta$  thymocyte subset with homogenous T-cell receptors to mucosal epithelia. *Nature* 1990;**343**:754–757.
20. Viney JL, MacDonald TT, Kilshaw PJ. T-cell receptor expression in intestinal intraepithelial lymphocyte subpopulations of normal and athymic mice. *Immunology* 1989;**66**:583–587.
21. Rocha B, Vassalli P, Guy-Grand D. Thymic and extrathymic origins of gut intraepithelial lymphocyte populations in mice. *J Exp Med* 1994;**180**:681–686.
22. Pereira P, Hermitte V, Lembezat MP, Boucontet L, Azuara V, Grigoriadou K. Developmentally regulated and lineage-specific rearrangement of T cell receptor Valpha/delta gene segments. *Eur J Immunol* 2000;**30**:1988–1997.
23. Davis MM, Bjorkman PJ. T cell antigen genes and T cell recognition. *Nature* 1988;**334**:395–402.
24. Tigelaar RE, Lewis JM. Immunobiology of mouse dendritic epidermal T cells: a decade later, some answers, but still more questions. *J Invest Dermatol* 1995;**105**:43S–49S.
25. Asarnow DM, Cado D, Raulet DH. Selection is not required to produce invariant T cell receptor  $\gamma$ -gene junctional sequences. *Nature* 1993;**362**:158–160.
26. Zhang Y, et al. The role of short homology repeats and TdT in generation of the invariant  $\gamma\delta$  antigen receptor repertoire in the fetal thymus. *Immunity* 1995;**3**:439–447.
27. Itoharu S, et al. T cell receptor  $\delta$  gene mutant mice: independent generation of  $\alpha\beta$  T cells and programmed rearrangements of  $\gamma\delta$  TCR genes. *Cell* 1993;**72**:337–348.
28. Korman A, Marusic-Galesic S, Spencer D, Kruisbeek A, Raulet DH. Predominant variable region gene usage by  $\gamma/\delta$  T cell receptor-bearing cells in the adult thymus. *J Exp Med* 1988;**168**:1021–1040.
29. Heilig JS, Tonegawa S. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature* 1986;**322**:836–840.
30. Takagaki Y, Nakanishi N, Ishida I, Kanagawa O, Tonegawa S. T-cell receptor- $\gamma$  and - $\delta$  genes preferentially utilized by adult thymocytes for the surface expression. *J Immunol* 1989;**142**:2112–2121.
31. Guy-Grand D, Cerf-Bensussan N, Malissen B, Malassis-Seris M, Briottet C, Vassalli P. Two gut intraepithelial CD8<sup>+</sup> lymphocyte populations with different T cell receptors: a role for gut epithelium in T cell differentiation. *J Exp Med* 1991;**173**:471–481.
32. Lefrançois L, Puddington L. Extrathymic intestinal T-cell development: virtual reality? *Immunol Today* 1995;**16**:16–21.
33. Ikuta K, et al. A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell* 1990;**62**:863–874.
34. Goldman J, Spencer D, Raulet D. Ordered rearrangement of variable region genes of the T cell receptor gamma locus correlates with transcription of the unrearranged genes. *J Exp Med* 1993;**177**:729–739.
35. Baker JE, Cado D, Raulet DH. Developmentally programmed rearrangement of T cell receptor V $\gamma$  genes is controlled by sequences immediately upstream of the V $\gamma$  genes. *Immunity* 1998;**9**:159–168.
36. Agata Y, et al. Histone acetylation determines the developmentally regulated accessibility for T cell receptor gamma gene recombination. *J Exp Med* 2001;**193**:873–879.
37. Yancopoulos GD, Desiderio SV, Paskind M, Kearney JF, Baltimore D, Alt FW. Preferential utilization of the most J<sub>H</sub>-proximal V<sub>H</sub> gene segments in pre-B-cell lines. *Nature* 1984;**311**:727–733.
38. Yancopoulos GD, Malynn BA, Alt FW. Developmentally regulated and strain-specific expression of murine V<sub>H</sub> gene families. *J Exp Med* 1988;**168**:417–435.
39. Chien Y-H, et al. T cell receptor  $\delta$  gene rearrangements in early thymocytes. *Nature* 1987;**330**:722–727.
40. Peschon JJ, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 1994;**180**:1955–1960.
41. Corcoran AE, Riddell A, Krooshoop D, Venkitaraman AR. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. *Nature* 1998;**391**:904–907.
42. Malynn BA, Yancopoulos GD, Barth JE, Bona CA, Alt FW. Biased expression of J<sub>H</sub>-proximal V<sub>H</sub> genes occurs in the newly generated repertoire of neonatal and adult mice. *J Exp Med* 1990;**171**:843–859.
43. Jung D, Giallourakis C, Mostoslavsky R, Alt FW. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annu Rev Immunol* 2006;**24**:541–570.
44. Connor AM, Fanning LJ, Celler JW, Hicks LK, Ramsden DA, Wu GE. Mouse VH7183 recombination signal sequences mediate recombination more frequently than those of VHJ558. *J Immunol* 1995;**155**:5268–5272.
45. Williams GS, et al. Unequal VH gene rearrangement frequency within the large VH7183 gene family is not due to recombination signal sequence variation, and mapping of the genes shows a bias of rearrangement based on chromosomal location. *J Immunol* 2001;**167**:257–263.
46. Xiong N, Baker JE, Kang C, Raulet DH. The genomic arrangement of T cell receptor variable genes is a determinant of the developmental rearrangement pattern. *Proc Natl Acad Sci U S A* 2004;**101**:260–265.
47. Tanimoto K, Liu Q, Bungert J, Engel JD. Effects of altered gene order or orientation of the locus control region on human beta-globin gene expression in mice. *Nature* 1999;**398**:344–348.
48. Xiong N, Kang CH, Raulet DH. Redundant and unique roles of two enhancer elements in the TCR gamma locus in gene regulation and gamma delta T cell development. *Immunity* 2002;**16**:453–463.
49. Greenbaum S, Zhuang Y. Regulation of early lymphocyte development by E2A family proteins. *Semin Immunol* 2002;**14**:405–414.

50. Bain G, Romanow WJ, Albers K, Havran WL, Murre C. Positive and negative regulation of V(D)J recombination by the E2A proteins. *J Exp Med* 1999;**189**:289–300.
51. Baker JE, Kang J, Xiong N, Chen T, Cado D, Raulet DH. A novel element upstream of the V $\gamma$ 2 gene in the murine T cell receptor  $\gamma$  locus cooperates with the 3' enhancer to act as a locus control region. *J Exp Med* 1999;**190**:669–679.
52. Sleckman BP, et al. Accessibility control of variable region gene assembly during T-cell development. *Immunol Rev* 1998;**165**: 121–130.
53. Seidl KJ, et al. Position-dependent inhibition of class-switch recombination by PGK-neor cassettes inserted into the immunoglobulin heavy chain constant region locus. *Proc Natl Acad Sci U S A* 1999;**96**:3000–3005.
54. Tough DF, Sprent J. Lifespan of gamma/delta T cells. *J Exp Med* 1998;**187**:357–365.
55. Zijlstra M, Bix M, Simister NE, Loring JM, Raulet DH, Jaenisch R.  $\beta$ 2-Microglobulin deficient mice lack CD4<sup>+</sup>8<sup>+</sup> cytolytic T cells. *Nature* 1990;**344**:742–746.
56. Grusby M, et al. Mice lacking major histocompatibility complex class I and class II molecules. *Proc Natl Acad Sci U S A* 1993;**90**:3913–3917.
57. Correa I, Bix M, Liao N-S, Zijlstra M, Jaenisch R, Raulet D. Most  $\gamma\delta$  T cells develop normally in  $\beta$ 2-microglobulin-deficient mice. *Proc Natl Acad Sci U S A* 1992;**89**:653–657.
58. Bigby M, et al. Most  $\gamma\delta$  T cells develop normally in the absence of MHC class II molecules. *J Immunol* 1993;**151**: 4465–4475.
59. Ito K, Van KL, Bonneville M, Hsu S, Murphy DB, Tonegawa S. Recognition of the product of a novel MHC TL region gene (27b) by a mouse gamma delta T cell receptor. *Cell* 1990;**62**:549–561.
60. Schild H, et al. The nature of MHC recognition by  $\gamma\delta$  T cells. *Cell* 1994;**76**:29–37.
61. Bonneville M, et al. Recognition of a self major histocompatibility complex TL region product by  $\gamma\delta$  T-cell receptors. *Proc Natl Acad Sci U S A* 1989;**86**:5928–5932.
62. Bluestone JA, Cron RQ, Cotterman M, Houlden BA, Matis LA. Structure and specificity of T cell receptor  $\gamma/\delta$  on major histocompatibility complex antigen-specific CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocytes. *J Exp Med* 1988;**168**:1899–1916.
63. Crowley MP, et al. A population of murine gammadelta T cells that recognize an inducible MHC class Ib molecule. *Science* 2000;**287**:314–316.
64. Dent AL, Matis LA, Hooshmand F, Widacki SM, Bluestone JA, Hedrick SM. Self-reactive  $\gamma\delta$  T cells are eliminated in the thymus. *Nature* 1990;**343**:714–719.
65. Bonneville M, et al. Blockage of  $\alpha\beta$  T-cell development by TCR  $\gamma\delta$  transgenes. *Nature* 1989;**342**:931–934.
66. Pereira P, Zijlstra M, McMaster J, Loring JM, Jaenisch R, Tonegawa S. Blockade of transgenic gamma delta T cell development in beta-2-microglobulin deficient mice. *EMBO J* 1992;**11**:25–31.
67. Wells F, Tatsumi Y, Bluestone J, Hedrick S, Allison J, Matis L. Phenotypic and functional analysis of positive selection in the  $\gamma/\delta$  T cell lineage. *J Exp Med* 1993;**177**:1061–1070.
68. Schweighoffer E, Fowlkes BJ. Positive selection is not required for thymic maturation of transgenic gd T cells. *J Exp Med* 1996;**183**:2033–2041.
69. Ferrick DA, et al. T cell function and expression are dramatically altered in T cell receptor V $\gamma$ 1.1J $\gamma$ 4C $\gamma$ 4 transgenic mice. *Cell* 1989;**57**:483–492.
70. Bonneville M, et al. Transgenic mice demonstrate that epithelial homing of  $\gamma/\delta$  T cells is determined by cell lineages independent of T cell receptor specificity. *J Exp Med* 1990;**171**:1015–1026.
71. Ferrero I, Wilson A, Beermann F, Held W, MacDonald HR. T cell receptor specificity is critical for the development of epidermal gammadelta T cells. *J Exp Med* 2001;**194**:1473–1483.
72. Mallick-Wood C, Lewis JM, Richie LI, Owen MJ, Tigelaar RE, Hayday AC. Conservation of T cell receptor conformation in epidermal  $\gamma\delta$  cells with disrupted primary V $\gamma$  gene usage. *Science* 1998;**279**:1729–1733.
73. Hara H, et al. Development of dendritic epidermal T cells with a skewed diversity of gamma delta TCRs in V delta 1-deficient mice. *J Immunol* 2000;**165**:3695–3705.
74. Xiong N, Kang C, Raulet DH. Positive selection of dendritic epidermal  $\gamma\delta$  T cell precursors in the fetal thymus determines expression of skin-homing receptors. *Immunity* 2004;**21**:121–131.
75. Leclercq G, Plum J, Nandi D, De Smedt M, Allison J. Intrathymic differentiation of V $\gamma$ 3 T cells. *J Exp Med* 1993;**178**:309–315.
76. Van Beneden K, De Creus A, Stevenaert F, Debacker V, Plum J, Leclercq G. Expression of inhibitory receptors Ly49E and CD94/NKG2 on fetal thymic and adult epidermal TCR V gamma 3 lymphocytes. *J Immunol* 2002;**168**:3295–3302.
77. Lewis JM, Girardi M, Roberts SJ, Barbee SD, Hayday AC, Tigelaar RE. Selection of the cutaneous intraepithelial gammadelta(+) T cell repertoire by a thymic stromal determinant. *Nat Immunol* 2006;**7**:843–850.
78. Minagawa M, Ito A, Shimura H, Tomiyama K, Ito M, Kawai K. Homogeneous epithelial gamma delta T cell repertoire of the skin is shaped through peripheral selection. *J Dermatol Sci* 2001;**25**:150–155.
79. Havran WL, Chien Y, Allison JP. Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors. *Science* 1991;**252**:1430–1432.
80. Lin T, et al. Autospecific gammadelta thymocytes that escape negative selection find sanctuary in the intestine. *J Clin Invest* 1999;**104**:1297–1305.
81. Iwashima M, Green A, Bonyhadi M, Davis MM, Allison JP, Chien YH. Expression of a fetal gamma delta T-cell receptor in adult mice triggers a non-MHC-linked form of selective depletion. *Int Immunol* 1991;**3**:385–393.
82. Itohara S, Tonegawa S. Selection of  $\gamma\delta$  T cells with canonical T-cell antigen receptors in fetal thymus. *Proc Natl Acad Sci U S A* 1990;**87**:7935–7938.
83. Matloubian M, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 2004;**427**:355–360.
84. Greaves DR, et al. CCR6, a CC chemokine receptor that interacts with macrophage inflammatory protein 3alpha and is highly expressed in human dendritic cells. *J Exp Med* 1997;**186**:837–844.
85. Baba M, et al. Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. *J Biol Chem* 1997;**272**:14893–14898.
86. Liao F, Alderson R, Su J, Ullrich SJ, Kreider BL, Farber JM. STRL22 is a receptor for the CC chemokine MIP-3alpha. *Biochem Biophys Res Commun* 1997;**236**:212–217.
87. Varona R, et al. Molecular cloning, functional characterization and mRNA expression analysis of the murine chemokine receptor CCR6 and its specific ligand MIP-3alpha. *FEBS Lett* 1998;**440**:188–194.
88. Morales J, et al. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc Natl Acad Sci U S A* 1999;**96**:14470–14475.
89. Homey B, et al. Cutting edge: the orphan chemokine receptor G protein-coupled receptor-2 (GPR-2, CCR10) binds the skin-associated chemokine CCL27 (CTACK/ALP/ILC). *J Immunol* 2000;**164**: 3465–3470.
90. Jarmin DI, et al. Cutting edge: identification of the orphan receptor G-protein-coupled receptor 2 as CCR10, a specific receptor for the chemokine ESKine. *J Immunol* 2000;**164**:3460–3464.
91. De Creus A, Van Beneden K, Stevenaert F, Debacker V, Plum J, Leclercq G. Developmental and functional defects of thymic and epidermal V gamma 3 cells in IL-15-deficient and IFN regulatory factor-1-deficient mice. *J Immunol* 2002;**168**:6486–6493.

92. Schimpl A, Hünig T, Elbe A. Development and Function of the Immune System in Mice with Targeted Disruption of the Interleukin 2 Gene. San Diego: Academic Press, 1994.
93. Kawai K, Suzuki H, Tomiyama K, Minagawa M, Mak TW, Ohashi PS. Requirement of the IL-2 receptor beta chain for the development of Vgamma3 dendritic epidermal T cells. *J Invest Dermatol* 1998;**110**:961–965.
94. Zhou D, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 2004;**306**:1786–1789.
95. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol* 2005;**23**:877–900.
96. Tilloy F, et al. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med* 1999;**189**:1907–1921.
97. Treiner E, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* 2003;**422**:164–169.
98. Yamagata T, Mathis D, Benoist C. Self-reactivity in thymic double-positive cells commits cells to a CD8 alpha alpha lineage with characteristics of innate immune cells. *Nat Immunol* 2004;**5**:597–605.
99. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science* 1998;**279**:1737–1740.
100. Ebert LM, Meuter S, Moser B. Homing and function of human skin gammadelta T cells and NK cells: relevance for tumor surveillance. *J Immunol* 2006;**176**:4331–4336.
101. Parker CM, et al. Evidence for extrathymic changes in the T cell receptor gamma/delta repertoire. *J Exp Med* 1990;**171**:1597–1612.