T Cell Receptor Specificity Is Critical for the Development of Epidermal γδ T Cells

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Abstract

A particular feature of γδ T cell biology is that cells expressing T cell receptor (TCR) using specific Vγ/Vδ segments are localized in distinct epithelial sites, e.g., in mouse epidermis nearly all γδ T cells express Vγ3/Vδ1. These cells, referred to as dendritic epidermal T cells (DETC) originate from fetal $V\gamma3^+$ thymocytes. The role of γδ TCR specificity in DETC's migration/localization to the skin has remained controversial. To address this issue we have generated transgenic (Tg) mice expressing a TCR δ chain (Vδ6.3-Dδ1-Dδ2-Jδ1-Cδ), which can pair with Vγ3 in fetal thymocytes but is not normally expressed by DETC. In wild-type (wt) Vδ6.3Tg mice DETC were present and virtually all of them express Vδ6.3. However, DETC were absent in TCR-$\delta^{-/-}$ Vδ6.3Tg mice, despite the fact that Vδ6.3Tg γδ T cells were present in normal numbers in other lymphoid and nonlymphoid tissues. In wt Vδ6.3Tg mice, a high proportion of in-frame Vδ1 transcripts were found in DETC, suggesting that the expression of an endogenous TCR-δ (most probably Vδ1) was required for the development of $V\delta6.3^+$ epidermal γδ T cells. Collectively our data demonstrate that TCR specificity is essential for the development of γδ T cells in the epidermis. Moreover, they show that the TCR-δ locus is not allelically excluded.

Key words: γδ T cells • repertoire selection • migration • epidermis • allelic exclusion

Introduction

γδ T cells represent a small proportion of T cells in peripheral blood and lymphoid tissues, but they comprise the majority of T cells in certain epithelia, especially in those that cover the internal and external surfaces of the body, such as skin, reproductive tract, gastrointestinal tract, and lung. While the majority of intestinal intraepithelial γδ T cells arise independently from the thymus, it is generally accepted that γδ T cells develop from lymphoid precursors that require the thymic microenvironment for their differentiation. Compared with αβ T cells, however, little is known with regard to the differentiation steps and selection events that take place during intrathymic maturation of γδ T cells. Moreover, the relationship between αβ and γδ T cell precursors and the mechanisms underlying αβ versus γδ T cell lineage commitment have not been clarified (1–4).

The analysis of the γδ TCR repertoire has demonstrated an intriguing correlation between the usage of specific TCR Vγ/Vδ combinations and the anatomical site where these cells are found. This is particularly striking in the epithelia of the skin and reproductive tract in the mouse, where nearly all γδ T cells use Vγ3/Vδ1 and Vγ4/Vδ1 receptors, respectively (5–7). In addition, most human peripheral blood γδ T cells express Vγ9/Vδ2 TCR (8). This restricted TCR usage has suggested the existence of specific ligands for different γδ T cell subsets in particular sites. However, neither the nature of these ligands nor the mechanisms responsible for homing and/or maintenance of different γδ T cell subsets in particular tissues have been identified.

T cells in mouse epidermis are referred to as dendritic epidermal T cells (DETC)* because of their characteristic cellular shape. Initially identified by the expression of Thy-1 and CD3, these cells form a continuous network among the basal layer of keratinocytes (9, 10). Although little is known about the function of DETC, several stud-

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***Abbreviations used in this paper:* DETC, dendritic epidermal T cells; HS, horse serum; iIEL, intestinal intraepithelial lymphocytes; RT, reverse transcription; Tg, transgenic; wt, wild-type.

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ies indicate a biological relationship between DETC and other epidermal cells. For example, activated DETC can secrete keratinocyte growth factor and promote the growth of epithelial cells in vitro, suggesting a role for DETC in tissue repair (11, 12). In addition, it has been reported that DETC can be activated by an unidentified factor secreted by heat-stressed keratinocytes (5, 13–15). Furthermore, their capacity to lyse in vitro different skin-tumor cell lines (16) and to prevent in vivo tumor growth has been demonstrated (17).

TCR sequence analysis of DETC clones generated from different mouse strains has shown a very high TCR homogeneity, not only because all of them use a Vγ3Jγ1Cγ1/Vδ1Dδ2Jδ2Cδ TCR, but also because they lack junctional diversity (5). This TCR homogeneity presumably constricts DETC responses to a limited number of stimuli.

DETC originate from fetal thymocytes (18). Fetal thymic γδ T cell differentiation is characterized by the sequential appearance of γδ T subsets with TCR composed of canonical γ and δ chains (19, 20). This is due to programmed rearrangement of TCR γ and δ chains and the absence of significant exonucleolytic nibbling or N nucleotide insertions. The first thymic T cell population is detected around day E14 and is composed almost exclusively of Vγ3/Vδ1 γδ T cells. These cells are considered to be DETC precursors, which migrate to the skin. A second fetal thymic γδ T cell subset expressing Vγ4/Vδ1 migrates to the epithelium of the reproductive tract. The role of the γδ TCR in the migration and/or localization of γδ T cell subsets to specific tissues has remained controversial. For example, Bonneville et al. found DETC expressing a transgenic (Tg) γδ TCR (Vγ2/Vδ5) that is different from the normal DETC γδ TCR (Vγ3/Vδ1; reference 21), and Iwashima et al. found cells expressing a DETC Tg TCR (Vγ3/Vδ1) in tissues other than the skin (22), suggesting that the migration of γδ T cells to specific tissues may not be dictated by TCR specificity. On the other hand, it has recently been shown that DETC developing in either Vγ3- or Vδ1-deficient mice express TCR with a very limited repertoire and particular conformations, which emphasize the importance of the TCR specificity in the localization of γδ T cells in the skin (23, 24).

The recent analysis of TCR-δ rearrangements in hybridomas derived from splenic γδ T cells has demonstrated that TCR-δ gene expression is not subjected to allelic exclusion (25). This possibility has not been considered previously when DETC development was examined in γδ TCR Tg mice (21, 22). To reevaluate the role of the TCR specificity in DETC development we have generated Tg mice expressing a TCR δ chain (Vδ6.3-Dδ1-Dδ2-Jδ1-Cδ) which can pair with Vγ3 in the fetal thymus but is not normally used by DETC. We show that DETC expressing the Tg TCR δ chain arise in wild-type (wt) Tg mice, but fail to develop in the absence of endogenous TCR-δ expression. These findings suggest that the specificity of the TCR is critical for the development of epidermal γδ T cells. Our results are discussed in the context of TCR-δ allelic inclusion.

Materials and Methods

Mice. C57BL/6 (wt) and C57BL/6 TCR-$\delta^{-/-}$ (TCR-$\delta^{-/-}$) mice were originally purchased from Harlan Netherlands and The Jackson ImmunoResearch Laboratories, respectively. All mice were used at 4–8 wk of age. Fetal mice were obtained from timed matings where the day of finding a vaginal plug was designated as day 0 of embryonic development.

Generation of Vδ6.3Tg Mice. A TCR-δ cDNA clone was isolated from the RL6.14 hybridoma obtained from C57BL/6 DN HSA$^-$ thymocytes (26) using reverse transcriptase and the reverse transcription (RT)-PCR. The forward primer (ATC GGT CGA CGT CAC ATG CCT CCT CAC AGC containing a BamHI site) and the reverse primer (ATC GGG ATC CCC GTA GTC TCC TCA TGT CAG containing a SalI site) used in this reaction are specific for the leader sequence of the Vδ6 TCR segment and the Cδ segment, respectively. DNA sequence determination of several independent clones revealed that this TCR δ chain is composed of Vδ6.3 (ADV7S1), Dδ1, Dδ2, Jδ1, and Cδ TCR segments, according to the Vδ designation proposed by Arden et al. (27). Vδ6.3, which is the allele expressed in C57BL/6 mice, is recognized by mAb 8F4H7B7. This Vδ6.3 cDNA was inserted into the class I promoter expression cassette containing a genomic fragment of the human β globin gene and the Ig heavy chain enhancer element (see Fig. 3 A; reference 28). Subsequently, the Tg construct was excised from the vector and microinjected into fertilized (C57BL/6 × DBA.2)F_2 eggs. Tg founders were identified by PCR screening of genomic tail DNA. The primers used were the forward primer 5′-GCC AAA CCA TCT GTT TTC ATC-3′ (specific for the Cδ segment) and the reverse primer 5′-CTG GTG GGG TGA ATT CTT TGC C-3′ (specific for the β globin exonIII). A single founder was able to transmit the TCR-δ transgene to the progeny. This male was backcrossed to C57BL/6 females to obtain Vδ6.3Tg mice expressing endogenous TCR-δ and to TCR-$\delta^{-/-}$ females to obtain Vδ6.3Tg mice lacking endogenous TCR-δ expression (29). Data shown herein are derived from mice which have been backcrossed at least four times.

Cell Preparations. Single-cell suspensions were prepared from fetal thymi, adult liver, and spleen. The lymphocyte fraction in the total liver cell suspension was recovered by centrifugation (900 *g* for 20 min at room temperature), through a Percoll (Amersham Pharmacia Biotech) gradient (total liver cells were resuspended in 8 ml 40% isotonic Percoll which was layered over 8 ml 80% isotonic Percoll). After harvesting the lymphocyte fraction at the 40–80% interface, the cells were washed two times with PBS containing 2% FCS before staining and flow cytometric analysis.

Epidermal Cell Suspensions. Epidermal cell suspensions were prepared from ear skin after the protocol described by Schuler and Steinman (30). Briefly, 8-wk-old mice were killed, the ears were cut off, and mechanically split into dorsal and ventral sides, then placed in 0.5% Trypsin (Life Technologies) in PBS containing 5% FCS. After a 30-min incubation at 37°C, the epidermis was peeled off as a single sheet and epidermal cell suspensions were obtained by filtering the trypsinized epidermal sheets through a stainless-steel sieve. Subsequently, epidermal cell suspensions were stained and analyzed by flow cytometry.

Isolation of Intestinal Intraepithelial Lymphocytes. Intestinal intraepithelial lymphocytes (iIEL) were isolated from individual mice by standard methods (31) as detailed by Wilson et al. (32). Briefly, mice were killed and the small intestines were removed into cold PBS. Peyer's patches were removed and the intestines were then opened longitudinally, flushed with cold PBS to remove detritus, cut into small pieces, and washed twice in Ca^{2+}-

and Mg^{2+}-free HBSS (Life Technologies) supplemented with 2% horse serum (HS; Life Technologies). Intestinal pieces were incubated two times in 50 ml HBSS/1mM Hepes/1 mM DTT/2.5 mM $NaHCO_3$/10% HS for 20 min at 37°C with constant stirring in a bottle precoated with HS to minimize cell loss by adhesion. Cells released into the supernatant were harvested by filtration through a stainless-steel sieve and washed once in HBSS/Hepes/5% HS. The lymphocyte fraction was subsequently recovered by centrifugation at 900 *g* for 15 min through a Percoll gradient (5 ml 44% isotonic Percoll layered over 5 ml 67.5% isotonic Percoll) at room temperature. After harvesting the lymphocyte fraction at the 44–67.5% interface the cells were washed twice in HBSS/5% HS before analysis.

Antibodies. The following mAb conjugates were used: anti–CD3ε-PE (clone 17A2), anti–TCR-δ–FITC (clone GL3), anti-Thy-1–FITC or Cy5 (clone AT15), anti-Vγ1.1–FITC (clone 2.11), anti-Vγ2–FITC (clone UC3–10A6), anti-Vγ3–FITC or biotinylated (clone F536), anti-Vδ4–FITC or –PE (clone GL2), anti-Vδ5–FITC or –PE (clone 45.152), anti-Vδ6.3–FITC or –PE (clone 8F4H7B7), anti-B220–APC (clone RA3.6B2), anti-TCR-β–APC (clone H57), anti-TCR-δ-Cy5 (GL3), anti-F4/80–Cy5 (clone F4/80), anti-CD45.2–Cy5 (clone 104), and anti-CD24–FITC (clone M1/69). Anti-Thy-1–FITC and -Cy5, anti–TCR-δ-Cy5, anti-F4/80–Cy5, anti-Vγ1.1–FITC, anti-Vγ2–FITC, anti-Vδ4–FITC and -PE, and anti-Vδ5–FITC and -PE antibodies were purified and conjugated in this laboratory; 2.11 and 45.152 clones (33) were provided by P. Pereira (Institut Pasteur, Paris, France). Anti-CD45.2-Cy5 was conjugated in this laboratory from protein purchased from BD PharMingen. The rest of the mAb conjugates were purchased from BD PharMingen.

Flow Cytometry and Sorting. Cells were preincubated with 2.4G2 culture supernatant to block Fcγ receptors, then washed and incubated with the indicated mAb conjugates for 30 min at 4°C in a final volume of 100 μl PBS containing 2% FCS. Cells were washed and analyzed on a FACSCalibur™ flow cytometer using CELLQuest™ software (Becton Dickinson). Dead cells were gated out by their forward and side scatter profile. Electronic sorting of adult thymic γδ T cells and of fetal thymic $V\gamma3^+$ $V\delta6.3^+$ cells (populations A and B) was performed on a FACStar™ flow cytometer (Becton Dickinson).

RT-PCR, Cloning, and Sequencing of Vδ1. Total RNA was extracted from 10^6 epidermal cells with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. The first-strand cDNA from extracted RNA was synthesized with oligo(dT) (Amersham Pharmacia Biotech) in a final volume of 20 μl using AMV reverse transcriptase. PCR was performed in a final volume of 50 μl containing 1 μl cDNA, $MgCl_2$ (1.5 mM), PCR buffer (1×), Vδ1 and Cδ primers (1 μM each), dNTPs mixture (0.2 mM each), and 1 U of cloned *Pfu* DNA polymerase (Stratagene). Each of the 35 cycles consisted of 1 min at 94°C, 1 min at 60°C, and then 1 min at 72°C. Before the first cycle, a 2-min 94°C denaturation step was included, and after the 35th cycle the extension at 72°C was prolonged for 5 min. PCR for β-actin was performed (using the same PCR conditions) as control. The sequence of the oligonucleotides used as primers for the PCR are as follows: Vδ1 forward primer, 5′-GGA ATT CAG AAG GCA ACA AT-3′; Cδ reverse primer, 5′-GGA ACC GTA GTC TCC TCA TG-3′; β-actin forward primer, 5′-GTG GGC CGC TCT AGG CAC CAA-3′; β-actin reverse primer 5′-CTC TTT GAT GTC AGC CAC GAT TTC-3′. After purification (StrataPrep; Stratagene) PCR products were cloned into the PCR-Script™ Amp Cloning Kit (Stratagene). Clones were sequenced on both strands in a LI-COR 4200L automatic sequencer (Lincoln) using the Excell II Sequitherm kit (Inotech). Sequences were aligned using Sequencher software (GeneCodes Corporation).

RT-PCR Primers for TCR-δ Expression. In addition to the forward Vδ1 primer and reverse Cδ primer mentioned above, the following forward primers were used: Vδ4 primer, 5′-CCG CTT CTG TGT GAA CTT CC-3′; Vδ5 primer, 5′-CAG ATC CTT CCA GTT CAT CC-3′; and Vδ6 primer, 5′-TCA AGT CCA TCA GCC TTG TC-3′.

Results

TCR Vγ and Vδ Usage of DETC. We have taken advantage of newly available mAbs against different Vγ and Vδ TCR segments to further characterize DETC TCR diversity in wt mice using flow cytometry. DETC were identified among epidermal cells by the expression of CD45.2 and CD3. A $CD45.2^+$ $CD3^+$ population represents 2–3% of the total epidermal cell suspension in adult mice (Fig. 1). Most cells expressing CD45.2 but not CD3 (∼2% of the total epidermal suspension) express MHC class II molecules (data not shown) and represent the other major compartment of bone marrow–derived cells in the skin: the Langerhans cells. DETC are $Thy\text{-}1^+$ and express Vγ3, as described previously, but do not express Vγ1.1 or Vγ2 TCR segments (Fig. 1). In addition, DETC do not express any of the TCR Vδ segments preferentially used by thymic and splenic γδ T cells in adult mice (Vδ4, Vδ5, or Vδ6.3), consistent with previous data demonstrating that Vγ3 preferentially pairs with Vδ1 in DETC clones (5). As shown in Fig. 1, $CD3^+$ cells are not detected in epidermal suspensions from $TCR\text{-}\delta^{-/-}$ mice, which demonstrates that the normal DETC population depends on a γδ TCR.

TCR Vδ Usage of $V\gamma3^+$ γδ T Cells in the Fetal Thymus. It has been demonstrated that DETC originate from an early wave of lymphoid precursors that colonize the fetal thymus (18), which are characterized by the exclusive rearrangement and expression of Vγ3. To determine whether the TCR homogeneity found in DETC is shared by putative DETC precursors we have analyzed the TCR Vδ usage of fetal $V\gamma3^+$ thymocytes by flow cytometric analysis at different stages of embryonic development. For that purpose, thymi of wt fetuses were analyzed at different days of embryonic development. The results are summarized in Fig. 2. γδ T cells were identified in the $Thy1^+$ fraction of fetal thymocytes. In agreement with previous results (19) $V\gamma3^+$ γδ T cells are detected as early as E14 and E15 (data not shown), but they constitute a very small population at these stages. For that reason, we have analyzed the TCR Vδ usage of $V\gamma3^+$ thymocytes at E16, E17, and E18, when $V\gamma3^+$ thymocytes are particularly abundant. A clear population of γδ T cells representing ∼6% of $Thy1^+$ thymocytes can be identified at these embryonic stages (data not shown). As indicated in Fig. 2, the percentage of $V\gamma3^+$ γδ T cells progressively decreases as embryonic development progresses (62% at E16, 42.5% at E17, and 24% at E18), becoming nearly undetectable by the first week after birth (data not shown) consistent with earlier data (19, 34).

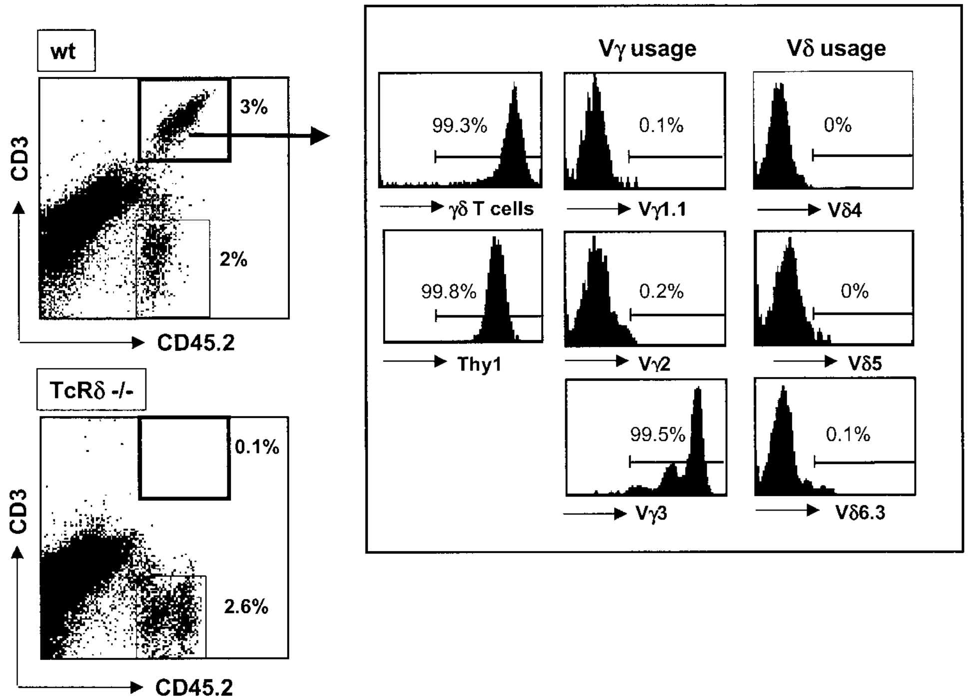


Figure 1. TCR Vγ and Vδ usage of DETC. Epidermal cell suspensions were prepared from wt and TCR-$\delta^{-/-}$ mice. The cells were analyzed after three color staining with anti-CD45.2 (Cy5 conjugate), anti-CD3 (PE conjugate), and FITC-conjugated mAbs specific for different T cell markers. DETC were identified as CD45.2$^+$ CD3$^+$ epidermal cells as indicated in the dot plots. The percentage of DETC and the percentage of the remaining bone marrow–derived (CD45.2$^+$) epidermal cells in the total epidermal suspension are indicated. Histograms show the expression of Thy-1, TCRδ, Vγ, and Vδ TCR segments by DETC. The percentage of DETC positive for each one of these markers is indicated. These data are representative of three independent experiments with similar results.

Vδ6.3, Vδ5, or Vδ4 expressing thymocytes can first be detected on E16. As shown in Fig. 2, in contrast to Vγ3, the percentages of Vδ6.3, Vδ5, or Vδ4 expressing thymocytes increase throughout embryonic development. Interestingly, between E16 and E18, Vγ3 pairs to a significant extent with Vδ6.3 and Vδ5, but not with Vδ4. These results demonstrate that a large proportion of fetal thymic Vγ3$^+$ γδ T cells in normal mice use TCR Vδ segments other than Vδ1 (such as Vδ5 and Vδ6.3), which is in sharp contrast to what is observed in the skin (Fig. 1). This raises the possibility that intrathymic selection and/or selective migration of γδ T cells from the fetal thymus to the skin, prevents the development of Vγ3$^+$Vδ6.3$^+$ or Vγ3$^+$Vδ5$^+$ DETC in normal mice.

Vδ6.3 Tg Mice. Based on the finding that among fetal thymic γδ T cells Vγ3 pairs with TCR Vδ segments other than Vδ1 (for example Vδ6.3), we have generated Vδ6.3Tg mice in order to study the role of γδ TCR specificity in DETC development. A mouse TCRδ cDNA clone composed of Vδ6.3, Dδ1, Dδ2, Jδ1, and Cδ gene segments was isolated from the RL6.14 hybridoma derived from C57BL/6 DN HSA$^-$ thymocytes (26) and inserted into the class I promoter/Ig enhancer expression cassette (28) (Fig. 3 A). This promoter drives the expression of the

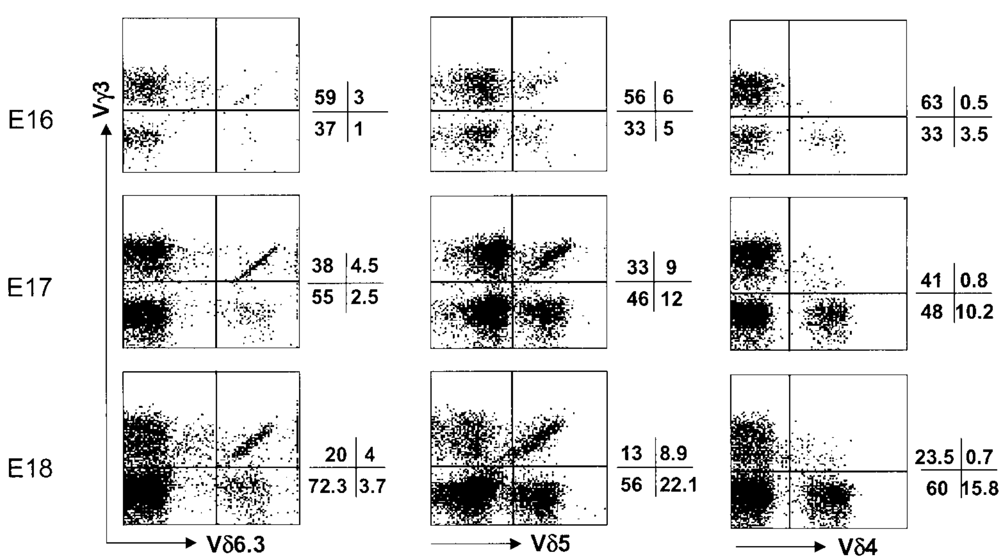


Figure 2. TCR Vδ usage of Vγ3$^+$ thymocytes during fetal thymic development. Thymocyte suspensions were obtained from wt C57BL/6 embryos at the indicated day of gestation. Four color staining was performed with anti-CD3 (CyChrome conjugated), a cocktail of APC/Cy5-conjugated mAbs (including anti–TCR-β, anti-B220, anti-F4/80, and anti-Gr1), anti-Vγ3 (FITC conjugate), and the indicated anti-Vδ (PE conjugate). Coexpression of Vγ3 and each of the Vδs by E16, E17, or E18 thymic γδ T cells was analyzed by gating on CD3$^+$ APC/Cy5 cocktail$^-$ thymocytes. Numbers indicate the percentage of cells in the respective quadrants. The data are representative of two independent analyses with similar results.

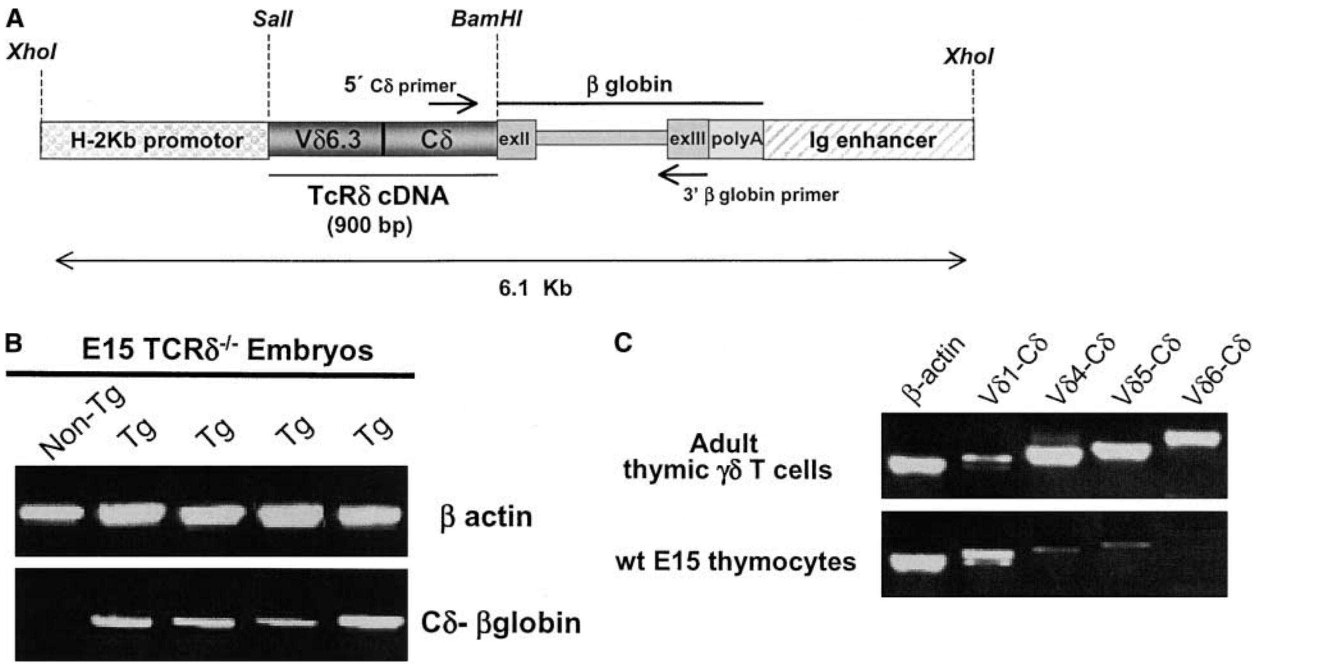


Figure 3. (A) DNA construct for Vδ6.3Tg mice. The detailed DNA construction is described in Materials and Methods. A mouse TCRδ6.3 cDNA clone was isolated from the RL6.14 hybridoma (from C57BL/6 DN thymocytes) and inserted into the class I promoter/Ig enhancer expression cassette. (B) Expression of Vδ6.3 transgene at an early stage of fetal thymic T cell development. E15 embryos were obtained from TCR-$\delta^{-/-}$ Vδ6.3Tg crosses. Each littermate was typed (data not shown) and analyzed individually. RNA was extracted from total thymocytes and RT-PCR was performed using Vδ6.3 transgene specific primers (Cδ primer and β-globin primer in A, as forward and reverse primers, respectively). β-actin RT-PCR was performed in parallel as a positive control. A 550-bp band specific for Vδ6.3 transgene expression was detected in the thymus of E15 TCR$\delta^{-/-}$ Vδ6.3Tg embryos but not in control (non-Tg) embryos. (C) Expression of endogenous Vδ−Cδ transcripts in wt adult thymic γδ T cells and wt E15 thymocytes. Wt E15 thymocytes were obtained from timed pregnant females. Adult thymic γδ T cells were obtained by electronic sorting after CD4/CD8 complement depletion. RNA was extracted and RT-PCR was performed using specific primers for the indicated Vδ-Cδ transcripts. β-actin RT-PCR was performed in parallel as a positive control.

Vδ6.3 transgene as early as E15 of fetal thymic development in both TCR-$\delta^{-/-}$ Vδ6.3Tg embryos (Fig. 3 B) and wt Vδ6.3Tg embryos (data not shown), before detectable endogenous Vδ6.3 expression (Fig. 3 C).

Expression of the Vδ6.3 Transgene during Fetal Thymic Development. To assess whether the Vδ6.3 transgene is expressed at the protein level by Vγ3$^+$ putative DETC precursors early in fetal ontogeny, timed pregnant females from wt Vδ6.3Tg crosses were killed at E16-E18. Embryos were typed and fetal thymi were analyzed individually. A population of γδ T cells (3–5% of the total fetal thymocytes) was observed in all wt embryos (data not shown). In Vδ6.3Tg embryos 60–80% of the thymic γδ T cells expressed Vδ6.3 on E16-E18 as compared with 5–7% in non-Tg littermates (Fig. 4). Thus a considerable proportion (20–40%) of thymic γδ T cells in wt Vδ6.3Tg mice is Vδ6.3$^-$, suggesting that Vδ6.3 transgene expression does not efficiently suppress endogenous TCRδ in early fetal thymic γδ T cells. A large population (40–50%) of wt Vδ6.3Tg fetal thymocytes coexpressed Vγ3 and Vδ6.3 demonstrating that the Vδ6.3 transgene can pair with Vγ3 as early as E16 (Fig. 4).

Similarly, timed pregnancies were set up with TCR-$\delta^{-/-}$ Vδ6.3Tg mice. A population of γδ T cells was detected as early as E16 in the thymus of TCR-$\delta^{-/-}$ Vδ6.3Tg embryos but not in TCR-$\delta^{-/-}$ non-Tg littermate controls (Fig. 4 and data not shown). As expected, in the absence of endogenous TCR-δ, all fetal thymic γδ T cells were Vδ6.3$^+$ in the Tg embryos. Importantly, a large fraction of Vδ6.3$^+$ fetal thymocytes (50–70%) paired with Vγ3 (Fig. 4). These results demonstrate that the Vδ6.3 transgene is expressed by Vγ3$^+$ putative fetal thymic DETC precursors as early as E16 in the presence or absence of endogenous TCR-δ expression.

Interestingly, we noted two distinct populations coexpressing Vγ3 and Vδ6.3 at different levels (populations A and B in Fig. 4) in wt Vδ6.3Tg embryos. Further analysis of these two populations will be presented below.

Role of the TCR in the Development of Epidermal γδ T Cells. To examine whether fetal γδ T cells that do not express the canonical Vγ3/Vδ1 TCR can give rise to DETC we analyzed DETC from Vδ6.3Tg mice. In contrast to what is found in wt non-Tg mice, virtually all DETC from wt Vδ6.3Tg mice express Vδ6.3 and Vγ3 (Fig. 5). Additionally, DETC in these mice do not express Vδ5, Vδ4, Vγ1.1, or Vγ2 TCR segments (data not shown). These results indicate that Tg fetal thymic Vγ3$^+$Vδ6.3$^+$ γδ T cells have migrated to the skin. However, when we analyzed epidermal preparations from TCR-$\delta^{-/-}$ Vδ6.3Tg mice, no DETC were found (Fig. 5).

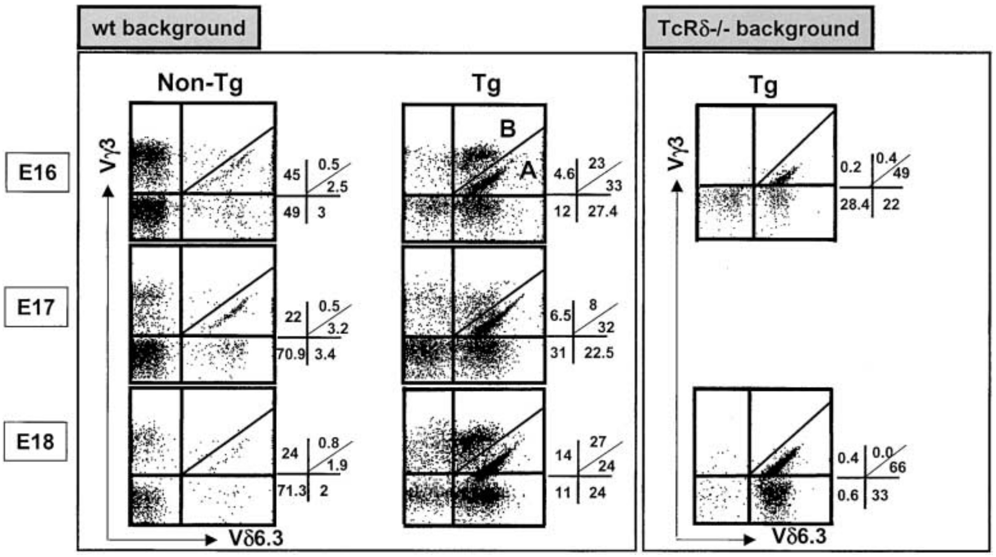


Figure 4. Analysis of Vδ6.3 transgene expression by fetal thymic DETC precursors. E16–E18 fetuses were obtained from wt Vδ6.3Tg or TCRδ$^{-/-}$ Vδ6.3Tg crosses. Embryos were typed and analyzed individually. The dot plot corresponding to E16 TCRδ$^{-/-}$ Vδ6.3Tg thymocytes shows the Vγ3 versus Vδ6.3 expression of Thy-1^{+} CD3^{+} thymocytes analyzed after four color staining using anti-Thy1 (APC conjugate), anti-CD3 (CyChrome conjugate), anti-Vγ3 (FITC conjugate), and anti-Vδ6.3 (PE conjugate). For the rest of the cases, four color staining was performed on thymocyte suspensions using anti-CD3 (CyChrome conjugate), an APC/Cy5-conjugated mAb cocktail (described in Fig. 2 legend), anti-Vγ3 (FITC conjugate), and anti-Vδ6.3 (PE conjugate). γδ T cells, identified as CD3^{+} APC/Cy5 cocktail^{-} thymocytes, were analyzed for Vγ3 and Vδ6.3 expression. Data shown here are representative of individual fetal thymi. In two separate experiments, two or more fetuses of each type (Tg or non-Tg) were analyzed yielding similar results.

These results show that the Vδ6.3 transgene is not able to promote the development of DETC. They suggest rather that the migration and/or localization of fetal γδ T cells to the skin in wt Vδ6.3Tg mice is dependent on an endogenous TCR-δ. It is likely that this endogenous TCR-δ uses Vδ1, the expression of which has been demonstrated to be restricted to fetal thymocytes and DETC (5, 18). As there are no mAbs against Vδ1, we have tested this hypothesis by RT-PCR, cloning and sequencing of Vδ1-Cδ DETC transcripts. As shown in Fig. 6 A, a Vδ1-Cδ PCR product was observed using cDNA templates prepared from epidermal cells of both Vδ6.3Tg and non-Tg mice. In contrast, no product was detected in epidermal cells from TCR-δ$^{-/-}$ mice (Fig. 6 A). To determine whether the Vδ1-Cδ PCR product corresponded to in-frame TCR δ chains, multiple independent clones were sequenced (Fig. 6 B). Indeed, 17 out of 20 clones (85%) analyzed were in-frame, with 82% of them having the published canonical sequence for Vδ1-Cδ (5, 35, 36). These findings indicate that DETC present in wt Vδ6.3Tg mice express Vδ1 in addition to the Vδ6.3 transgene. Therefore, it is likely that the endogenous Vδ1 is responsible for the development of Vδ6.3^{+} DETC in wt Vδ6.3Tg mice, which would imply that fetal thymic

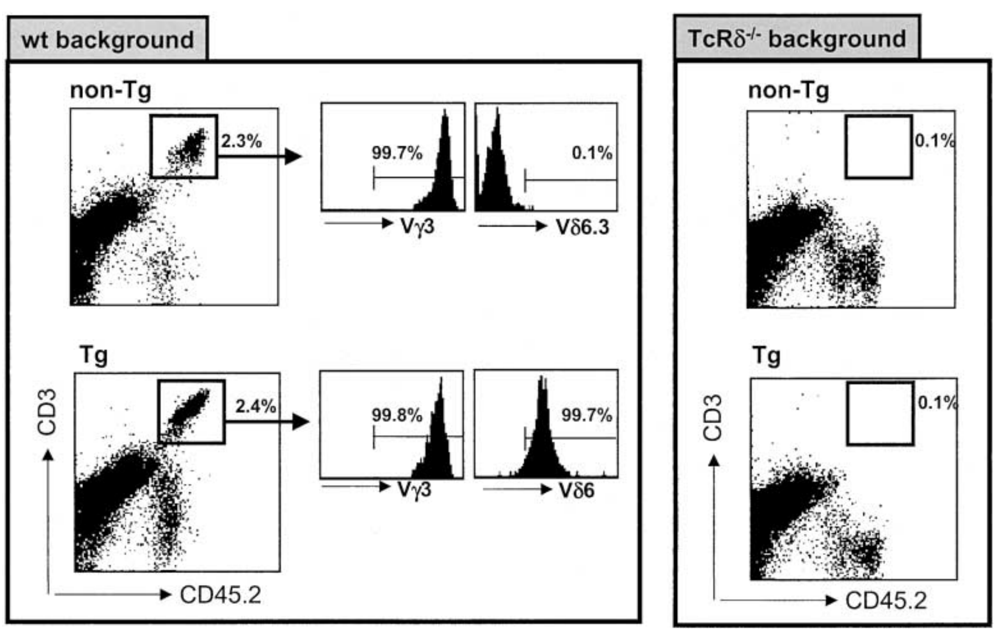


Figure 5. The development of epidermal γδ T cells is dependent upon TCR specificity. Epidermal cell suspensions obtained from Vδ6.3Tg and non-Tg mice on either wt or TCR-δ$^{-/-}$ backgrounds, were three color stained using anti-CD45.2 (Cy5 conjugate), anti-CD3 (PE conjugate), and either anti-Vγ3 or anti-Vδ6.3 (FITC conjugates). The percentage of DETC (CD45^{+} CD3^{+} cells) in the total epidermal suspension from each mouse is indicated. Histograms show Vγ3 or Vδ6.3 expression by DETC. Left and right panels correspond to analyses performed on wt and TCR-δ$^{-/-}$ backgrounds respectively. Data shown are representative of three independent experiments with identical results.

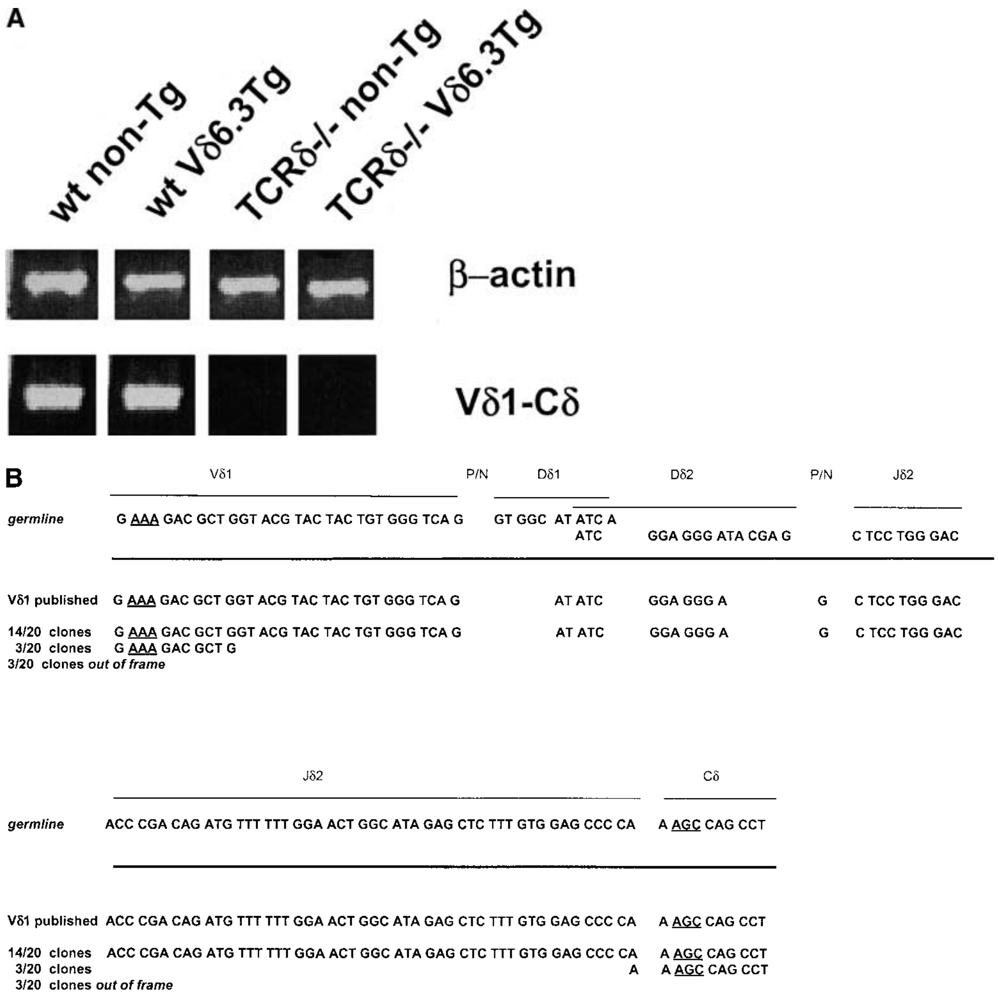


Figure 6. Analysis of Vδ1 expression by Vδ6.3 Tg DETC. (A) RT-PCR for Vδ1-Cδ transcripts in DETC. RNA was extracted from epidermal cell suspensions of the indicated mice, and RT-PCR was performed using specific primers for Vδ1-Cδ transcripts (see Materials and Methods). β-actin was used as an amplification control. (B) Vδ1-Cδ junctions of 20 independent PCR clones derived from epidermal cells from wt Vδ6.3Tg mice were sequenced and compared with the published canonical sequence (reference 5). 17 clones had in-frame Vδ1-Cδ sequences, 14 of which matched the canonical DETC TCRδ. Three clones lacked part of the Vδ1 segment as well as the Dδ and Jδ regions. These may represent aberrantly spliced Vδ1-Cδ transcripts.

DETC precursors express two TCR δ chains on the same cell in these mice.

Vδ6.3Tg γδ T Cells Are Present in other Lymphoid and Nonlymphoid Tissues. To exclude the possibility that Vδ6.3 transgene expression prevented the emigration of γδ T cells from the thymus, we analyzed γδ T cell populations in various anatomical sites. As shown in Fig. 7 A, Vδ6.3$^+$ γδ T cells in wt non-Tg mice represent 17, 20, and 33% of total γδ T cells in iIEL, liver, and spleen, respectively. When we analyzed TCR-δ$^{-/-}$ Vδ6.3Tg mice, in contrast to the skin, we were able to find normal numbers of γδ T cells in the iIEL, spleen, and liver (Fig. 7 B). As expected, all of these γδ T cells were Vδ6.3$^+$ due to the absence of endogenous TCR-δ expression. These results show that Vδ6.3Tg γδ T cells can migrate to and reconstitute organs other than the skin. Therefore, the failure to reconstitute DETC in TCR-δ$^{-/-}$ Vδ6.3Tg mice is not due to an impediment to migration of the Tg γδ T cells, but rather to the lack of expression of a permissive TCR, most probably Vγ3/Vδ1.

Role of TCR Specificity in Intrathymic Maturation of Putative DETC Precursors. A likely candidate for Vδ6.3$^+$ DETC precursors in wt Vδ6.3Tg mice could be Vγ3$^+$ fetal thymocytes included in population B (Fig. 4), as this population is absent in TCR-δ$^{-/-}$Vδ6.3Tg mice (Fig. 4) which also lack DETC (Fig. 5). Population B was already prominent as early as E16 (Fig. 4). Since this population was selectively absent in TCR-δ$^{-/-}$ Vδ6.3Tg embryos (Fig. 4) it seemed likely that it may correspond to Vγ3$^+$Vδ6.3$^+$ cells expressing endogenous TCR δ chains. Cells in population B did not express surface Vδ4 or Vδ5 (data not shown), thus raising the interesting possibility that they might express the canonical Vγ3/Vδ1 TCR. This hypothesis was directly tested by sorting populations A and B from E18 wt Vδ6.3Tg thymi and analyzing Vδ1-Cδ transcripts by semiquantitative RT-PCR. As shown in Fig. 8 A population B contained ~10-fold higher levels of Vδ1-Cδ transcripts than population A consistent with expression of a Vγ3/Vδ1 TCR in addition to the Vγ3/Vδ6.3 TCR detected by surface staining.

Previous studies (37) have shown that the maturation of fetal Vγ3$^+$ thymocytes in normal mice is accompanied by upregulation of TCR expression and concomitant downregulation of CD24 (HSA). Since population B expressed higher levels of Vγ3 than population A, we also analyzed these subsets for expression of CD24. As shown in Fig. 8 B

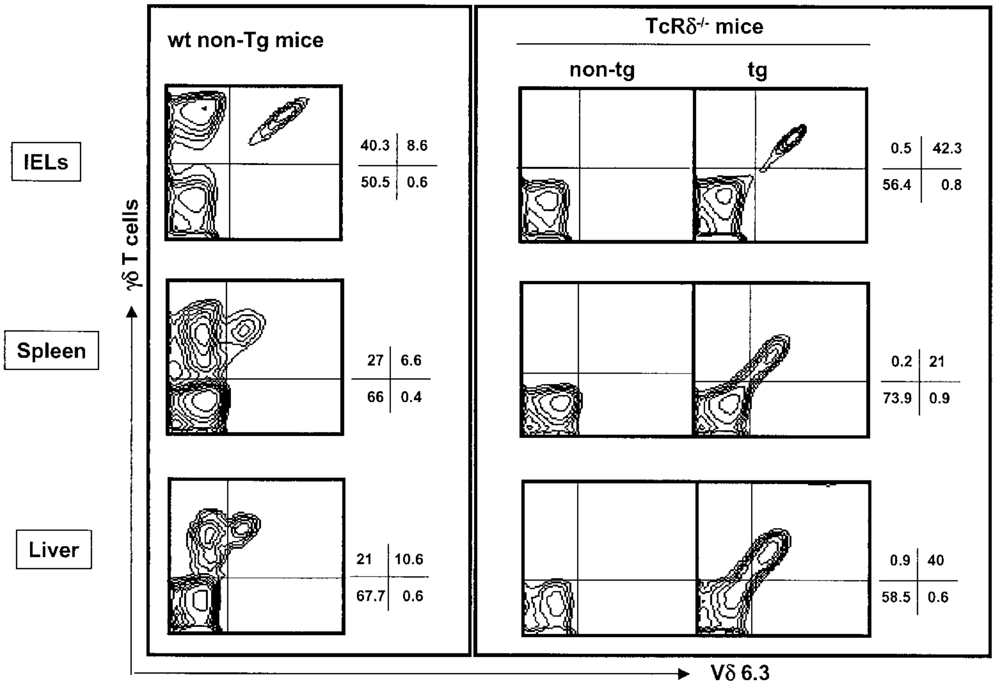
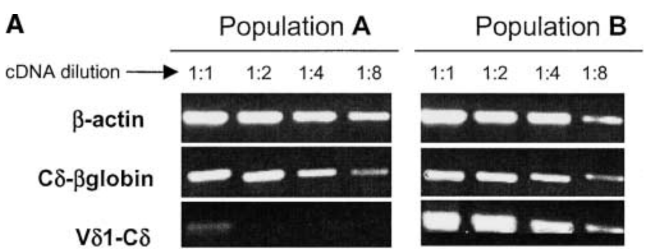


Figure 7. Vδ6.3 Tg γδ T cells can reconstitute the γδ T cell compartment in the intestinal epithelium, liver, and spleen. iIEL were stained with anti-CD45.2 (Cy5 conjugate), anti-TCRδ (FITC conjugate), and anti-Vδ6.3 (PE conjugate). iIEL contour plots show the TCR-δ versus Vδ6.3 expression pattern after gating on CD45.2$^+$ cells. Spleen and liver cell suspensions were stained using anti-CD3 (PE conjugate), anti-Vδ6.3 (FITC conjugate), and a cocktail including anti–TCR-β, anti-B220, anti-F4/80, and anti-Gr1 mAbs (APC or Cy5 conjugates). Spleen and liver contour plots correspond to CD3 versus Vδ6.3 expression after gating out APC-cocktail$^+$ cells. Numbers indicate percentage of cells in the respective quadrant.

population B showed significant CD24 downregulation as compared with population A.

Collectively, these data raise the intriguing possibility that the intrathymic maturation of Vγ3/Vδ1 DETC precursors involves selection events based on TCR specificity.



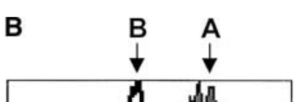


Figure 8. Characterization of two populations of Vγ3$^+$ Vδ6.3$^+$ fetal thymocytes in wt Vδ6.3Tg mice. (A) Thymocytes in populations A or B (see Fig. 4) were isolated by electronic sorting from E18 wt Vδ6.3Tg fetuses. RNA was extracted from equal numbers of cells and semi-quantitative RT-PCR was performed using primers specific for Vδ1-Cδ transcripts (see Material and Methods). RT-PCR for β-actin and for the Vδ6.3 transgene (Cδ-β globin) were included as controls. (B) Four color staining was performed in thymocytes from E18 wt Vδ6.3Tg embryos using in a first step anti-CD3 (CyChrome conjugate), anti-Vγ3 (biotin conjugate), anti-Vδ6.3 (PE conjugate), and anti-CD24 (FITC conjugate), followed by a second step with streptavidin-APC. Overlapping histograms correspond to CD24 levels of Vγ3$^+$ Vδ6.3$^+$ thymocytes in population A (gray profile) or in population B (empty profile).

Discussion

Since they were first identified, mouse DETC have been considered a homogeneous population of T cells expressing Vδ1/Vγ3 TCR (5, 6). TCR sequence analysis and the generation of a mAb against Vγ3 has demonstrated that the expression of Vγ3 bearing TCR is restricted to the epidermis and to fetal thymocytes (5, 6). These results, together with the finding that repopulation of DETC in adult mice is only possible upon transplantation of both fetal lymphoid precursors and fetal thymus (38), have led to the assumption that fetal Vγ3$^+$ thymocytes are DETC precursors. We have examined whether the TCR homogeneity found in DETC, is a consequence of a restricted γδ TCR pattern of expression by putative fetal thymic DETC precursors. We found, as previously shown by other authors (19), that Vγ3$^+$ cells are the predominant T cell subset at early stages of fetal intrathymic T cell differentiation (~E15–17). Interestingly, as early as E16 we found Vγ3 paired with both Vδ5 and Vδ6.3, but not with Vδ4 (despite the fact that this chain is already expressed at these stages), showing that Vγ3 can pair with Vδs other than Vδ1. These results clearly demonstrate that the TCR diversity of putative intrathymic DETC precursors is much greater than that observed in DETC in the skin thereby raising the possibility that only fetal thymic γδ T cells expressing an appropriate TCR will localize later in the epidermis.

We further investigated the importance of γδ TCR specificity for DETC migration/localization in the skin by generating a new TCR-δ Tg mouse. For this purpose we chose a TCR δ chain containing the Vδ6.3 segment because this segment can pair with Vγ3 in putative DETC precursors of wt mice. Our results demonstrate that fetal thymocytes indeed express the Vδ6.3 transgene in association with Vγ3 as early as E15–E16 on both wt and TCR-$\delta^{-/-}$ backgrounds. As expected, all thymic γδ T cells expressed Vδ6.3 in TCR-$\delta^{-/-}$ Vδ6.3Tg embryos. Interestingly, only ~75% of thymic γδ T cells expressed Vδ6.3 on the cell surface in wt Vδ6.3Tg embryos, suggesting that a considerable fraction of γδ T cells expressed endogenous TCR δ chains (see below).

DETC from Vδ6.3Tg mice were analyzed in order to examine whether enforced Vδ6.3 expression on putative DETC precursors would affect their development. In wt Vδ6.3Tg mice we found a normal percentage of DETC in the epidermis. Interestingly, virtually all DETC from these mice express Vγ3 and Vδ6.3 segments, in contrast to non-Tg littermates. But surprisingly no $CD3^{+}$ cells are found in epidermal cell suspensions from TCR-$\delta^{-/-}$ Vδ6.3Tg mice. The fact that Vδ6.3Tg γδ T cells reconstitute the γδ T cell compartment in the iIEL, liver, and spleen of TCR-$\delta^{-/-}$ Vδ6.3Tg mice, demonstrates that the absence of DETC is not because of a general deficiency in migration of the Vδ6.3Tg γδ T cells, but rather to the lack of expression of a TCR that is permissive for skin localization. These results strongly suggest that a γδ TCR, in which an endogenous TCR δ chain takes part, has directed the migration and/or localization of DETC to the epidermis of wt Vδ6.3Tg mice. As Vδ1 is most frequently associated with Vγ3 in DETC (5), we investigated whether Vδ1 was expressed in wt Vδ6.3Tg DETC. Indeed, PCR and sequence analysis showed a large proportion (85%), of in-frame Vδ1 transcripts, most of which corresponded to the canonical Vδ1 DETC sequence (5). Therefore, we can conclude that wt Vδ6.3Tg DETC express a second TCR-δ, most probably Vδ1, and that the expression of this second TCR-δ is a prerequisite for their development. The expression of two different TCR δ chains by the same cell can also be inferred from the analysis of Vγ3 and Vδ6.3 expression in wt Vδ6.3Tg embryos. There, cells expressing both Vγ3 and Vδ6.3 are distributed into two different populations. One population (A in Fig. 4) presumably corresponds to cells expressing exclusively Vγ3/Vδ6.3 TCR as suggested by its presence in TCR-$\delta^{-/-}$ Vδ6.3 Tg mice. In contrast, population B (which is absent in TCR-$\delta^{-/-}$ Vδ6.3Tg embryos), would correspond to cells expressing two different types of γδ TCR: one of them Vγ3/Vδ6.3, and the other Vγ3 paired with Vδ1. We believe that expression of this second γδ TCR is a prerequisite for DETC maturation and/or localization in the skin. In wt non-Tg embryos, population B represents only 0.3%, which could explain the fact that $V\delta6.3^{+}$ DETC are not detected in normal mice. Taken together our results provide strong evidence for the simultaneous expression of two different TCR δ chains at the cell surface of primary γδ T cells, thus confirming and extending the concept of allelic inclusion of the TCR δ locus as proposed initially for γδ T cell hybridomas by Sleckman et al. (25).

Collectively, our results strongly suggest that TCR specificity is critical for fetal γδ T cells to migrate/localize in the skin. This conclusion is in apparent disagreement with an earlier study by Bonneville et al. (21) who investigated DETC migration in Vγ2/Vδ5 double Tg mice. They found that most DETC expressed this Tg γδ TCR and, as they did not consider the expression of endogenous TCR δ and TCR γ chains, they concluded that TCR specificity was not essential for the normal migration of γδ T cells to the epidermis. These authors rather proposed that intrinsic properties of DETC precursors were responsible for migration to the skin. However, if that were the case, we should have found Vγ3/Vδ6.3 DETC in TCR-$\delta^{-/-}$ Vδ6.3Tg mice.

Recently, mice deficient for either Vγ3 or Vδ1 expression have been reported (23, 24). In both types of mice it is possible to observe DETC expressing TCR other than the prototypic Vγ3/Vδ1. However, in $V\gamma3^{-/-}$ mice DETC preferentially expressed Vδ1-bearing TCR which were recognized in large proportion by the mAb 17D1. Since this mAb was originally described as recognizing a conformational epitope found exclusively in Vγ3/Vδ1 DETC (39), the authors speculated that a limited number of TCR conformations are permissive for DETC development. Interestingly in $V\delta1^{-/-}$ mice, relatively normal numbers of DETC developed and the most frequently used TCR δ chain was Vδ6. However, in contrast to the Tg TCR δ chain used in our study (Vδ6.3-Dδ1-Dδ2-Jδ1-Cδ) most of the Vδ6 chains in DETC of $V\delta1^{-/-}$ mice lacked Dδ1 and had relatively few nucleotide additions in the CDR3 region, suggestive of a fetal thymic origin. Thus, the failure of our Tg Vδ6.3 chain to support DETC development (even though it is paired with Vγ3 in the fetal thymus) may reflect the absence of an (as yet unidentified) critical CDR3 motif that allows the migration and/or localization of DETC in the skin. Alternatively it is possible that most (or all) Vγ3/Vδ6 TCR are able to support DETC development, but only very inefficiently. According to this scenario, the presence of polyclonal Vδ6 populations in $V\delta1^{-/-}$ mice would collectively allow relatively efficient DETC generation, whereas monoclonal Vδ6.3 T cells present in TCR-$\delta^{-/-}$ Vδ6.3Tg mice would not be able to generate detectable numbers of DETC.

In conclusion, our data, as well as those obtained from Vγ3- and Vδ1-deficient mice, support the concept of an important role for the γδ TCR in DETC development. Several possibilities could be envisaged to explain these results. First, an intrathymic process could positively select only γδ T cells with a particular TCR specificity, which would subsequently migrate to the skin independently of this specificity. Migration in this case could be directed by the expression of homing receptors as has been shown for Langerhans cell migration to the skin in humans (40, 41). This interesting hypothesis is further supported by our finding that two populations of $V\gamma3^{+}V\delta6.3^{+}$ thymocytes can be defined at E16–E18 in wt Vδ6.3Tg mice. Whereas population A does not express endogenous Vδ1 and has a TCR^{lo} $CD24^{hi}$ phenotype, population B expresses endogenous Vδ1 and is TCR^{hi} $CD24^{lo}$. Since TCR^{hi} $CD24^{lo}$ $V\gamma3^{+}$ thymocytes represent the mature progeny of TCR^{lo}

CD24[hi] Vγ3+ precursors in normal mice (37) our data raise the intriguing possibility that population B has matured as a result of selection by specific "DETC selecting ligands" in the fetal thymus and thus that γδ T cells may pass through an intrathymic selection process as αβ T cells do. Alternatively, it cannot be excluded that TCR specificity could by itself direct the migration of γδ T cells to the skin or that γδ T cells expressing diverse TCR specificities could migrate to the skin, but only those having a permissive TCR would be retained and/or locally expand, perhaps due to the recognition of a ligand expressed specifically by keratinocytes in the skin. Future experiments will be required to distinguish between these possibilities.

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