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Positive Selection of Dendritic Epidermal γδ T Cell Precursors in the Fetal Thymus Determines Expression of Skin-Homing Receptors

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Summary

The role of cellular selection in the development of γδ T cells remains unclear. Knockout mice lacking a subset of Vγ genes, including Vγ3, contain abundant γδ T cells but are devoid of dendritic epidermal γδT cells (DETCs), which normally express an invariant Vγ3/Vδ1 γδ TCR. A rearranged Vγ2 transgene restored DETC development, but the restored DETCs selectively expressed a unique Vδ gene other than Vδ1, indicating that DETC development involves TCR-based selection. In both normal and transgenic/knockout mice, specific DETC precursors in the fetal thymus were activated and expressed the IL-15 receptor β chain, skin-homing receptors, and thymic exiting receptors. In vitro activation of irrelevant precursors also led to upregulation of the skin-homing receptor, providing an explanation for how thymic selection is coordinated with development of epidermal γδ T cells.

Introduction

T cells are divided into two populations based on their surface expression of αβ or γδ T cell receptors (TCR). While αβ T cells predominate in secondary lymphoid organs, many γδ T cells reside in various epithelial layers of tissues underlying internal and external surfaces of the body, such as the skin, intestinal epithelium, vagina, and tongue. These intraepithelial γδ T cells are thought to recognize stress-induced tissue-specific antigens in the local environment and play an important role in the first line of defense. For example, specific functions proposed for murine skin γδ T cells (dendritic epidermal T cells, DETCs) include immune surveillance against tumors, wound healing, and regulation of contact allergic responses (Dieli et al., 1997; Girardi et al., 2001, 2002; Weigmann et al., 1997; Witherden et al., 2000). DETCs, unlike other γδ T cells, are reactive with "stressed" keratinocytes (Havran and Allison, 1991), though the nature of the keratinocyte ligand is unknown. In normal mice, this specificity is conferred by an invariant skin-specific TCR composed of Vγ3/Vδ1 TCR chains lacking junctional diversity, called the "canonical" DETC TCR (Havran and Allison, 1990), which is expressed by nearly all DETCs.

γδ T cells in other tissues express different γδ TCRs (Allison, 1993; Haas et al., 1993; Raulet et al., 1991). γδ T cells expressing a canonical Vγ4-Jγ1Cγ1 chain predominate in the reproductive tract and tongue (Itohara et al., 1990). γδ T cells in the epithelium of the small intestine are more heterogeneous, but preferential usage of specific Vγ5$^+$ and Vγ1.1$^+$ γδ TCRs is evident (Pereira et al., 2000). γδ T cells in secondary lymphoid organs preferentially express Vγ2 and Vγ1.1 (Pereira et al., 2000; Raulet et al., 1991).

Development of tissue-specific γδ T cells is a complex process controlled by several layers of regulation. In the case of DETCs, generation of γδ T cells in the early fetal thymus has been shown to be essential for DETC development in the epidermis (Allison and Havran, 1991; Ikuta et al., 1990). Within the fetal thymus, a genetic program favors rearrangement of Vγ3 (and Vδ1) genes over other V genes, and the rearrangement mechanism at this stage tends to generate invariant V(D)J junctional sequences (Asarnow et al., 1993; Itohara et al., 1993; Raulet et al., 1991). Recently, we found that preferential Vγ3 rearrangement in the fetal thymus is determined largely by the genomic location of the Vγ3 gene segment in the TCRγ locus (Xiong et al., 2004).

On top of the evidence for genetically programmed rearrangement events, many studies have addressed the role of cellular selection in DETC development and the acquisition of an invariant TCR repertoire by these cells. Early studies reported normal DETC development in mice expressing various γδ TCR transgenes, suggesting that DETC development is not dependent on expression of Vγ3 or Vδ1 (Bonneville et al., 1990; Ferrick et al., 1989). These conclusions were undermined by the finding that DETCs develop in TCRδ6.3 transgenic mice only when the mice contain intact endogenous TCRδ genes (Ferrero et al., 2001). Thus, endogenous TCR genes can promote development of DETCs expressing other transgenic TCRγδ genes. Better evidence for plasticity in DETC Vγ usage came from the finding that DETC development was unimpaired in Vγ3 knockout mice, suggesting that Vγ3 is not essential for the process (Mallick-Wood et al., 1998). The DETCs in Vγ3$^{-/-}$ mice reportedly expressed Vγ1.1, Vγ2, and Vγ5 paired with Vδ1 and other Vδ genes. Vγ1.1$^+$ DETC clones reportedly retained reactivity with a keratinocyte cell line and contained an "idiotypic" determinant shared by canonical Vγ3/Vδ1 γδ TCR that is detected with the 17D1 mAb. It was concluded that, while Vγ3 is unnecessary for DETC development, the TCRγδ receptors capable of substituting for the canonical receptor must exhibit a specific conformation detected with the 17D1 mAb. Contradicting this conclusion, it was shown that DETCs developed normally in Vδ1 knockout mice, but the corresponding TCRs lacked the 17D1 determinant (Hara et al., 2000). Therefore, the role of TCR-specific selection in DETC development remains poorly defined. In this report we show that TCRs of TCRγ Cγ1 cluster is essential for DETC development but can be substituted with a different specific TCR that exhibits a certain specificity. Moreover, we provide evidence that positive selection within the thymus is essential for coordinating homing

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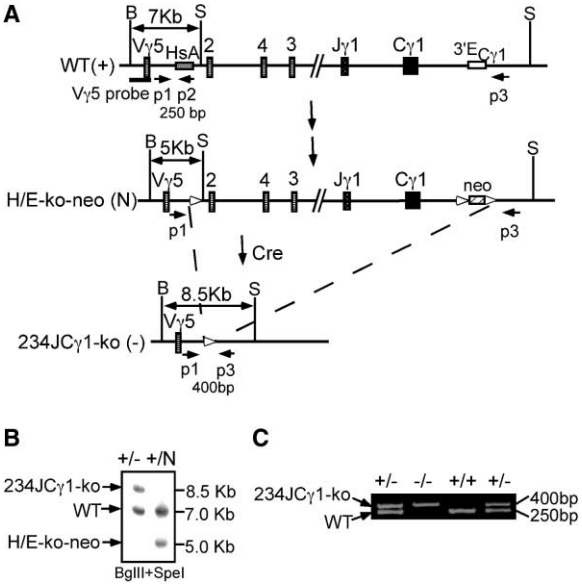

Figure 1. Generation of 234JCγ1 Knockout Mice

(A) Scheme for the generation of 234JCγ1 knockout ES cells. B, Bgl II; S, Spe I. Positions of Vγ5 probe and primers p1, p2, and p3 are shown. The number between each set of primers indicates size (bp) of PCR product amplified by the set of primers. Note that the *neo* cassette was deleted in the 234JCγ1-ko ES cells.

(B) Identification of 234JCγ1 knockout ES cell clones by Southern blot analysis with the Vγ5 probe. Refer to (A) for restriction sites and the position of the Vγ5 probe.

(C) Typing of 234JCγ1 knockout mice by PCR with the primers p1, p2, and p3, as illustrated in (A).

events necessary for thymic egress and homing to the epidermis, and eventual development of DETCs with specific γδ TCR composition.

Results

Defective Development of Epidermal γδ T Cells in Mice Lacking the Cγ1 Cluster

We recently reported that mice lacking two enhancer elements in the Cγ1 cluster of the TCRγ locus, HsA and $3'E_{C\gamma1}$, produced normal numbers of Vγ1.1$^+$ and Vγ5$^+$ cells in the fetal thymus but were impaired in the production of Vγ3$^+$ cells. Although Vγ3$^+$ cells represented only 10% of the fetal thymocytes, they predominated in the adult epidermis, consistent with a strong selection for these cells (Xiong et al., 2002, and data not shown). To address the consequences for DETC development of the absence of Vγ3 and other Vγ genes in the Cγ1 cluster (Vγ2, 4, and 5), we generated gene-targeted mice in which the Cγ1 cluster was ablated by deleting a 35 kb fragment from 3 kb upstream of Vγ2 to 3 kb downstream of ECγ1 (including Vγ2, Vγ4, Vγ3, Jγ1, and Cγ1, as well as both enhancer elements, HsA and $3'E_{C\gamma1}$) (Figure 1). Although the Vγ5 gene that remains in the knockout mice could theoretically rearrange to one of the remaining JγCγ genes of other Cγ clusters, we never observed Vγ5$^+$ γδ T cells in these animals, nor did we detect an increase in Vγ5 rearrangement to other Jγ genes (data not shown and Figure 2D). The knockout mice, named 234JCγ1 knockout mice, retain two intact functional TCRγ clus-

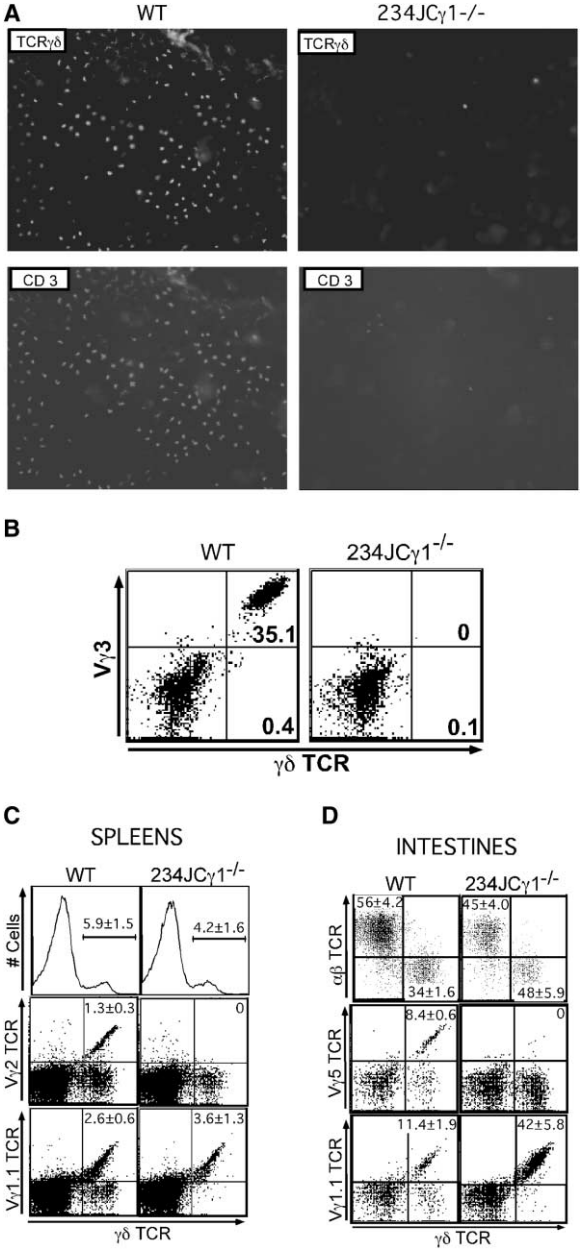

Figure 2. Absence of γδ T Cells in the Skin but Normal Development of γδ T Cells in the Spleen and Small Intestine of 234JCγ1-Deficient Mice

(A) Immunohistochemistry of stained epidermal sheets. Antibodies used are indicated in the panels.

(B) Flow cytometric analysis of epidermal cells for expression of γδ TCR and Vγ3. The numbers in the quadrants indicate the percentages of cells compared to total numbers of cells (including T cells and non-T cells).

(C) Flow cytometric analysis of nylon wool column-passed splenocytes. Cells that were gated for the absence of CD4 and CD8 expression were analyzed for γδTCR and Vγ expression. The numbers refer to percentages ± SD (n > 5 mice per sample).

(D) Flow cytometric analysis of intraepithelial lymphocytes (IEL) prepared from small intestines. Gated CD3$^+$ IELs were analyzed for the expression of αβ and γδ TCR (top panel), γδ TCR and Vγ5 (middle), and γδTCR and Vγ1.1 (bottom).

ters, Cγ2 and Cγ4, which encode two associated TCRγ gene rearrangements, Vγ1.2-Jγ2Cγ2 and Vγ1.1-Jγ4Cγ4.

Development of γδ T cells in the skin of adult 234JCγ1$^{-/-}$ mice was examined by in situ immuno-staining of skin epidermal sheets and flow cytometric analysis of skin epidermal cell preparations. Surprisingly, the skin sheets of the knockout mice were nearly devoid of TCRγδ^{+} cells, indicating that the subtypes of γδ cells remaining in the mice did not substitute for the missing Vγ3^{+} cells in the epidermis (Figures 2A and 2B). In contrast to the situation in the skin, the development of γδ T cells was essentially normal in the spleen or intestinal epithelium of 234JCγ1$^{-/-}$ mice (Figures 2C and 2D). In the spleen, Vγ2^{+} cells were absent, but other γδ cells made up most of the difference (Figure 2C). Vγ1.1^{+} cells accounted for more than 80% of the splenic γδ cells, with the remainder probably attributable to Vγ1.2^{+} cells. In the intestinal epithelium of 234JCγ1$^{-/-}$ mice, the normally abundant Vγ5^{+} γδ T cell population was absent, but a dramatic increase in Vγ1.1^{+} cells more than made up for the difference (Figure 2D). The cell surface levels of γδ TCR on splenic γδ T cells and IELs were unchanged in the knockouts. In conclusion, the Cγ1 cluster is critical for development of γδ T cells in the skin but not in the spleen or intestine.

The fact that Vγ1.1^{+} and other γδ T cells remaining in the 234JCγ1$^{-/-}$ mice failed to populate the epidermis was not due to a delay in the development of these cells. Compared to wild-type controls, the 234JCγ1$^{-/-}$ mice contained fewer γδ T cells in the early fetal thymus, largely due to the absence of Vγ3^{+} cells (Figure 3A). However, the absolute number of Vγ1.1^{+} γδ cells in the fetal thymus was similar or slightly higher in the 234JCγ1$^{-/-}$ than in wild-type mice (Figure 3B). Vγ1.1^{+} cells accounted for approximately 90% of total γδ T cells in the fetal thymus of the knockout mice (Figure 3C), whereas in normal mice approximately 70% of the early fetal thymic γδ T cells expressed Vγ3 (Figure 3D). The level of rearrangements of Vγ1.1 (to Jγ4) was normal in the knockout mice, as were rearrangements of the predominant fetal TCRδ gene, Vδ1-Jδ2 (Figure 3E). Together, the results indicate that thymic development of Vγ1.1^{+} γδ T cells is normal in 234JCγ1$^{-/-}$ mice, arguing that the absence of these cells from the epidermis reflects a specific inability of these cells to migrate to or expand in the epidermis. These findings clearly suggest that development of γδ T cells in the skin is dependent on expression of specific γδ TCRs.

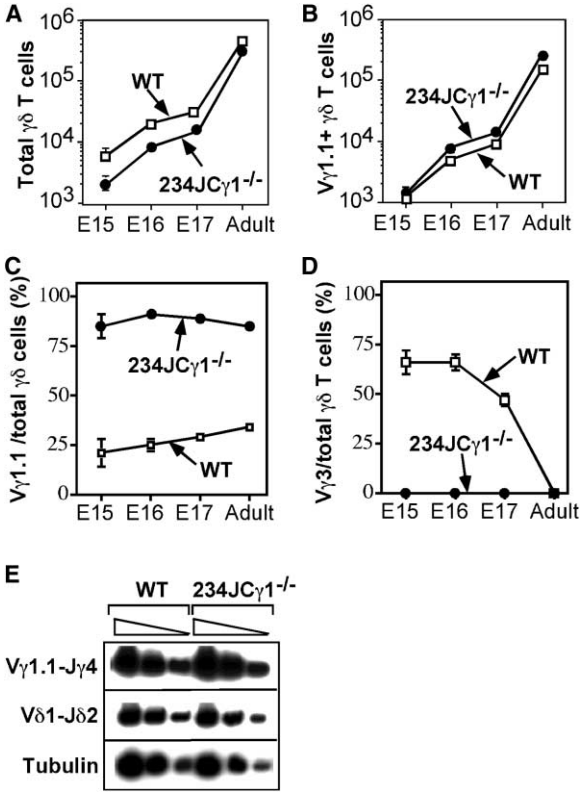

Figure 3. Development of γδ T Cells in Thymi of 234JCγ1$^{-/-}$ Mice

(A) The number of total γδ T cells per thymus as function of age.

(B) The number of Vγ1.1^{+} γδ T cells per thymus as function of age.

(C and D) Percentage of γδ T cells expressing Vγ1.1 (C) or Vγ3 (D) in the thymi of wild-type and 234JCγ1$^{-/-}$ mice of different ages.

(E) Comparison of Vγ1.1-Jγ4 or Vδ1-Jδ2 rearrangements in E16 fetal thymocytes between wild-type and 234JCγ1$^{-/-}$ mice by semiquantitative PCR. Semiquantitative PCR for β-tubulin was used as a loading control.

A Vγ2-Jγ1Cγ1 Transgene Restores Development of Functional DETCs in 234JCγ1$^{-/-}$ Mice

In mice with a Vγ2-Jγ1Cγ1 transgene whose transcription is driven by the natural TCRγ regulatory elements (Kang et al., 1998), nearly all the DETC expressed the Vγ2 transgene (Figure 4A), similar to results reported previously with a different Vγ2 transgene (Bonneville et al., 1990). However, costaining demonstrated that many or most of the DETCs in such mice coexpressed endogenous Vγ3 chains (Figure 4A), raising the possibility that Vγ3 was essential for DETC development in the transgenic mice. To explore whether transgenic Vγ2^{+} γδ T cells could develop in the skin in the absence of endogenous Vγ3 or Cγ1 gene expression, we crossed the TCRγ2 transgenic mice with the 234JCγ1 knockout mice to obtain transgenic/knockout TCRγ2Tg^{+}234JCγ1$^{-/-}$ mice. These mice developed abundant DETCs, essentially all of which expressed the TCRVγ2 transgene but did not express Vγ3 or endogenous Vγ1.1 (Figure 4B). Thus, the Vγ2 transgene complements the defect in 234JCγ1$^{-/-}$ mice, and γδ T cells expressing the Vγ2^{+} transgene and no other Vγ genes can develop into DETC in the absence of Vγ3^{+} γδ T cells.

To determine whether transgenic Vγ2^{+} DETCs that develop in TCRγ2Tg^{+}234JCγ1$^{-/-}$ mice possess functions similar to wild-type DETCs, we took advantage of previous findings that stressed keratinocyte cell lines stimulate wild-type Vγ3^{+} DETCs to secrete IL-2 (Havran and Allison, 1991). We used this assay to examine the specificity of Vγ2^{+} DETCs from TCRγ2Tg^{+}234JCγ1$^{-/-}$ mice. Interestingly, the transgenic Vγ2^{+} DETCs responded even more strongly than wild-type Vγ3^{+} DETCs to stimulation by a keratinocyte cell line, PDV (Figure 4C). Peritoneal γδ T cells from the mutant mice or wild-type mice, many or all of which also express Vγ2, failed to respond to PDV keratinocytes, confirming that only DETCs are responsive. Thus, the transgenic Vγ2^{+} DETCs, like the wild-type Vγ3^{+} DETCs, are specific for PDV keratinocytes.

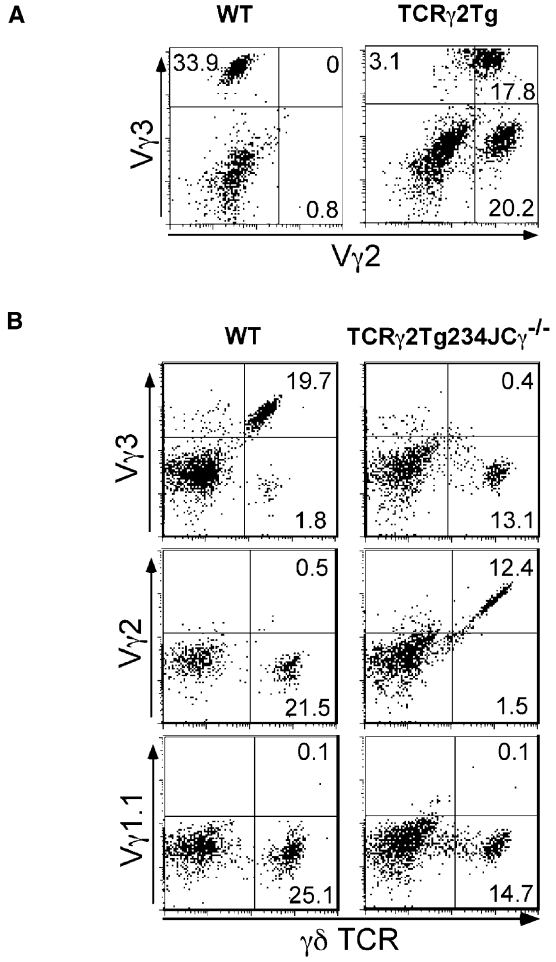

Figure 4. A Vγ2-Jγ1Cγ1 Transgene Restores Development of Functional DETCs in 234JCγ1$^{-/-}$ Mice

(A) Coexpression of endogenous TCRγ3 and transgenic TCRγ2 in epidermal γδ T cells of TCRγ2 transgenic mice as determined by flow cytometry.

(B) Flow cytometric analysis of epidermal cell preparations of TCRγ2Tg^{+}234JCγ1$^{-/-}$ mice for expression of individual subsets of Vγ^{+} versus total γδ T cells.

(C) Production of IL-2 by epidermal γδ T cells in response to a keratinocyte cell line PDV. DETCs from wild-type and TCRγ2Tg^{+} 234JCγ1$^{-/-}$ were incubated with the keratinocyte cell line PDV for 24 hr, and the supernatants were assayed for IL-2 by ELISA. Representative data from one of three experiments are shown.

Transgenic Vγ2^{+} DETCs Represent a Specific γδ Subset that Selectively Expresses Vδ7 but Lacks the 17D1 Idiotypic Determinant

It was previously reported that some TCRs compatible with DETC development are defined by a specific conformational TCR epitope detected by the 17D1 monoclonal antibody (Mallick-Wood et al., 1998). However, we did not detect any 17D1^{+} DETCs in the TCRγ2Tg^{+} 234JCγ1$^{-/-}$ mice although the majority of DETCs in normal mice stained with 17D1 mAb (Figure 5A). These data indicate that the 17D1 epitope is not required for DETC development, consistent with an earlier finding with Vδ1 knockout mice (Hara et al., 2000). Nevertheless, the Vγ2^{+} DETCs exhibited highly selective Vδ usage, consistent with selection of specific Vγ2^{+} precursors for differentiation into DETC. Semiquantitative RT-PCR analysis of RNA isolated from sorted DETCs revealed that Vδ1 transcripts dominated in wild-type Vγ3^{+} DETCs, but were more than 25-fold less abundant in the transgenic Vγ2^{+} DETCs. In contrast, Vδ7 transcripts were dramatically elevated in these cells, while other Vδ transcripts were either undetectable or only slightly elevated (Figure 5B). Therefore, selective TCR Vδ gene usage by DETCs occurs in the transgenic mice as it does in wild-type mice, but the dominant Vδ gene used (Vδ7) is different from the dominant Vδ present in wild-type mice (Vδ1). No mAb was available to test Vδ7 expression at the cell surface, but flow cytometric analyses confirmed the absence of Vδ4^{+} or Vδ5^{+} DETCs in the transgenic mice (Figure 5C), consistent with selective Vδ usage by these cells.

Wild-type DETCs originate in the early fetal thymus (Havran and Allison, 1990). To address whether the Vγ2^{+} DETC in the TCRγ2Tg^{+}234JCγ1$^{-/-}$ mice have the properties of cells that originate in the fetal thymus, TCRδ7 RT-PCR products from transgenic Vγ2^{+} DETCs were cloned and sequenced. In all five clones, Vδ7 was rearranged to Dδ2 and Jδ1, and the V(D)J junctions lacked N nucleotide additions (data not shown). Both of these properties are characteristic of rearrangements that occur in the fetal period (Asarnow et al., 1993; Chien et al., 1987; Feeney, 1990).

We then determined whether Vγ2^{+} Vδ7^{+} γδ T cells populate the skin preferentially compared to other γδ T cells in the fetal thymus of the TCRγ2Tg^{+}234JCγ1$^{-/-}$ mice. Strikingly, Vδ4^{+} and Vδ5^{+} γδ T cells constituted more than 40% of total γδ T cells in the E16 fetal thymus of the transgenic mice (Figure 5D), yet no Vδ4^{+} or Vδ5^{+} cells were detected among DETC in these mice (Figure 5C). Thus, DETC development is selective for cells expressing specific TCRδ even when substantial numbers of other types of γδ cells are generated in the fetal thymus.

DETC Developmental Potential Is Associated with Selective Activation of Fetal Thymic Precursors

As previously reported (Van Beneden et al., 2002), the majority of Vγ3^{+} γδ T cells in the E16 fetal thymus of wild-

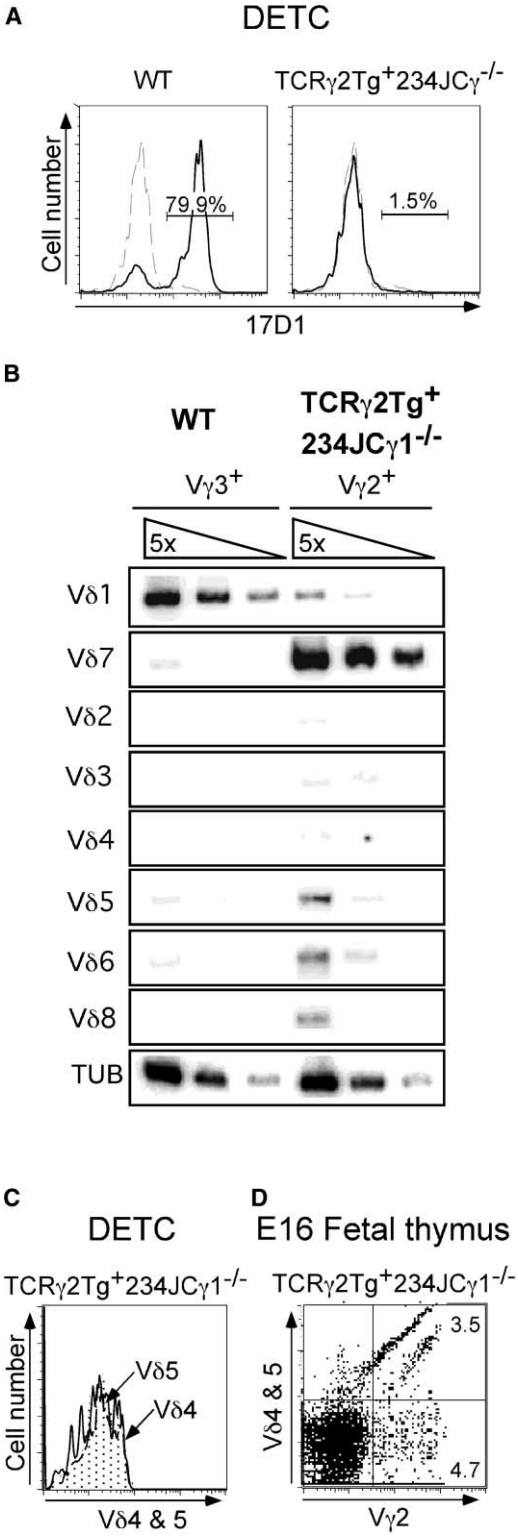

Figure 5. Selective Development of γδ T Cells Expressing Vγ2/Vδ7 TCRs in the Skin of TCRγ2Tg$^+$234JCγ1$^{-/-}$ Mice

(A) No expression of the 17D1 γδ TCR epitope by DETCs of TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice.

(B) RT-PCR analysis of TCR Vδ gene usage in sorted wild-type Vγ3$^+$ and transgenic Vγ2$^+$ skin γδ T cells. RNA samples were reverse transcribed with an oligo dT primer. The RT products were serially diluted and amplified by PCR with unique Vδ gene-specific primers and a common Cδ primer.

(C) Flow cytometric analysis of TCRδ4 and δ5 usage by gated Vγ2$^+$ skin γδ T cells of TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice. The shaded area is the negative control stained with a secondary antibody only.

(D) Flow cytometric analysis of TCRδ4 and δ5 usage by E16 fetal transgenic Vγ2$^+$ γδ thymocytes of TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice.

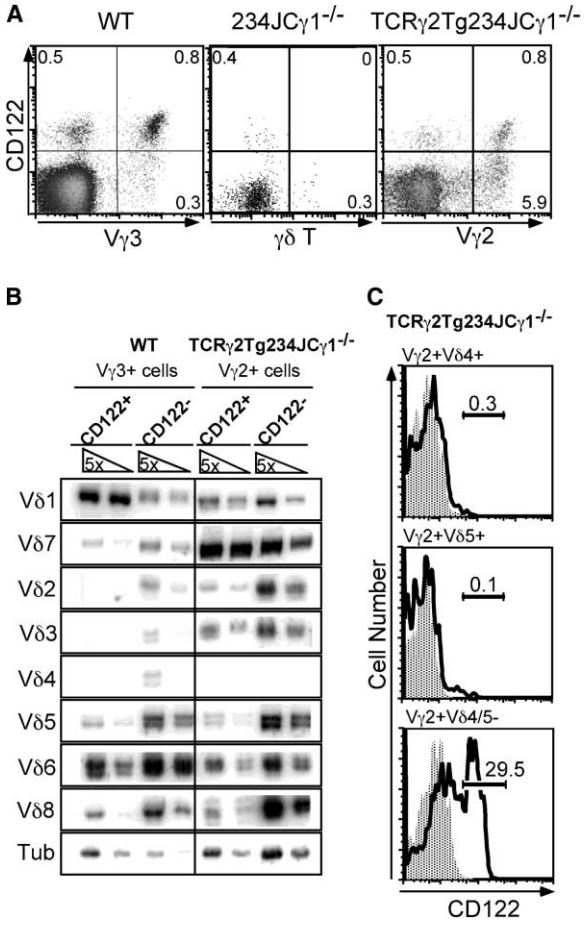

Figure 6. The Selective Activation of Specific Subsets of γδ T Cells in Fetal Thymus Correlates with Their Preferential Development in the Skin

(A) Flow cytometric analysis of CD122 expression by relevant γδ T cells in E16 fetal thymi of wild-type, 234JCγ1$^{-/-}$, and TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice.

(B) Vδ genes expressed by CD122$^+$ fetal thymic γδ T cells correspond to those expressed in corresponding DETCs. RT-PCR analysis of Vδ gene usage in sorted CD122$^+$ and CD122$^-$ populations of wild-type Vγ3$^+$ and transgenic Vγ2$^+$ fetal thymocytes was performed as described in Figure 5B.

(C) Flow cytometric analysis of CD122 expression by E16 Vγ2$^+$Vδ4&5$^+$ versus Vγ2$^+$Vδ4&5$^-$ fetal thymic γδ T cells from TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice. The shaded area is the negative control.

type mice (70.9% ± 10%, n = 5) display an activated or memory phenotype, including expression of CD122 (IL-2 receptor β chain) (Figure 6A). Interestingly, CD122$^+$ γδ T cells were essentially undetectable in the E16 fetal thymus of 234JCγ1$^{-/-}$ mice, which lack DETCs (Figure 6A). In contrast, in the TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice, in which DETC development was restored, CD122 was

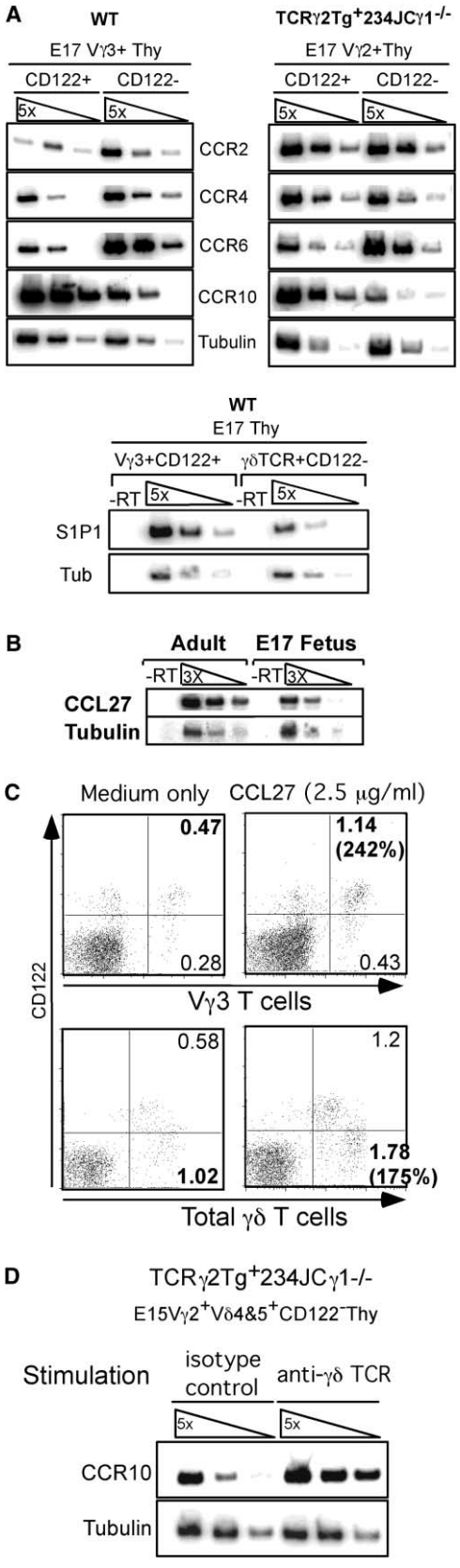

Figure 7. Coordinated Changes in Chemokine Receptor Expression in Activated Fetal Thymic γδ T Cells

(A) Expression of chemokine receptors CCR2, 4, 6, and 10 and the sphingosine-1-phosphate receptor 1 (S1P1) was determined by RT-PCR in RNA samples from sorted CD122$^+$ and CD122$^-$ populations of wild-type Vγ3$^+$ and transgenic Vγ2$^+$ E17 fetal thymocytes.

(B) Expression of CCL27, a CCR10 ligand, in both adult and fetal skin of wild-type mice, as determined by semiquantitative RT-PCR.

(C) CCL27 preferentially chemo-attracts fetal thymic CD122$^+$Vγ3$^+$ γδ T cells. Fetal thymocytes of wild-type mice were resuspended in the upper chamber of a Transwell culture insert and incubated with CCL27 in the bottom chamber. Four hours later, migrated cells of the bottom chamber were collected and analyzed for TCRδ, Vγ3, and CD122 expression by flow cytometry. The numbers in the quadrants indicate the percentages of all migrated cells. The numbers in parentheses represent the ratios of the number of cells that migrated at a specific concentration of CCL27 to the number of cells that migrated to medium alone. The chemotaxis experiments were performed in duplicate three times, and a representative experiment is shown.

(D) Upregulation of expression of CCR10 in the in vitro anti-γδ TCR antibody-stimulated Vγ2$^+$Vδ4&5$^+$CD122$^-$ γδ T cells from E15 fetal thymi of TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice, compared to the isotype control antibody-stimulated cells, as determined by RT-PCR.

expressed on 12.0% ± 2.7% (n = 5) of the Vγ2$^+$ cells in the E16 fetal thymus (Figure 6A). Hence, the capacity to generate DETCs in these mice was correlated with the presence of a significant CD122$^+$ γδ T cell population in the fetal thymus.

To investigate whether the activated phenotype is correlated with the expression of TCRs compatible with DETC development, we examined Vδ usage by the CD122$^+$ and CD122$^-$ subpopulations of γδ T cells in E16 fetal thymi (Figure 6B). The CD122$^-$ populations in both wild-type mice (Vγ3$^+$ cells) and TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice (Vγ2$^+$ cells) exhibited a relatively diverse pattern of Vδ gene expression. Strikingly, the CD122$^+$ populations were highly enriched in cells expressing the Vδ region found in DETC in the corresponding mice, Vδ1 in the case of wild-type mice, and Vδ7 in the case of TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice. Conversely, cells expressing other Vδ regions were selectively depleted in the respective CD122$^+$ populations. Flow cytometric analysis corroborated the repertoire analysis by demonstrating that Vδ4$^+$ and Vδ5$^+$ fetal thymocytes in TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice do not express CD122 (Figure 6C), in line with the absence of these cells among DETCs (Figure 5C). These striking correlations represent clear-cut evidence that the development of DETCs requires initial activation of a specific subset of fetal thymic TCRγδ cells, suggesting encounters with a thymic ligand.

Activation of Fetal Thymic γδ T Cells Results in Altered Homing Receptor Expression

To investigate whether DETC potential in the fetal thymus is related to expression of molecules that control cell homing, we examined expression of various chemokine receptors and other receptors in the CD122$^+$ and CD122$^-$ populations of fetal thymocytes. There were dramatic differences in chemokine receptor expression in activated versus unactivated γδ T cells in the fetal thymus (Figure 7A). Most notably, expression of the chemokine receptor CCR10 was dramatically upregulated

in the CD122$^+$ population in both wild-type (Vγ3$^+$) and transgenic (Vγ2$^+$) mice. One of the ligands for CCR10, CCL27, is specifically expressed in the adult skin (Homey et al., 2000; Jarmin et al., 2000; Morales et al., 1999), as well as in fetal skin (Figure 7B), suggesting that CCR10 may represent a skin-homing receptor selectively upregulated on activated fetal thymic γδ T cells, which thus determines their preferential migration to the skin. Interestingly, expression of the sphingosine-1-phosphate receptor 1 (S1P1), which has been shown to be necessary for exit of thymocytes from the thymus (Matloubian et al., 2004), was also upregulated by 8- to 10-fold in Vγ3$^+$CD122$^+$ thymocytes compared to CD122$^-$ γδ TCR$^+$ thymocytes (Figure 7A).

In contrast to CCR10 and S1P1, expression of CCR6 was downregulated 10- to 25-fold in CD122$^+$ fetal thymic γδ T cells compared to CD122$^-$ cells (Figure 7A). Since a ligand for CCR6, CCL20, is expressed in the thymus (Baba et al., 1997; Greaves et al., 1997; Liao et al., 1997; Varona et al., 1998), downregulation of CCR6 in the activated γδ T cells, along with upregulation of S1P1, may allow fully developed DETC precursors to exit the thymus while retaining immature γδ T cells in the thymus.

To address whether CCR10 expression is functional, we determined whether fetal thymocytes migrate toward CCL27 with the use of a cell migration assay in which fetal thymocytes are allowed to migrate from the upper chamber of a Transwell culture insert into a bottom chamber that contains CCL27 or medium alone. In cultures containing CCL27 in the bottom well, 2.42-fold more Vγ3$^+$ CD122$^+$ cells migrated to the bottom well than in the control cultures containing only medium in the bottom well (Figure 7C). Although some CD122$^-$ fetal thymic γδ T cells also showed enhanced migration to CCL27, they migrated less efficiently than CD122$^+$Vγ3$^+$ fetal thymic γδ T cells (1.75-fold more CD122$^-$ γδ T cells migrated to CCL27 than to medium alone). The preferential migration to CCL27 of the CD122$^+$ cells as compared to CD122$^-$ cells was a consistent finding in three experiments. Although the CD122$^-$ cells exhibit some capability to migrate toward CCL27, their relatively high expression of CCR6 and low expression of S1P1 may contribute to their relative inability to populate the skin.

The results suggested that CCR10 may be specifically upregulated in γδ precursors in response to ligand engagement in the fetal thymus. To test this idea directly, we determined whether TCR stimulation induces CCR10 upregulation in fetal thymic precursors bearing irrelevant γδ TCRs. Sorted Vδ4$^+$ and Vδ5$^+$ CD122$^-$ precursors from the E15 fetal thymus of TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice dramatically upregulated CCR10 mRNA when stimulated with anti-γδ TCR mAb in comparison to the levels in the starting population (data not shown). Because unstimulated cultured cells died, we were unable to compare stimulated and unstimulated cells maintained in parallel. When viability was maintained by adding IL-7 to the cultures, similar results were obtained: TCRγδ stimulation induced 5- to 25-fold upregulation of CCR10 in the cultured Vδ4$^+$ and Vδ5$^+$ CD122$^-$ precursors in comparison to cells cultured with an isotype control antibody (Figure 7D). Thus, engagement of TCRs on fetal thymic γδ T cells induces upregulation of chemokine receptors that are likely involved in homing of fetal thymic precursors to the epidermis.

Discussion

The basis for the predominance of Vγ3/Vδ1$^+$ γδ T cells in the epidermis has been investigated over a decade with conflicting results. Although generation of γδ T cells in the fetal thymus is clearly important in DETC development (Allison and Havran, 1991; Ikuta et al., 1990), the role of the γδ TCR itself in this process is less clear. Using gene-targeted mice in which the generation of Vγ3$^+$ cells was impaired, we reported that Vγ3 γδ T cells selectively develop into DETC even when they are not preferentially generated in the early fetal thymus (Xiong et al., 2002). We now report that the development of DETCs is absolutely dependent on TCRγ genes within the Cγ1 cluster despite the fact that other γδ precursor cells arise normally in the fetal thymus. These data demonstrated a critical role of specific γδ TCRs in DETC development.

Interestingly, a Vγ2-Jγ1Cγ1 transgene restored the development and functions of DETCs in the 234Jγ1$^{-/-}$ mice. This finding did not undermine the conclusion that a selective process restricts TCR usage among DETCs because the resulting Vγ2$^+$ DETCs were highly selective in usage of Vδ7, a Vδ chain not used by DETCs in normal mice. Furthermore, other Vδ chains that associated with Vγ2 in the transgenic fetal thymus were not represented among the resulting DETCs. Finally, the Vγ2$^+$ DETCs, like Vγ3$^+$ DETCs in normal mice, respond specifically to a keratinocyte cell line, while Vγ2$^+$ peritoneal γδ T cells from the same mice did not. These results firmly establish that development of DETCs is critically dependent on expression of specific γδ TCRs and therefore involves a cellular selection process. The failure of Vγ2$^+$ DETC to develop in normal mice may reflect the inefficiency of Vγ2 rearrangement in the normal fetal thymus and/or a competitive disadvantage of the cells as compared to Vγ3$^+$ DETC precursors.

The putative thymic ligand(s) for the Vγ3/Vδ1 and Vγ2/Vδ7 DETC-specific TCRs remains to be identified, and it remains unclear whether the two receptors recognize the same ligand. A previous study characterized Vγ2/Vδ7 T cells in the periphery of normal mice and demonstrated that the frequency of these cells is strain dependent, being high in B6 mice but low in DBA/2 mice, suggesting Vγ2/Vδ7 TCR is stimulated by endogenous ligands (Sperling et al., 1997). Those findings are consistent with our data, which also suggest that Vγ2/Vδ7 DETC precursors react with an endogenous ligand, in this case in the fetal thymus.

Previous evidence for positive thymic selection of TCRγδ transgenic T cells by MHC class I-related molecules (Ito et al., 1990; Wells et al., 1991, 1993) was later attributed to negative selection or genetic background effects (Schweighoffer and Fowlkes, 1996). Furthermore, while the expression of an invariant TCR by DETCs was initially suggestive of positive selection (though not necessarily thymic selection), subsequent studies demonstrated that the assembly of the invariant DETC receptor is favored by programmed rearrangement of Vγ3 and Vδ1 in the fetal thymus and mechanisms that restrict V(D)J junctional diversity (Asarnow et al., 1993; Itohara et al., 1993; Xiong et al., 2004). The demonstration here that fetal thymic DETC precursors bearing either of the two distinct TCRs are selectively activated in the fetal

thymus represents compelling evidence that γδ T cells, in this case DETC precursors, undergo thymic positive selection.

Considering previous results, it was surprising that deletion of the TCRγ Cγ1 cluster abolished all DETC development and especially surprising that Vγ1.1$^+$ DETCs failed to substitute for Vγ3$^+$ DETCs. Reportedly, Vγ3 knockout mice had large numbers of DETCs, many of which were Vγ1.1$^+$ cells (Mallick-Wood et al., 1998). The Vγ1.1 gene contains its own regulatory elements, and expression and rearrangement of this Vγ gene occurred normally in mice lacking the two known Cγ1 regulatory elements (H/E$^{-/-}$ mice) (Xiong et al., 2002). Furthermore, rearrangement of Vγ1.1 was normal in the 234JCγ1$^{-/-}$ mice studied here (Figure 3E), and Vγ1.1$^+$ cells developed normally in the thymus of 234JCγ1$^{-/-}$ mice, populated the spleen and the intestinal epithelium, and expressed normal levels of cell surface TCR. The possibility that variable development of Vγ1.1$^+$ DETC is due to genetic differences in the background of the Vγ3$^{-/-}$ and 234JCγ1$^{-/-}$ mice is raised by previous evidence that genetic polymorphisms influence the DETC repertoire (Girardi et al., 2002).

Using the 234JCγ1$^{-/-}$ and TCRγ2 transgenic mice, we elucidated molecular events underlying the selective developmental process of DETCs. Comparing the development of wild-type Vγ3$^+$ and transgenic Vγ2$^+$ DETCs, we demonstrated that, despite completely different TCR usage, both Vγ3$^+$ and Vγ2$^+$ DETC precursors in the fetal thymus underwent selective activation, which led to several events that likely equip the cells to migrate to the skin: upregulation of S1P1, which is necessary for exiting the thymus, upregulation of the potential skin homing chemokine receptor CCR10, and downregulation of the potential thymus-retaining chemokine receptor CCR6. These findings are consistent with previous studies showing that Vγ3$^+$ cells acquire the capacity to migrate to the skin while still in the fetal thymus (Allison and Havran, 1991; Haas et al., 1993). Coordinated switches in the expression of chemokine receptors have been reported previously to play roles in developmental maturation of αβ T cells and inflammatory responses of immune cells (Campbell et al., 1999; Sallusto et al., 1998).

In addition to their preferential skin-homing ability, the activated fetal thymic γδ DETC precursors downregulate CD24 (Ferrero et al., 2001) and upregulate CD122, the β chain of the IL-15 (and IL-2) receptor. CD122, as well as its cognate cytokine IL-15, are absolutely required for DETC development (De Creus et al., 2002; Kawai et al., 1998), presumably reflecting the requirement of IL-15 for survival and expansion of the cells. Thus, ligand-induced changes in gene expression that occur during DETC development in the fetal thymus equip DETC precursors with the homing molecules necessary for epidermal localization, as well as cytokine receptor chains that are likely necessary for subsequent survival and expansion of the cells. These changes were not inherently restricted to cells with DETC-specific TCRs, because in vitro activation of irrelevant fetal thymic γδ T cells also led to upregulation of CCR10 and CD122 (Figure 7 and data not shown). Thus, cellular selection, dependent on engagement of a ligand in the fetal thymus, is a primary determining factor in DETC development. Some evidence also suggests that continued TCR/ligand interactions are necessary for the continued survival/expansion of DETCs once they arrive in the epidermis (Minagawa et al., 2001).

While the present data provide strong evidence that DETC development involves cellular selection, previous findings established that intrinsic molecular programming events within fetal thymocytes are also involved. Initial studies showed that fetal precursor cells, and not adult precursor cells, can give rise to Vγ3$^+$ cells in fetal thymic organ cultures (Ikuta et al., 1990). Subsequent studies showed that, in the absence of the possibility of cellular selection, fetal thymocytes are much more likely than adult thymocytes to rearrange the Vγ3 and Vδ1 gene segments that are expressed by nearly all DETCs in normal mice and to create the canonical V(D)J junctional sequences lacking N regions (Asarnow et al., 1993; Itohara et al., 1993; Raulet et al., 1991; Zhang et al., 1995). Importantly, fetal thymic precursors are not sufficient for the development of Vγ3$^+$ cells because such cells failed to develop from fetal precursors that repopulated an adult thymus lobe. An intriguing hypothesis to integrate these findings is that fetal (as opposed to adult) thymic stromal cells specifically express the cognate ligand for the DETC receptor, while fetal (as opposed to adult) thymocyte precursors are specifically endowed with the capacity to assemble the canonical DETC receptor. In this manner, a selective process may be superimposed on a programmed scheme of thymic development, leading to systematic production of DETCs during the fetal period.

Experimental Procedures

Mice

To generate 234JCγ1 knockout mice, we used a previously reported H/E-ko-neo ES clone in which one loxP site replaced HsA, a regulatory element, 3 kb upstream of Vγ2, and a loxP-flanking Neo cassette replaced the Cγ1-associated enhancer element, 3′E$_{Cγ1}$, 3 kb downstream of Cγ1 (Figure 1A) (Xiong et al., 2002). Transient transfection of these cells with a *Cre* expression plasmid resulted in the 234JCγ1-ko subclones, in which the intervening 35 kb fragment was deleted, including the *neo* cassette. Deletion of the 35 kb fragment was confirmed by Southern blot analysis with a Vγ5 probe (Figures 1A and B). The 234JCγ1-ko ES clones therefore lack Vγ2, Vγ4, Vγ3, Jγ1, and Cγ1, as well as the two regulatory elements, HsA and 3′E$_{Cγ1}$. The confirmed 234JCγ1-ko ES clones were injected into fertilized blastocysts of C57BL/6 (B6) mice, from which chimeric mice were generated. The chimeric mice were crossed with B6 or 129 mice to generate 234JCγ1 knockout mice on either a mixed 129/B6 background or a pure 129 background, respectively. The experiments in Figures 1–3 were performed with mixed background mice, but similar results were obtained in pure 129 background knockout mice. TCRγ2 transgenic mice have been described before (Kang et al., 1998). TCRγ2Tg$^+$234JCγ1$^{-/-}$ mice were generated by breeding the TCRγ2 transgenic mice (B6-backcrossed) with 234JCγ1$^{+/-}$ that had been backcrossed once already to B6. These mice were used in Figures 4–7.

Cell Preparations

Epidermal cells were prepared from skin samples as described (Sullivan et al., 1985). For in vitro expansion of DETCs, the initial epidermal cell preparation (2–3 × 10^6 cells, of which 3%–5% were T cells) was cultured in DMEM medium/10% fetal calf serum, supplemented with 1.25 μg/ml Con A and 100 units/ml rIL2 for 1 week, and then cultured for at least several additional weeks in the same medium supplemented with 100 units/ml rIL2. The γδ T cells were further purified from the expanded DETCs by magnetic sorting using anti-γδ

TCR antibody according to the manufacturer's instructions (Miltenyi Biotec Inc., Auburn, CA). Intraepithelial lymphocytes were prepared from small intestines as described (Chu et al., 1999). Thymocytes and nylon wool column-passed splenocytes were prepared by standard methods (Coligan et al., 1991). The specific subsets of γδ T cells used for RT-PCR were sorted with an EPICS Elite ESP sorter (Beckman-Coulter, Miami, FL) following staining with appropriate antibodies. The keratinocyte cell line PDV was generously provided by Wendy Havran (The Scripps Research Institute, La Jolla, CA).

Antibodies and Flow Cytometry

Purified anti-γδ TCR (GL4), FITC-, biotin-conjugated anti-γδ TCR (GL3), Cy-Chrome or APC-conjugated anti-CD3 antibodies were purchased from Pharmingen (San Diego, CA). Biotin- and FITC-conjugated anti-Vγ3 TCR (F536) and anti-Vγ2 TCR (UC3) antibodies were prepared in our lab. FITC-conjugated anti-Vγ1.1 TCR antibody, FITC-conjugated anti-Vγ5 antibody, and antibody 17D1 were generously provided by Pablo Pereira (Pasteur Institute, Paris), Leo Lefrancois (University of Connecticut, Farmington, CT) and Robert Tigelaar (Yale University, New Haven, CT), respectively. Red 613-conjugated anti-CD4, tricolor-conjugated anti-CD8 and biotin-conjugated anti-αβ TCR (H57) antibodies were purchased from Gibco BRL (Gaithersburg, MD). Alexa 488- and PE-conjugated streptavidin were purchased from Molecular Probes (Eugene, OR). Expression of TCR and other markers on cells were analyzed by multiple color flow cytometry on an EPICS XL instrument (Coulter, Hialeah, FL), and the data were analyzed by FlowJo software (Tree Star Inc, San Carlos, CA).

Immunohistochemical Analysis of Skin Epidermal Sheets

Skin epidermal sheets were prepared as described (Miyauchi and Hashimoto, 1987). The sheets were stained with APC-conjugated anti-CD3 and biotin-conjugated anti-TCRγδ antibodies (or biotin-conjugated anti-Vγ3 antibody) followed with Alexa 488-conjugated streptavidin and analyzed on a fluorescence microscope.

Primers, Probes, and Typing of 234JCγ1-Knockout Mice

The sequences of primers used for PCR are as follows: p1, CTATA CATCATATGACTTGG; p2, CTGGGCTATGTTAGAGATGC; p3, CTA ATGGTTATCCTAGAACTGG; pVγ1, GGGCTTGGGCAGCTGGAGCA; pJγ4, GGGGAATTACTACGAGCTTTGTC; pVδ1, ATGCTTTGGAGAT GTCCAGT; pVδ2, AGTTCCCTGCAGATCCAAGC; pVδ3, TCCTGGC TATTGCCTCTGAC; pVδ4, CCGCTTCGCTGTGAACTTCC; pVδ5, CAG ATCCTTCCAGTTCATCC; pVδ6, TCAAGTCCATCAGCCTTGTC; pVδ7, CGCAGAGCTGCAGTGTAACT; pVδ8, AAGGAAGATGGACGATTC; pJδ2, CTCCACAAAGAGCTCTATGC; pCδ, CGAATTCCACAATCTTCTT (Fujise et al., 1997). CCR2-5′, TGATAGGTACTTGGCTATTG; CCR2-3′, CAGTGTGTCATTCCAAGAG; CCR4-5′, CCTCTTGTTCAGCACTTG CTAC; CCR4-3′, TTACAAAGCGTCACGGAAGTCATG; CCR6-5′, GTTTTCAGCGATGCACTGTG; CCR6-3′, GTAGTTTCTGAATTTCTG TCC; CCR10-5′, AGAGCTCTGTTACAAGGCTGATGTC; CCR10-3′, CAGGTGGTACTTCCTAGATTCCAGC; CCL27-5′, GCTGTTACTGTT GCTTCTG; CCL27-3′, TGGCGTTCTAACCACCGAG; S1P1-5′, GTA CTTCCTGGTTCTGGCTGTGC; S1P1-3′, CGTTTCCAGACGACATAA TGG. The tubulin 5′ and tubulin 3′ primers have been described (Goldman et al., 1993). The positions of primers p1, p2, p3, and Vγ5 probe are indicated in Figure 1A. To type 234JCγ1-ko mice, PCR was performed on genomic DNA with primers p1, p2, and p3, and PCR products were separated on 1.5% agarose gel to distinguish wild-type from knockout band by size (Figure 1C).

Semiquantitative PCR and RT-PCR

The semiquantitative PCR and RT-PCR assays were performed essentially as described before (Baker et al., 1998). For the RT-PCR assay, RNA samples were reverse transcribed (RT) with Superscript II RNase H$^-$ reverse transcriptase using oligo-dT primers. The RT products were serially diluted (indicated in the figures) and subjected to semiquantitative PCR using different sets of primers. In all cases, RNA samples without RT were included in PCR reaction to assure that there was no genomic DNA contamination (data not shown).

IL-2 Production by Epidermal γδ T Cells in Responses to Keratinocytes

The experiment was performed essentially as described (Havran and Allison, 1991). In brief, 10^5 in vitro expanded epidermal γδ T cells (about 95% are TCRγδ positive) were incubated with 5×10^5 irradiated PDV cells for 24 hr, and the supernatants of the cultures were assayed for IL-2 by ELISA according the manufacturer's instruction (Pharmingen, San Diego, CA).

Chemotaxis Assay

The chemotaxis assay was performed in 24-well plate tissue culture inserts with 5 μm pore filters (Costar Corp., Cambridge, MA). $5–10 \times 10^5$ fetal thymocytes in chemotaxis medium (DMEM/10% fetal bovine serum) were resuspended in the upper chamber of a Transwell culture insert and incubated with CCL27 (R&D Systems, Minneapolis, MN) in the bottom chamber at 37°C and 6.5% CO_2. Four hours later, migrated cells in the bottom chamber were collected and analyzed for TCRδ, Vγ3, and CD122 expression by flow cytometry.

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