

Recombination Signal Sequence-Associated Restriction on TCR δ Gene Rearrangement Affects the Development of Tissue-Specific $\gamma\delta$ T Cells¹

Uzodinma N. Uche,^{2*} Christopher R. Huber,^{*} David H. Raulet,[†] and Na Xiong^{3*}

Assembly of TCR α and TCR δ genes from the TCR α/δ locus is tightly controlled for the proper generation of $\alpha\beta$ and $\gamma\delta$ T cells. Of >100 shared variable gene segments in the TCR α/δ locus, only a few are predominantly used for the TCR δ gene assembly, while most are for TCR α . However, the importance and mechanisms of the selective variable gene rearrangement for T cell development are not fully understood. We report herein that the development of a tissue-specific $\gamma\delta$ T cell population is critically affected by recombination signal sequence-associated restriction on the variable gene usage for TCR δ assembly. We found that the development of substitute skin $\gamma\delta$ T cells in mice deficient of the TCR $\gamma 3$ gene, which is used in wild-type skin $\gamma\delta$ T cells, was drastically affected by the strain background. A V $\gamma 2^+$ skin $\gamma\delta$ T cell population developed in mice of the B6 but not the 129 strain backgrounds, due to a difference in the rearrangement of endogenous V $\delta 7^+$ TCR δ genes, which paired with the V $\gamma 2^+$ TCR γ gene to generate the V $\gamma 2/V\delta 7^+$ skin $\gamma\delta$ T cell precursors in fetal thymi of the B6 background mice. The defective TCR δ rearrangement of the 129-“V $\delta 7$ ” gene was associated with specific variations in its recombination signal sequence, which renders it poorly compatible for rearrangement to D δ genes. These findings provide the first direct evidence that recombination signal sequence-associated restriction on the variable gene usage for TCR α/δ gene assembly plays an important role in T cell development. *The Journal of Immunology*, 2009, 183: 4931–4939.

T cells can be distinguished by their expression of distinct forms of TCRs. $\alpha\beta$ T cells express the $\alpha\beta$ TCR, a heterodimer of TCR α - and TCR β -chains, and $\gamma\delta$ T cells express the $\gamma\delta$ TCR of TCR γ and TCR δ chains. Although both $\alpha\beta$ and $\gamma\delta$ T cells are generated in the thymus, they have different peripheral tissue distribution and function. While $\alpha\beta$ T cells are predominantly localized in secondary lymphoid organs after exiting the thymus, $\gamma\delta$ T cells are more often localized to epithelial tissues, such as the skin and reproductive tracts (1, 2). The epithelial tissue-specific $\gamma\delta$ T cells usually display limited diversities in their TCR sequences and function as innate immune cells in the first line of defense (3–5). In mice, nearly all $\gamma\delta$ T cells in the skin epidermis, called dendritic epidermal T cells (DETCs),⁴ express canonical V $\gamma 3/V\delta 1^+$ TCRs while vaginal epithelial $\gamma\delta$ T cells express V $\gamma 4/V\delta 1^+$ TCRs. In comparison, $\gamma\delta$ T cells in secondary

lymphoid organs usually express more diverse TCRs, predominantly of V $\gamma 2$ and V $\gamma 1.1$ associated with several V δ -chains.

Development of the various tissue-specific $\gamma\delta$ T cell subsets is orchestrated at different stages of ontogeny, presumably to optimize the production of different specialized sets of T cells required for host defense. The first subsets to arise in the early embryonic day 15 fetal thymus assemble V $\gamma 3$ and V $\gamma 4$ gene segments (1, 6, 7). The resulting T cells include precursors of DETCs (V $\gamma 3/V\delta 1$) and the vaginal intraepithelial T cells (V $\gamma 4/V\delta 1$), which subsequently migrate to their peripheral destinations. After birth, V $\gamma 3$ and V $\gamma 4$ rearrangements are suppressed and rearrangements of V $\gamma 2$, V $\gamma 1.1$, and others predominate (8–10). The resulting T cells preferentially emigrate to secondary lymphoid organs, among other tissues. TCR δ genes are rearranged coordinately with the TCR γ genes. The V $\delta 1$ gene is predominantly rearranged and expressed at the same early fetal thymic stage as V $\gamma 3$ and V $\gamma 4$ genes (11), enabling the production of V $\gamma 3/V\delta 1$ and V $\gamma 4/V\delta 1$ TCRs, with the forms being expressed in DETCs and vaginal intraepithelial $\gamma\delta$ T cells, respectively. In the adult stage, a distinct set of V δ gene segments is predominantly rearranged (11).

The TCR δ locus is embedded within the TCR α locus, and the two loci share the same set of variable gene segments (V α/δ). Although all the variable gene segments could potentially rearrange to D $\delta/J\delta$ or J α segments for assembly of TCR δ or TCR α genes, the actual usage of V α/δ genes in the TCR δ and TCR α assembly is very restricted. Of >100 V α/δ gene segments in the murine TCR α/δ loci, <10 of them (so-called V δ genes) are predominantly used in the TCR δ gene assembly, while most (V α genes) are preferentially in rearrangements of TCR α genes (12). The preferential usage of different V α/δ gene segments for the TCR δ and TCR α gene rearrangement is genetically programmed and independent of cellular selection processes, as it applies even in CD3 $\epsilon^{-/-}$ mice, in which cellular selection is defective (13, 14). However, importance and mechanisms of the regulated V α/δ gene rearrangement for the T cell development are not fully understood.

*Center for Molecular Immunology and Infectious Diseases, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802; and [†]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720

Received for publication June 10, 2009. Accepted for publication August 11, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants from the National Institutes of Health (5R01AI071043 to N.X. and 5R01AI031650 to D.H.R.) and, in part, under a grant with the Pennsylvania Department of Health using Tobacco Settlement Funds (to N.X.). The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions.

² Current address: Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591.

³ Address correspondence and reprint requests to Dr. Na Xiong, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, 115 Henning Building, University Park, PA 16802. E-mail address: nux1@psu.edu

⁴ Abbreviations used in this paper: DETC, dendritic epidermal T cell; RSS, recombination signal sequence.

Multiple mechanisms underlie the regulated TCR gene rearrangements, including gene accessibility, germline transcription, V gene competition, and preferential pairing of specific recombination signal sequences (RSSs). The accessibility of V, D, and J segments in chromatin to the recombinase apparatus is regulated at different ontogenic and developmental stages and mediated by transcription factors through *cis*-acting regulatory elements located in the TCR loci. Recent evidence suggests that the capacity of the gene segments to undergo germline transcription, which may in turn be regulated by the gene accessibility, is a direct determinant of rearrangement efficiency (15, 16). Accessibility of V γ gene segments has been found to be an important determinant of the rearrangement pattern in the adult thymus, where the frequently rearranged V γ 2 segment resides in acetylated (active) chromatin, and the rarely rearranged V γ 3 segment resides in inactive chromatin (17). This difference is determined in large part by the promoter regions of the V γ 3 and V γ 2 gene segments (9, 18).

Gene accessibility also plays a role in the selective V α/δ gene usage for TCR δ and TCR α gene assembly. At the early CD4⁻CD8⁻ (double-negative) stage of thymic T cell development, J α gene segments are inaccessible for rearrangement due to the inactivity of their associated *cis*-regulatory elements such as the E α enhancer and TEA promoter, while D δ and J δ gene segments are rendered accessible by their associated E δ enhancer. Therefore, only TCR δ genes are assembled at the double-negative stage (19, 20). Most (but not all) of the “V δ ” genes are located at the 3' region of the V α/δ gene cluster, near the D δ -J δ region and within the effect range of the E δ enhancer (21, 22). Consistent with this, many of the proximal V δ genes were present in a hyperacetylated (active) chromatin status at the double-negative stage (23). At the later CD4⁺CD8⁺ (double-positive) stage (for $\alpha\beta$ T cell development), the more powerful and $\alpha\beta$ T lineage-specific E α enhancer becomes active and promotes the accessibility of J α genes and more distal “V α ” genes and TCR α gene assembly (21). However, several V δ genes are located as far as 1 Mb away from the D δ /J δ region, while some V α genes lie within 100 kb from the D δ -J δ region, suggesting that the enhancers-regulated accessibility alone could not account completely for the selective usage of V α/δ genes in the TCR δ and TCR α gene assembly.

Competition among different V gene segments is another mechanism that regulates their usage in the TCR gene assembly. In the TCR γ locus, both V γ 2 and V γ 3 segments are accessible at the early fetal stage, yet V γ 3 is preferentially rearranged to J γ 1 gene. Transgenic and gene-targeting studies showed that the preference for V γ 3 is due in large part to its proximal location to the J γ 1 gene (10, 18). A competition mechanism might also play a role in determining V δ gene usage in fetal thymocytes, especially considering that the fetal-specific V δ 1 gene is most proximal to the D δ -J δ segments.

An RSS-mediated restriction might also play a role in the regulated TCR gene arrangement. An RSS is composed of a semi-conserved heptamer and nonamer, separated by a poorly conserved 23- or 12-bp spacer, and it is the target site of Rag1/2 recombinases for cleaving and joining of the VDJ segments. Rearrangements occur only between an RSS with a 12-bp spacer (12-RSS) and another with a 23-bp spacer (23-RSS), the so-called 12/23 rule. However, certain rearrangements, such as those between the 23-RSS-flanked V β segments and the 12-RSS-flanked J β segments, occur rarely if not at all, even though they are allowed by the 12/23 rule. The restriction beyond the 12/23 rule was suggested to be important for the ordered V β -D β -J β rearrangement to generate vast TCR β repertoires and was reportedly enforced by the incompatibilities of the V β -RSSs with J β -RSSs for RAG-mediated pairing and cleavage (24–26). The RSS-mediated restriction might

also play a role in the selective V gene usage for the TCR δ/α gene rearrangement. A “motif” found in spacers of many V α -RSSs was suggested for their preferential rearrangement to J α fragments (27). However, this motif was found only in 80% of known V α genes and has not been directly demonstrated to be involved in restricting the V α genes for TCR α rearrangement. No motif has been discerned in the RSSs of V δ genes, and there is currently no direct evidence that rearrangement of V δ segments is restricted by the RSS beyond the 12/23 rule. However, considering that no motifs have been defined in RSSs of V β /D β /J β fragments, where the restriction was demonstrated, the possibility of RSS-restricted V δ usage remains open.

Cellular selection further shapes the development of T cell populations resulting from the regulated TCR rearrangement. Although $\gamma\delta$ T cell selection processes are not as well understood as are those of $\alpha\beta$ T cells, recent studies found that a positive selection is critical, at least, for development of the skin-specific DETCs (28–30). We reported that positive selection of the DETC precursors V γ 3/V δ 1⁺ $\gamma\delta$ T cells in the fetal thymus resulted in a coordinate switch in expression of several chemokine receptors and cytokine receptors important for their localization and expansion in the skin (28). In absence of the positive selection, such as in a substrain of FVB mice (Taconic), few of the fetal thymic V γ 3/V δ 1⁺ $\gamma\delta$ T cells developed into DETCs even though they are generated in normal numbers in the fetal thymus (29).

Although the V γ 3/V δ 1⁺ $\gamma\delta$ T cells are the dominant population of DETCs in wild-type mice, other (but not all) subsets of $\gamma\delta$ T cells could substitute in the skin, especially in mice deficient of the native V γ 3/V δ 1⁺ DETCs (31, 32). We reported that a rearranged TCR γ 2 transgene (TCR γ 2Tg) could restore the development of DETCs in 234JC γ 1 knockout (C γ 1^{-/-}) mice that are deleted of the C γ 1 cluster of TCR γ locus (including the DETC-specific V γ 3, as well as V γ 2, V γ 4, and V γ 5 genes) and, therefore, lack the native V γ 3⁺ DETCs (28). The transgenic V γ 2⁺ DETCs predominantly expressed endogenous TCR δ genes of V δ 7-D δ 2-J δ 1 rearrangements (TCR δ 7) (28), suggesting that they were selected for the DETC development. Supporting this, of multiple subsets of fetal thymic transgenic V γ 2⁺ $\gamma\delta$ T cells that paired with different endogenous TCR δ -chains, only the V γ 2/V δ 7⁺ subset preferentially underwent the positive selection-associated cytokine and chemokine receptor expression, which was important for their homing and expansion in the skin (28). However, factors that affect the DETC development are not fully understood. For example, although the V γ 3^{-/-} mice reportedly had abundant substitute DETCs, of which many expressed V γ 1.1⁺ $\gamma\delta$ TCRs (31), nearly no DETCs developed in the C γ 1^{-/-} mice that generated the V γ 1.1⁺ $\gamma\delta$ T cells normally (28). These observations suggested the existence of additional factors that affect the development of DETCs.

We report herein that differential TCR δ gene rearrangement of different strain backgrounds affect the DETC development significantly. Particularly, we found that variations in recombination signal sequences of the V δ 7 genes of different strains were associated with their selective usage for the TCR δ gene rearrangement, which in turn had a dramatic effect on development of a skin-specific $\gamma\delta$ T cell population. These findings provide the first compelling evidence that the RSS-associated restriction on TCR δ gene assembly plays an important role in the T cell development.

Materials and Methods

Mice

C γ 1^{-/-}, V γ 3^{-/-}, and TCR γ 2Tg mice have been described (28, 31). C57BL/6 (B6) and 129 mice were purchased from The Jackson Laboratory. The C γ 1^{-/-} mice were originally generated on a pure 129 background (C γ 1^{-/-}129) (28, 31). To move C γ 1^{-/-} on B6 background (C γ 1^{-/-}B6),

the $C\gamma 1^{-/-}$ 129 mice were backcrossed to B6 mice for seven to eight generations. $V\gamma 3^{-/-}$, TCR $\gamma 2Tg$, and TCR $\gamma 2Tg^{+}C\gamma 1^{-/-}$ mice, originally on the mixed 129 \times B6 background (28, 31), were backcrossed to B6 or 129 mice for seven to eight generations to place them on the dominant B6 or 129 backgrounds.

Cell preparation

Epidermal cells were prepared from the skin as described (33). Thymocytes were prepared by a standard method (34). The specific subsets of skin $\gamma\delta$ T cells of TCR $\gamma 2Tg$ mice used for RT-PCR were sorted from the epidermal cells on an EPICS Elite ESP sorter (Beckman Coulter) following staining with appropriate Abs.

Abs and flow cytometry

FITC-, biotin-conjugated anti- $\gamma\delta$ TCR (GL3), PE-Cy5-conjugated anti-CD3, FITC-conjugated anti- $V\gamma 3$ TCR (F536), and anti- $V\gamma 2$ TCR (UC3) Abs were purchased from BD Biosciences. PE- or biotin-conjugated CD122 Ab was from eBioscience. Biotin-conjugated anti- $V\gamma 3$ TCR (F536) and anti- $V\gamma 2$ TCR (UC3) Abs were prepared in-house. PE-Cy5- and PE-conjugated streptavidin were purchased from Invitrogen. Expression of TCR and other markers on cells were analyzed by multiple color flow cytometry on an Epics XL instrument (Beckman Coulter) following staining with appropriate Abs. The flow cytometry data were analyzed by FlowJo software (Tree Star).

DNA and RNA preparation

Thymic genomic DNA was prepared as described (9). Total RNA was prepared from sorted $\gamma\delta$ T cells by TRIzol according to the manufacturer's instructions (Invitrogen). To prevent genomic DNA contamination, the RNA samples were treated with RNase-free DNase I before being used for RT-PCR analysis.

Semiquantitative PCR and RT-PCR

The semiquantitative PCR and RT-PCR assays were performed essentially as described (18, 28). To assess TCR δ rearrangements of $V\delta 7$ and its TRAV13 family members, thymic genomic DNA was serially diluted and subjected to PCR with primers TRAV13 (forward primer) (CTTGGTTCTGCAGGAGGGGAGAACGCAGAGC) and J $\delta 1$ (reverse primer) (AATGACTTACTTGGTTCCACAGTCACTTGGGT). The sequence of TRAV13 (forward primer) primer complements all TRAV13 ($V\delta 7$) family members, while the sequence of J $\delta 1$ (reverse primer) complements the J $\delta 1$ gene.

For the RT-PCR assessment of $V\delta$ gene usage in sorted skin $\gamma\delta$ T cells, RNA samples were reverse-transcribed with SuperScript II RNase H⁻ reverse transcriptase using oligo-dT primers. The reverse transcription products were serially diluted (indicated in figures) and subjected to PCR with proper sets of primers. PCR primers for detection of transcripts of various rearranged TCR δ genes and tubulin were described (7, 28).

Sequencing analysis of TCR δ gene rearrangements of the $V\delta 7$ genes

PCR products with the TRAV13 (forward primer)/J $\delta 1$ (reverse primer) primer set were inserted into TOPO TA cloning vectors (Invitrogen), which were used to transform competent *Escherichia coli* cells. Plasmids containing inserts of the PCR products were extracted from subclones of the transformed *E. coli* and sequenced for the inserts. Based on the sequence alignment, frequencies of $V\delta 7$ and the other family members in the subclones were calculated. Additionally, frequencies of in-frame and out-of-frame rearrangements in the rearranged TCR $\delta 7$ genes were also obtained based on the sequencing. Sequence analysis was conducted using National Center for Biotechnology Information BLAST, ExpAsy, and ClustalW.

Computation analysis of TRAV13 ($V\delta 7$) family members of TCR $\alpha\delta$ loci

Genomic DNA sequences of TCR $\alpha\delta$ loci of both B6 and 129 strains are available in the Mouse Genomic Database (<http://blast.ncbi.nlm.nih.gov>). The call number for the TCR $\alpha\delta$ sequences of B6 strain is NT_039606 and for 129 is NT_039614. To identify all the members of the $V\delta 7$ (TRAV13) family on TCR $\alpha\delta$ loci of B6 and 129 strains, a sequence probe corresponding to the coding sequence of the B6- $V\delta 7$ gene segment was used to blast the genomic sequences of both strains. The hits were aligned to each other for comparison of coding and recombination signal sequences with ClustalW, ExpAsy, and the National Center for Biotechnology Information sequence alignment programs.

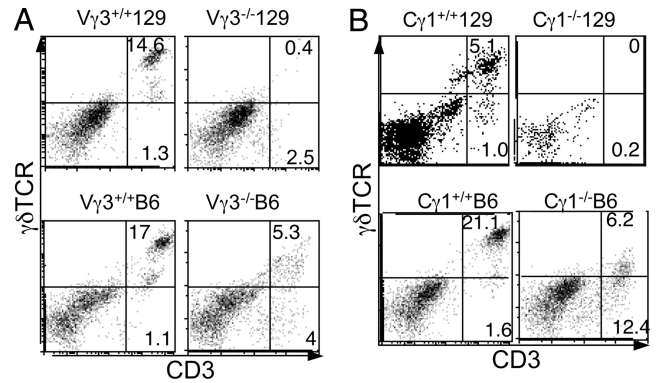


FIGURE 1. Different developmental potentials of skin $\gamma\delta$ T cells in $V\gamma 3^{-/-}$ or $C\gamma 1^{-/-}$ mice of 129 vs B6 background. Epidermal cells were prepared from the skin of $V\gamma 3$ knockout (A) or 234JC $\gamma 1$ ($C\gamma 1$) knockout mice (B) of 129 and B6 background and analyzed by flow cytometry for CD3 and $\gamma\delta$ TCR expression. The data shown in this figure, as well as in the other figures, are representatives of at least two independent experiments with similar results.

Construction of recombinant substrates and cell-based in vitro recombination assay

Recombinant substrates that contain nucleotide sequences corresponding to B6- $V\delta 7$ RSS and 129- $V\delta 7$ RSS, along with one of 5' $D\delta 2$, 5' $D\delta 1$, J $\alpha 48$, and J $\alpha 56$ RSSs, were constructed from a pCMV-based competitive recombinant substrate (35). The basic structure of the competitive recombinant substrate is same as the original construct except that the original RSSs were replaced with RSSs of the two competing $V\delta 7$ and $D\delta 2$ (or $D\delta 1$, J $\alpha 48$, J $\alpha 56$) genes, each with six nucleotides of their respective endogenous coding flank. There are several nucleotide variations in $V\delta 7$ -RSSs of B6 and 129 strains while RSSs of $D\delta 2$, $D\delta 1$, J $\alpha 48$, and J $\alpha 56$ genes of the two strains are identical (refer to Results for details). All of the substrates were sequence-confirmed before being used in a cell-based in vitro recombination assay.

The cell-based recombination assay was also performed as previously reported (35). Briefly, HEK293T cells were transiently transfected with the recombinant substrate and Rag1- and Rag2-expressing constructs. Two to 3 days after the transfection, plasmid DNAs were recovered from the transfected cells and used in a PCR reaction to detect rearranged $V\delta 7$ genes. The two primers used in the PCR are complementary to common sequences of the pCMV vector that flank the recombinant substrates (35). The PCR products were run on 2% agarose gel, and intensities of the bands corresponding to rearrangements of the two competing $V\delta 7$ RSSs were compared.

Results

Differential development of skin $\gamma\delta$ T cells in $V\gamma 3$ or $C\gamma 1$ knockout mice of the B6 vs 129 genetic background

To determine whether genetic background affects the development of substitute DETCs in mice deficient of the native skin-specific $V\gamma 3^{+}$ $\gamma\delta$ T cells, we backcrossed $V\gamma 3^{-/-}$ mice onto the C57BL/6 (B6) and 129 strain background and compared the development of their skin $\gamma\delta$ T cells. Similarly, we compared the development of skin $\gamma\delta$ T cells in $C\gamma 1^{-/-}$ mice of the B6 vs 129 background. Wild-type littermates of both the B6 and 129 backgrounds contained abundant $\gamma\delta$ TCR⁺ DETCs, as expected (Fig. 1). However, the development of the skin $\gamma\delta$ T cells was strikingly different in the $V\gamma 3^{-/-}$ (or $C\gamma 1^{-/-}$) mice of B6 and 129 backgrounds. Compared with those of their respective wild-type littermates, skin $\gamma\delta$ T cells were nearly absent (>30-fold reduction) in the $V\gamma 3^{-/-}$ (or $C\gamma 1^{-/-}$) mice of the 129 background (Fig. 1, top panels). In contrast, skin $\gamma\delta$ T cells were readily detectable in $V\gamma 3^{-/-}$ (or $C\gamma 1^{-/-}$) mice of the B6 background, whose numbers were only reduced 3- to 4-fold compared with their wild-type littermate controls (Fig. 1, bottom panels). Hence, substitute DETCs developed

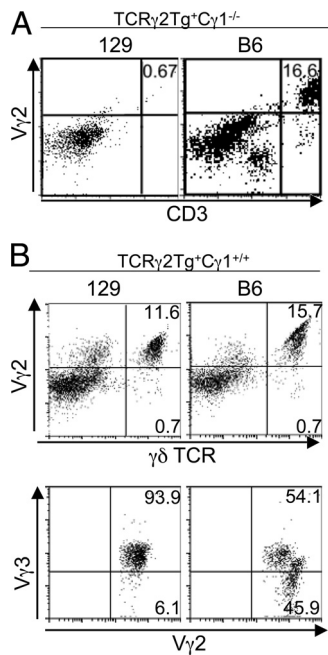


FIGURE 2. Differential development of transgenic V γ 2⁺ skin $\gamma\delta$ T cells in TCR γ 2 transgenic mice of the 129 vs B6 background. *A*, Development of transgenic V γ 2⁺ skin $\gamma\delta$ T cells is efficient in TCR γ 2Tg⁺C γ 1^{-/-} mice of B6 background but defective in those of 129 background. Epidermal cells were prepared from TCR γ 2Tg⁺C γ 1^{-/-} mice of the indicated backgrounds and assessed for percentages of the transgenic V γ 2⁺ $\gamma\delta$ T cells by flow cytometric analysis of anti-TCR δ and V γ 2 Ab staining. *B*, The V γ 2⁺V γ 3⁻ skin $\gamma\delta$ cells developed only in TCR γ 2Tg mice of B6 background while the V γ 2⁺V γ 3⁺ skin $\gamma\delta$ T cells developed in TCR γ 2Tg mice of both B6 and 129 backgrounds. Epidermal cell preparations were stained and analyzed by flow cytometry for V γ 2⁺ and total $\gamma\delta$ T cells (*top panels*). The V γ 2⁺ $\gamma\delta$ T cells were further gated for analysis of V γ 3 expression (*bottom panels*).

efficiently in V γ 3^{-/-} (or C γ 1^{-/-}) mice of the B6 but not 129 strain background, suggesting that the strain genetic background is an important factor in determining development of the skin-specific $\gamma\delta$ T cells.

Differential development of transgenic V γ 2⁺ skin $\gamma\delta$ T cells in mice of the B6 vs 129 background

Published results found that the substitute DETCs in V γ 3 knockout mice were somewhat heterogeneous populations (31). We reasoned that efforts to dissect the role of background genes in the development of substitute DETCs would be facilitated by simplifying the repertoire of these cells. Since the transgenic V γ 2⁺ $\gamma\delta$ T cells could develop into DETCs that predominantly coexpressed the endogenous TCR δ 7 gene in a mixed B6 \times 129 background (28), we compared development of the transgenic V γ 2⁺ DETCs in TCR γ 2Tg⁺C γ 1^{-/-} mice of the B6 and 129 background (TCR γ 2Tg⁺C γ 1^{-/-}B6 or TCR γ 2Tg⁺C γ 1^{-/-}129). The transgenic V γ 2⁺ DETCs developed efficiently in the TCR γ 2Tg⁺C γ 1^{-/-} mice of the B6 background but not in those of the 129 background (Fig. 2*A*). Thus, the strain background also affected development of the transgenic V γ 2⁺ DETCs.

Analysis of TCR γ 2Tg⁺C γ 1^{+/+} mice of the B6 or 129 background that retain the endogenous TCR γ locus provided additional information. Unlike in the TCR γ 2Tg⁺C γ 1^{-/-}129 mice, abundant V γ 2⁺ DETCs developed in the TCR γ 2Tg⁺C γ 1^{+/+} mice of the 129 background, which coexpressed the endogenous V γ 3 (Fig. 2*B*, *left panels*), suggesting that their development was dependent on

coexpression of the endogenous V γ 3, reminiscent of a previous report that the development of transgenic TCR δ 6.3⁺ DETCs depended on coexpression of endogenous TCR δ genes (36). In contrast, many V γ 2⁺ DETCs in TCR γ 2Tg⁺C γ 1^{+/+} mice of the B6 background did not coexpress the V γ 3, which could be also distinguished from the V γ 2⁺V γ 3⁺ DETC subset by their higher levels of V γ 2 staining (Fig. 2*B*, *right panels*). These data demonstrate that the V γ 3 expression is critical for the DETC development in the 129 strain background, but the transgenic V γ 2⁺ $\gamma\delta$ T cells can substitute V γ 3 cells for the DETC development in the B6 background.

Defective development of the transgenic V γ 2⁺ skin $\gamma\delta$ T cells on the 129 background results from absence of their positively selected precursors in the fetal thymus

Since DETCs originate from their precursor cells in the fetal thymus, which are positively selected to express the chemokine receptors, such as CCR10, and cytokine receptors, such as CD122, that promote their epidermal localization and proliferation (28), we analyzed selection status of the transgenic V γ 2⁺ fetal thymic cells of the B6 and 129 backgrounds, using the CD122 up-regulation as a positive selection marker (28).

In the TCR γ 2Tg⁺C γ 1^{-/-}129 mice, there was essentially no CD122 up-regulation on the transgenic V γ 2⁺ fetal thymic T cells (Fig. 3*A*, *left panel*), consistent with their absence in the skin (Fig. 2*A*). In contrast, CD122 was up-regulated in a significant fraction of the transgenic V γ 2⁺ fetal thymocytes of the TCR γ 2Tg⁺C γ 1^{-/-}B6 mice (Fig. 3*A*, *right panel*), in which the transgenic V γ 2⁺ DETCs developed (Fig. 2*A*).

Analysis of the TCR γ 2Tg⁺C γ 1^{+/+} mice further confirmed the correlation between positive selection of the specific subsets of transgenic V γ 2⁺ fetal thymic T cells and their development into DETCs. Although CD122 was up-regulated on significant percentages of V γ 2⁺ fetal thymic $\gamma\delta$ T cells in the TCR γ 2Tg⁺C γ 1^{+/+} mice of both B6 and 129 backgrounds, nearly all of the CD122⁺ fetal thymic V γ 2⁺ cells of the 129 background had intermediate levels of V γ 2 expression and coexpressed V γ 3 (Fig. 3*B*, *left panels*, population B), consistent with development of the V γ 2⁺V γ 3⁺ DETCs in these mice (Fig. 2*B*). On the other hand, in the TCR γ 2Tg⁺C γ 1^{+/+}B6 mice, besides the CD122⁺ V γ 2^{medium}⁺V γ 3⁺ fetal thymic T cell population, there was an additional CD122⁺ V γ 2^{high}⁺V γ 3⁻ population (Fig. 3*B*, *right panels*, population C), consistent with the development of such a subset of DETCs in these mice (Fig. 2*B*). The fact that the V γ 2⁺V γ 3⁺ but not V γ 2⁺V γ 3⁻ fetal thymic population underwent the positive selection and developed into DETCs in the TCR γ 2Tg⁺129 mice indicates that the defective DETC development of the latter is not likely due to any defective signaling process downstream of the TCR-mediated positive selection of the fetal thymic DETC precursors. Instead, these data suggest that the defect is in generation of the V γ 2⁺V γ 3⁻ DETC precursors or/and molecules that mediate their selection.

Mice of the 129 background have an intrinsic defect in the rearrangement of TCR δ 7 genes that were expressed in the transgenic V γ 2⁺ skin $\gamma\delta$ T cells of the B6 background

Since the transgenic V γ 2⁺ (V γ 3⁻) skin $\gamma\delta$ T cells in the TCR γ 2Tg⁺C γ 1^{-/-} mice on a mixed B6 \times 129 background predominantly expressed an endogenous TCR δ 7 gene and originated from their positively selected fetal thymic precursors (28), we decided to test the possibility that the differential development of these cells in the B6 and 129 background mice is due to differential generation or selection of the fetal thymic V γ 2/V δ 7⁺ $\gamma\delta$ T cells between the two backgrounds. First, we confirmed association of TCR δ 7 expression with the V γ 2⁺V γ 3⁻ DETCs in the B6 background

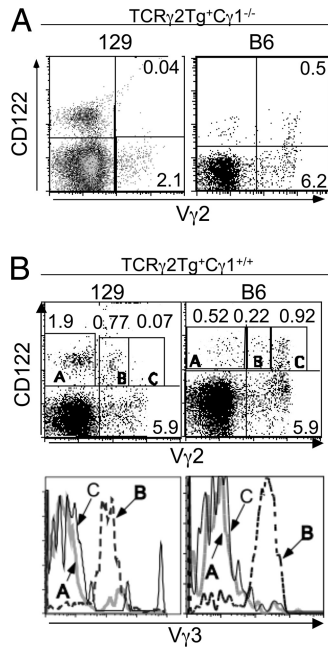


FIGURE 3. Different positive selection processes of the transgenic $V\gamma 2^+$ $\gamma\delta$ T cells in fetal thymus of 129 vs B6 background. *A*, Presence of positively selected transgenic $V\gamma 2^+$ $\gamma\delta$ T cells in the $TCR\gamma 2Tg^+C\gamma 1^{-/-}$ mice of the B6 but not 129 background. Embryonic day 16 fetal thymocytes of the $TCR\gamma 2Tg^+C\gamma 1^{-/-}$ mice of the 129 or B6 background were analyzed by flow cytometry for expression of CD122 on the transgenic $V\gamma 2^+$ $\gamma\delta$ T cells. *B*, The positively selected $CD122^+V\gamma 2^{high+}V\gamma 3^-$ fetal thymic T cells were detected only in the $TCR\gamma 2Tg$ mice of the B6 background while the $V\gamma 2^{medium+}V\gamma 3^+$ fetal thymic T cells were positively selected in the $TCR\gamma 2Tg$ mice of both B6 and 129 backgrounds. Embryonic day 16 fetal thymocytes of the $TCR\gamma 2Tg$ mice of both backgrounds were analyzed by flow cytometry for expression of $V\gamma 2$, $V\gamma 3$, and CD122. The histograms in the *top panels* were gated on total thymocytes, while the histograms in the *bottom panels* were gated on the $CD122^+V\gamma 2^-$ (A), $CD122^+V\gamma 2^{medium+}$ (B), or $CD122^+V\gamma 2^{high+}$ (C) population of the *top panels* (as indicated) and analyzed for $V\gamma 3$ TCR expression. Note that only the $CD122^+V\gamma 2^{medium+}$ population coexpressed $V\gamma 3^+$ TCR. The $CD122^+V\gamma 2^-$ population (A) was mostly NK cells.

mice by analyzing $TCR\delta$ gene usage in sorted skin $\gamma\delta$ T cell subsets from $TCR\gamma 2Tg^+C\gamma 1^{+/+}$ mice of the B6 or 129 background (Fig. 4A). The $V\gamma 2^+V\gamma 3^+$ skin $\gamma\delta$ T cells of both backgrounds expressed same levels of $TCR\delta 1$ transcripts as did the $V\gamma 3^+$ DETCs of wild-type B6 mice but rarely any other $V\delta$ transcripts (Fig. 4A), suggesting that the development of this DETC subset is mediated by the endogenous $V\gamma 3/V\delta 1^+$ TCR even though the transgenic $TCR\gamma 2$ was coexpressed. In contrast, the $V\gamma 2^+V\gamma 3^-$ DETC subset of the B6 background had at least a 20-fold reduction in $TCR\delta 1$ transcripts but predominantly expressed $TCR\delta 7$ (Fig. 4A). These data confirm that the $V\gamma 2/V\delta 7^+$ $\gamma\delta$ T cell population is selected for development into skin $\gamma\delta$ T cells in the B6 background mice and are consistent with the idea that absence of such a population in the 129 background mice is due to defective $TCR\delta 7$ rearrangement/expression or failed positive selection of the $V\gamma 2/V\delta 7^+$ population if they are ever generated.

To distinguish these two possibilities, we compared levels of $TCR\delta 7$ rearrangements in fetal thymus of the $TCR\gamma 2Tg^+$ mice of 129 and B6 backgrounds by semiquantitative PCR. The $V\delta 7$ gene belongs to a closely related family of $V\alpha/\delta$ genes (TRAV13 family according to the international ImMunoGeneTics database nomenclature), of which only $V\delta 7$ (also designated TRAV13-4)

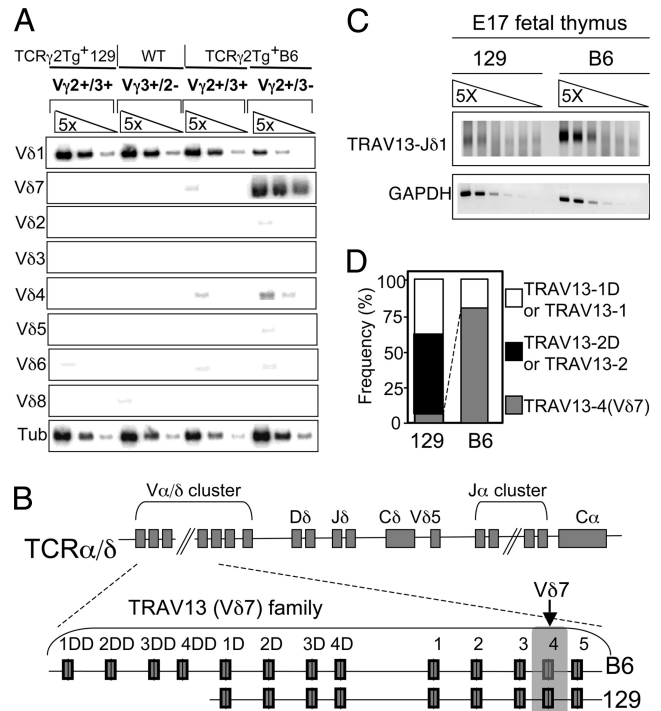


FIGURE 4. Intrinsically defective rearrangement of $TCR\delta 7$ gene in mice of the 129 background. *A*, $V\delta 7^+$ $TCR\delta$ gene is predominantly expressed only in the transgenic $V\gamma 2^+V\gamma 3^-$ skin $\gamma\delta$ T cells of the $TCR\gamma 2Tg^+B6$ mice, while the $V\delta 1^+$ $TCR\delta$ gene is predominantly expressed in the $V\gamma 2^+V\gamma 3^+$ skin $\gamma\delta$ T cells of both B6 and 129 backgrounds. RNA was prepared from sorted populations of skin $\gamma\delta$ T cells with different $V\gamma 2$ and $V\gamma 3$ expression of the indicated mouse strains and analyzed for $V\delta$ gene usage by semiquantitative RT-PCR. RT-PCR for β -tubulin (tub) was used as loading controls. *B*, Genomic organization of TRAV13 ($V\delta 7$) family members in the $TCR\alpha/\delta$ loci of the B6 vs 129 strain. The $V\delta 7$ gene is the second from the right in the family, as indicated. *C*, Comparison of $TCR\delta$ gene rearrangements of the TRAV13 ($V\delta 7$) family members in the $TCR\gamma 2Tg$ mice of the 129 and B6 background. The genomic DNAs of embryonic day 17 fetal thymocytes were serially diluted and analyzed by semiquantitative PCR. GAPDH was used as loading controls. *D*, 129- $V\delta 7$ was rarely used in $TCR\delta$ rearrangements of the TRAV13 family members, while the B6- $V\delta 7$ gene was predominantly used. Subcloned PCR products of 129 or B6 genomic DNAs in *C* were sequenced and frequencies of the $TCR\delta$ rearrangements of $V\delta 7$ genes and the other family members were plotted.

is known to rearrange predominantly to $D\delta$ - $J\delta$ gene segments for the $TCR\delta$ assembly in adult wild-type B6 mice, while all the other family members are preferentially used for the $TCR\alpha$ rearrangements (14, 22). The TRAV13 family consists of five founding members, located at the 3' region of the $V\alpha/\delta$ gene cluster, four of which were duplicated once in the 129 strain and twice in the B6 strain (Fig. 4B, assembled based on mouse genomic database, refer to *Materials and Methods*; nucleotide sequences of these family members are also compiled in an online database of <http://imgt.cines.fr>). The $V\delta 7$ gene is the second to last member of the founding group and is therefore the second most proximal of the entire family in position to the $D\delta/J\delta$ (Fig. 4B). The coding sequence of the B6- $V\delta 7$ gene is 98% identical to that of the 129- $V\delta 7$ gene, and all the family members are at least 81% identical to each other in both strains (supplemental Fig. 1).⁵ We used a forward primer complementary to all members of the TRAV13 family (TRAV13 primer) and a common reverse $J\delta 1$ primer for the PCR, which

⁵ The online version of this article contains supplemental material.

Table I. Sequence alignment of recombination signal sequences of V δ 7 and the other TRAV13 family members of B6 and 129 strains

Strain	TRAV13-	Heptamer	Spacer ^a	Nonamer
B6	1DD	CACAGTG	CT CCCCACACACCTGCAGCC CGC	ACACA AA ACC
	2DD	CACAGTG	CT CCCCACACACCTGCAGCCT GT	ACACA AA ACC
	3DD	CACAGTG	CT CCCCACACACCTGCAGCC CAA	ACACA AA ACC
	4DD	CACAGTG	CT CCCCACACAC CA GCAGCC CGC	ACACA AA ACC
	1D	CACAGTG	CT CCCCACACACCTGCAGCC CGC	ACACA AA ACC
	2D	CACAGTG	CT CCCCACACACCTGCAGCCT GT	ACACA AA ACC
	3D	CACAGTG	CT CCCCACACACCTGCAGCC CGA	ACACA AA ACC
	4D	CACAGTG	CT CCCCACACACCTGCAGCC CGC	ACACA AA ACC
	1	CACAGTG	CT CCACACACACCTGCAGCC CGA	ACACA AA ACC
	2	CACAGTG	CT CCCCACACACCTGCAGCCT GT	ACACA AA ACC
	3	CACAGTG	CT CCCCACACACCTGCAGCC CGA	ACACA AA ACC
	5	CACAGTG	CT CCCCAGGCACCTGCAGCCT GT	ACACA AA ACC
	4 (V δ 7)	CACAGTG	ATCCCCACACACCTGCAGCCTGC	ACATA AA ACC
	129	4 ("V δ 7")	CACAGTG	CT CCCCACACAC CA GCAGCC CGC
1D		CACAGTG	CT CCCCACACACCTGCAGCC CGC	ACACA AA ACC
2D		CACAGTG	CT CCCCACACACCTGCAGCCT GT	ACACA AA ACC
3D		CACAGTG	CT CCCCACACACCTGCAGCC CGC	ACACA AA ACC
4D		CACAGTG	CT CCCCACACACCTGCAGCC CGC	ACACA AA ACC
1		CACAGTG	CT CC A CACACACCTGCAGCC CGC	ACACA T ACC
2		CACAGTG	CT CCCCACACACCTGCAGCCT GT	ACACA AA ACC
3		CACAGTG	CT CCCCACACACCTGCAGCC CGA	ACACA AA ACC
5		CACAGTG	CT CCCC AGG CACCTGCAGCCT GT	ACACA AA ACC

^a Boldface letters represent nucleotides that differ from those of the B6-V δ 7 RSS.

would amplify rearrangements of all the TRAV13 family members, including V δ 7. The rearrangements to J δ 1 were assessed because the V γ 2/V δ 7⁺ DETCs expressed TCR δ of V δ 7-D δ 2-J δ 1 rearrangements (28). Remarkably, the TRAV13-J δ 1 rearrangements were ~25-fold less abundant in the 129 background mice than in the B6 (Fig. 4C), suggesting a severe defect in the TCR δ gene rearrangement of TRAV13 family members, most likely of the V δ 7, in the fetal thymi of 129 background mice.

There was a possibility that the difference in TRAV13-J δ 1 rearrangements between the B6 and 129 strains was due not to their differential rearrangement efficiencies, but was rather due to differences in the positive selection of the transgenic V γ 2⁺ fetal thymic cells expressing the rearranged TCR δ 7. If this is the case, the higher level of rearrangements in the B6 background mice should result from the positive selection-mediated enrichment of the V γ 2/V δ 7⁺ population, and, consequently, most of the rearranged TCR δ 7 genes should be in frame and productive. On the other hand, if the positive selection does not result in the enrichment of the V γ 2/V δ 7⁺ fetal thymic population, it is predicted that only a third of the rearrangements should be in frame. Therefore, we sequenced subcloned PCR products of the TRAV13-J δ 1 rearrangements to determine frequencies of in-frame vs out-of-frame rearrangements. The sequencing also allowed us to determine percentages of the rearrangements of V δ 7 gene vs the other members of the TRAV13 family. Based on the sequencing, a vast majority of the rearrangements, ~83% (10 out of 12 subclones), of the B6 genomic DNA were of V δ 7 (Fig. 4D, right column), demonstrating that V δ 7 is the only member of the TRAV13 family that predominantly rearranges for TCR δ genes in the fetal thymus. Importantly, 70% (7 out of 10) of the V δ 7-J δ 1 rearrangements were out of frame, arguing strongly against the possibility that the higher level of V δ 7 rearrangements in B6 fetal thymocytes is due to the positive selection-mediated enrichment of cells bearing this receptor chain. Instead, the data suggest that the V δ 7 rearrangement efficiency for TCR δ gene assembly in the 129 fetal thymocytes is intrinsically lower than in the B6 fetal thymocytes. Confirming this, we sequenced subcloned PCR products of TRAV13-J δ 1 rearrangements of the 129 background mice and found that only 1

out of 24 subclones (4%) of the rearrangements was of the 129-V δ 7 gene (Fig. 4D, left panel). Taken together, these data demonstrate that the absence of V γ 2/V δ 7⁺ DETCs in the 129 background mice is due to an intrinsic defect in the rearrangement of TCR δ 7 genes and, consequently, the failed generation of such a $\gamma\delta$ T cell population in the fetal thymus.

Association of variations in recombination signal sequences of V δ 7 genes of the B6 and 129 strains with their different preferences for TCR δ rearrangement

The impaired TCR δ rearrangement of the V δ 7 gene in the 129 background mice is likely due to local V δ 7 gene variations but not global TCR δ gene rearrangement deficiency, since percentages of total transgenic V γ 2⁺ fetal thymocytes were not significantly different in the TCR γ 2Tg⁺ mice of 129 and B6 backgrounds (Fig. 3). Therefore, we compared genomic sequences of the V δ 7 genes of 129 and B6 strains for variations. Interestingly, there were significant differences in their RSSs, which became very striking when aligned with RSSs of other members of the TRAV13 family of both strains (Table I). Compared with those of the 129-V δ 7 and all the other TRAV13 family members, the RSS of the B6-V δ 7 is remarkably unique. Specifically, the first nucleotide of the 23-bp spacer in the B6-V δ 7 RSS is an adenine, whereas the corresponding site of the 129-V δ 7 and other family members is a cytosine. Additionally, the fourth nucleotide in the nonamer of the B6-V δ 7 RSS is a thymine, while the corresponding site is a cytosine in all of the others. The RSS of the 129-V δ 7 gene is more similar to those of the other TRAV13 family members than that of the B6-V δ 7. In contrast to the significant variations in their V δ 7 RSSs, there were no differences at all in RSSs of D δ and J δ gene segments between the 129 and B6 strains (supplemental Fig. 2). Since the recombination signal nonamer and spacer have been reported to be able to mediate restriction on the TCR β gene rearrangement (37), the variations in the 129-V δ 7 RSS might be responsible for its impaired TCR δ rearrangement.

To test this, we used a cell-based in vitro recombination assay to directly compare rearrangement efficiencies of the 129-V δ 7 RSS and B6-V δ 7 RSS to D δ 2 gene segment, which was used in

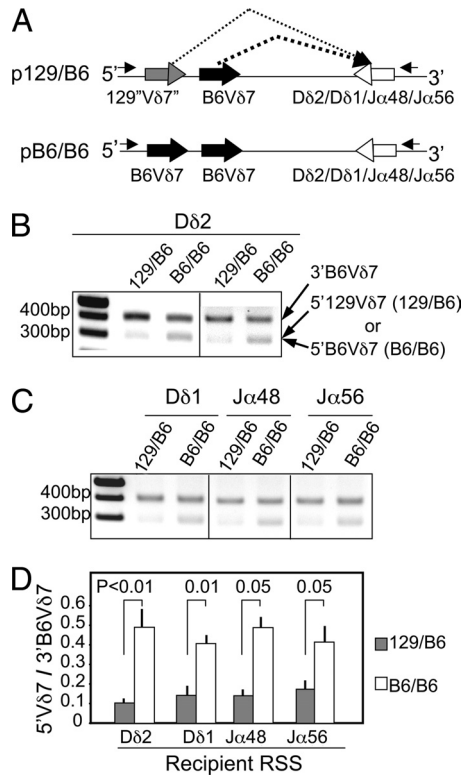


FIGURE 5. Comparison of rearrangement efficiencies of B6-V δ 7 RSS vs 129-V δ 7 RSS in an in vitro recombination assay. *A*, Schematic depiction of competitive recombination substrate constructs. Two V δ 7 RSSs were assembled in the same construct with a recipient RSS of D δ 2, D δ 1, J α 48, or J α 56 gene and would compete for rearrangement to the recipient RSS when the construct was transfected into cells expressing Rag1/2 genes. Rearrangements of the two competing V δ 7 RSSs were assessed by PCR with primers located in 5' and 3' flanking regions of the substrate (indicated by the two small arrows), which gave different sizes of products. *B*, Analysis of rearrangements of the two competing V δ 7 RSSs to the D δ 2 RSS. Plasmid DNAs recovered from recombinant substrate-transfected cells were subject to the PCR as depicted in *A* and gel analyzed. Bands of 400 bp correspond to rearrangements of the 3'B6-V δ 7, while those of 300 bp correspond to rearrangements of the 5' 129-V δ 7 (129/B6) or 5' B6-V δ 7 (B6/B6). *C*, Analysis of rearrangements of two competing V δ 7 RSSs to the recipient RSS of D δ 1, J α 48, or J α 56 gene, similar as in the *B*. *D*, Quantification of relative rearrangement efficiencies of the 129-V δ 7 RSS vs B6-V δ 7 RSS of the same position to the D δ 2, D δ 1, J α 48, or J α 56 RSS based on analyses of the *B* and *C*. The efficiency was expressed as a ratio of intensities of the 5'V δ 7 (129 or B6) vs the common 3'B6V δ 7 rearrangement bands.

all TCR δ 7 rearrangements of the transgenic V γ 2/V δ 7⁺ skin γ δ T cells (28). To this purpose, we assembled a competitive recombinant substrate (p129/B6) that contains the 129-V δ 7 RSS, B6-V δ 7 RSS, and the 5'RSS of D δ 2 gene, subcloned into a pCMV-based vector (Fig. 5*A*, top panel) (35). To correct any potential effect of the position on rearrangement efficiencies of the two competing V δ 7 genes, a recombinant substrate containing two identical B6-V δ 7 RSSs (pB6/B6) were used as a control (Fig. 5*A*, bottom panel). The substrates, along with Rag1- and Rag2-expressing vectors, were transiently transfected into 293 cells, and rearrangements of the two competing V δ 7 RSSs to D δ 2 were assessed by PCR with a primer set located outside the recombinant substrates, which distinguish rearrangements of the two competing V δ 7 RSSs based on their different sizes (Fig. 5, *A* and *B*).

In cells transfected with substrates containing the competing 129 and B6-V δ 7 RSSs, the distal 5' 129-V δ 7 RSS rearranged much less efficiently (\sim 10-fold) to the D δ 2 RSS than did the proximal 3'B6-V δ 7 RSS (Fig. 5, *B* and *D*). In contrast, in cells transfected with substrates containing the two identical B6-V δ 7 RSSs, the distal B6-V δ 7 RSS rearranged to the D δ 2 RSS only slightly less efficiently (\sim 2-fold) than did the proximal B6-V δ 7 RSS (Fig. 5, *B* and *D*), likely due to a minor position effect that was observed previously (37) and reportedly affected by distances between different RSSs (38), among other factors. Normalized on rearrangements of the common proximal B6-V δ 7 RSS to factor in the position effect, the 129-V δ 7 RSS still rearranged significantly less efficiently than did the B6-V δ 7 RSS of same distal position (4.7 ± 1.3 -fold reduction, $n = 3$), demonstrating that the variations in 129-V δ 7 RSS impair its rearrangement efficiency for TCR δ gene assembly.

To test further whether the reduced rearrangement efficiency of 129-V δ 7 RSS is restricted to the D δ 2 RSS, we also constructed competitive recombinant substrates containing the two competing V δ 7-RSSs with an RSS of D δ 1, J α 48, or J α 56 gene fragment (Fig. 5*A*). The D δ 1, J α 48, or J α 56 RSSs are identical between 129 and B6 strains (supplemental Fig. 2). As for the rearrangement to D δ 2 RSS, the 129-V δ 7 RSS also rearranged less efficiently than did the B6-V δ 7 RSS of same position to the D δ 1 RSS (3.0 ± 0.7 , $n = 2$) (Fig. 5, *C* and *D*). Additionally, the variations also rendered the 129-V δ 7 RSS less compatible for rearrangement to the tested J α RSSs (3.5 ± 0.7 - and 2.4 ± 0.2 -fold reduction to J α 48 and J α 56, respectively, $n = 2$ each), suggesting that the reduced TCR δ gene rearrangement of 129-V δ 7 RSS is probably not due to restriction beyond the 12/23 rule. Taken together, these results demonstrate that a dysfunctional 129-V δ 7 RSS is at least in part responsible for the defective TCR δ 7 rearrangement and transgenic V γ 2/V δ 7⁺ DETC development in 129 background mice.

Discussion

The proper rearrangement of TCR genes is critical for T cell development. Since both TCR α and TCR δ loci share the same V α / δ gene segments, unique questions with these loci are how the shared V α / δ segments are selectively used for the TCR α / δ gene assembly and its effect on the development of $\alpha\beta$ / $\gamma\delta$ T cells. Here, we report that differential TCR δ 7 gene rearrangement in fetal thymi of the B6 and 129 backgrounds profoundly affected development of a skin-specific $\gamma\delta$ T cell population. Furthermore, we found that the variations in RSSs of the V δ 7 genes of the two strains were associated with their preferential usage for the TCR δ gene assembly, demonstrating for the first time a biologically important role of the RSS-associated restriction of V α / δ rearrangement in the T cell development. Additionally, our findings provided further support for a critical role of positive selection in development of the skin-specific $\gamma\delta$ T cells.

Although it has been suggested for a long time that the selective V α / δ usage plays an important role in T cell development, the evidence is lacking. Our findings provide the first compelling evidence that the selective V δ gene usage for TCR δ rearrangement affects development of the skin-specific T cell population. However, effects of selective TCR δ gene rearrangement on the development of different tissue-specific T cell populations might vary. Unlike the skin $\gamma\delta$ T cells, development of $\gamma\delta$ T cells in secondary lymphoid organs such as spleens was not significantly different in the V γ 2 transgenic mice of the 129 and B6 backgrounds (data not shown). Possibly, innate T cell populations, such as the skin-specific $\gamma\delta$ T cells that use restricted TCR composition, are more easily affected by the selective V δ gene usage than are T cell populations with diverse TCR composition, such as the splenic $\gamma\delta$

T cells. However, even in the latter, the differential usage of V δ genes might still affect the T cell repertoire formation and possibly immune functions.

The finding that the differential rearrangement of the V δ 7 genes significantly affected the skin-specific $\gamma\delta$ T cell development also provides further support for a critical role of positive selection in the development of this tissue-specific $\gamma\delta$ T cell population. Despite the defective TCR δ 7 rearrangement, abundant transgenic V γ 2⁺ $\gamma\delta$ T cells were still generated in the TCR γ 2Tg⁺C γ 1^{-/-} 129 mice. However, they were not positively selected and did not develop into the skin $\gamma\delta$ T cells (Figs. 2 and 3), demonstrating that only specific $\gamma\delta$ T cell populations could be positively selected in the fetal thymus and develop into the skin $\gamma\delta$ T cells. Events that impair the generation of these specific $\gamma\delta$ T cells in the fetal thymus, as is the case for the V γ 2/V δ 7⁺ $\gamma\delta$ T cells in the TCR γ 2Tg⁺C γ 1^{-/-} 129 mice, will therefore impair the skin-specific $\gamma\delta$ T cell development.

Cellular selection has been also suggested to explain relatively abundant V γ 2/V δ 7⁺ $\gamma\delta$ T cells in spleens of the adult B6 than in BALB/c mice (39). However, the difference in thymic TCR δ 7 gene expression between B6 and BALB/c mice (<2-fold) is much smaller than that of the TCR δ 7 rearrangements between B6 and 129 mice, suggesting that different underlying mechanisms might operate in these two settings. Likely, the difference in the splenic V γ 2/V δ 7⁺ $\gamma\delta$ T cells between the B6 and BALB/c mice was resulting from the postthymic selection events, while the impaired TCR δ 7 rearrangement and generation of the corresponding V δ 7⁺ $\gamma\delta$ T cells in the fetal thymus are predominantly responsible for the failed development of skin V γ 2/V δ 7⁺ $\gamma\delta$ T cells in the 129 background mice.

Interestingly, the impaired rearrangement of TCR δ 7 genes in the 129 background mice is associated with the variations in recombination signal sequences of the V δ 7 gene segments, suggesting a role of the RSS in their restricted usage for TCR δ gene rearrangement. Supporting this, an in vitro rearrangement assay demonstrated directly that the 129-V δ 7 RSS rearranges to the RSS of the D δ 2 segment much less efficiently than does the B6-V δ 7 RSS. This also suggests a possibility that the variations in the B6-V δ 7 RSS may represent a nucleotide motif in restricting the variable gene usage for TCR δ assembly. However, such variations were not found in many other known V δ genes (data not shown). In fact, the B6-V δ 7 RSS, but not the 129-V δ 7 RSS, contains a palindrome sequence (CTGCAG) in its 23-bp spacer that was previously found in 80% of V α gene segments (27), suggesting that no common V δ or V α motif exists in determining their preferential TCR α/δ rearrangements. Consistent with this, there was no apparent correlation between reported TCR α/δ rearrangement preferences and RSSs among members of several other V α/δ gene families (data not shown).

In contrast to the stringent restriction imposed by RSSs on V β and J β genes to prevent the direct V β -J β rearrangement, the selective usage of V α/δ genes for the TCR α and TCR δ gene rearrangement is relatively loose. Although there are apparent preferences in the usage of share V α/δ segments for TCR α and TCR δ rearrangement, many of the V α/δ genes could be used for both (22), suggesting compatibility of their RSSs with those of both D δ /J δ and J α segments. However, such promiscuity might make them less fully compatible to a specific type of RSSs and more easily affected by individual variations, which could alter rearrangement preferences of specific V α/δ genes and affect the development of T cells, especially those that use limited TCR composition, as we report herein. Therefore, the variations in RSSs of the V α/δ gene segments might represent a major factor in determining $\gamma\delta$ and $\alpha\beta$ T repertoire formation among individuals. Par-

alleling this, RSS variations were also reported to affect rearrangement efficiencies in Ig gene loci. In one report, it was found that a single base pair change in the spacers or nonamers could affect recombination frequency of a V_H gene with significant biological consequences (40).

Our data demonstrate that the variations in the 129-V δ 7 RSS affect its rearrangement efficiencies for TCR δ gene assembly, which likely contributes to the defective TCR δ 7 rearrangements and skin $\gamma\delta$ T cell development of the 129 background mice. However, the exact contribution of the RSS variations in these processes remains to be assessed in vivo, by using techniques such as the knocking-in of the B6-V δ 7 RSS into the 129 allele or vice versa. Additionally, its effect is superimposed on other regulation mechanisms, such as the gene accessibility, which was known to be a prerequisite for selective usage of V α/δ genes in the TCR α and TCR δ rearrangement at different developmental stages (23). It is possible that multiple factors operate to regulate the differential V δ 7 gene rearrangement in the different genetic backgrounds.

Acknowledgments

We thank Adrian Hayday for the V γ 3 knockout mice, Ferenc Livak for plasmids and advice on the in vitro recombination assay, Hector Nolla for assistance in cell sorting, and Christina Saylor for technical support.

Disclosures

The authors have no financial conflicts of interest.

References

- Raulet, D., D. Spencer, Y.-H. Hsiang, J. Goldman, M. Bix, N.-S. Liao, M. Zijlstra, R. Jaenisch, and I. Correa. 1991. Control of $\gamma\delta$ T cell development. *Immunol. Rev.* 120: 185–204.
- Xiong, N., and D. H. Raulet. 2007. Development and selection of $\gamma\delta$ T cells. *Immunol. Rev.* 215: 15–31.
- Girardi, M., D. E. Oppenheim, C. R. Steele, J. M. Lewis, E. Glusac, R. Filler, P. Hobby, B. Sutton, R. E. Tigelaar, and A. C. Hayday. 2001. Regulation of cutaneous malignancy by $\gamma\delta$ T cells. *Science* 294: 605–609.
- Jameson, J., K. Ugarte, N. Chen, P. Yachi, E. Fuchs, R. Boismenu, and W. L. Havran. 2002. A role for skin $\gamma\delta$ T cells in wound repair. *Science* 296: 747–749.
- Hayday, A., and R. Tigelaar. 2003. Immunoregulation in the tissues by $\gamma\delta$ T cells. *Nat. Rev. Immunol.* 3: 233–242.
- Havran, W. L., and J. P. Allison. 1990. Origin of Thy-1⁺ dendritic epidermal cells of adult mice from fetal thymic precursors. *Nature* 344: 68–70.
- Goldman, J., D. Spencer, and D. Raulet. 1993. Ordered rearrangement of variable region genes of the T cell receptor γ locus correlates with transcription of the unrearranged genes. *J. Exp. Med.* 177: 729–739.
- Itoharu, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson, A. R. Clarke, M. L. Hooper, A. Farr, and S. Tonegawa. 1993. T cell receptor δ gene mutant mice: independent generation of $\alpha\beta$ T cells and programmed rearrangements of $\gamma\delta$ TCR genes. *Cell* 72: 337–348.
- Baker, J. E., D. Cado, and D. H. Raulet. 1998. Developmentally programmed rearrangement of T cell receptor V γ genes is controlled by sequences immediately upstream of the V γ genes. *Immunity* 9: 159–168.
- Xiong, N., L. Zhang, C. Kang, and D. H. Raulet. 2008. Gene placement and competition control T cell receptor γ variable region gene rearrangement. *J. Exp. Med.* 205: 929–938.
- Weber-Arden, J., O. M. Wilbert, D. Kabelitz, and B. Arden. 2000. V δ repertoire during thymic ontogeny suggests three novel waves of $\gamma\delta$ TCR expression. *J. Immunol.* 164: 1002–1012.
- Krangel, M. S., J. Carabana, I. Abbarategui, R. Schlimgen, and A. Hawwari. 2004. Enforcing order within a complex locus: current perspectives on the control of V(D)J recombination at the murine T-cell receptor α/δ locus. *Immunol. Rev.* 200: 224–232.
- Gallagher, M., S. Candeias, C. Martinon, E. Borel, M. Malissen, P. N. Marche, and E. Jouvin-Marche. 1998. Use of TCR ADV gene segments by the δ chain is independent of their position and of CD3 expression. *Eur. J. Immunol.* 28: 3878–3885.
- Pereira, P., V. Hermitte, M. P. Lembezat, L. Boucontet, V. Azuara, and K. Grigoriadou. 2000. Developmentally regulated and lineage-specific rearrangement of T cell receptor V α/δ gene segments. *Eur. J. Immunol.* 30: 1988–1997.
- Abbarategui, I., and M. S. Krangel. 2006. Regulation of T cell receptor- α gene recombination by transcription. *Nat. Immunol.* 7: 1109–1115.
- Abbarategui, I., and M. S. Krangel. 2007. Noncoding transcription controls downstream promoters to regulate T-cell receptor α recombination. *EMBO J.* 26: 4380–4390.
- Agata, Y., T. Katakai, S. K. Ye, M. Sugai, H. Gonda, T. Honjo, K. Ikuta, and A. Shimizu. 2001. Histone acetylation determines the developmentally regulated

- accessibility for T cell receptor γ gene recombination. *J. Exp. Med.* 193: 873–879.
18. Xiong, N., J. E. Baker, C. Kang, and D. H. Raulet. 2004. The genomic arrangement of T cell receptor variable genes is a determinant of the developmental rearrangement pattern. *Proc. Natl. Acad. Sci. USA* 101: 260–265.
 19. Krangel, M. S., C. Hernandez-Munain, P. Lauzurica, M. McMurry, J. L. Roberts, and X. P. Zhong. 1998. Developmental regulation of V(D)J recombination at the TCR α/δ locus. *Immunol. Rev.* 165: 131–147.
 20. Monroe, R. J., B. P. Sleckman, B. C. Monroe, B. Khor, S. Claypool, R. Ferrini, L. Davidson, and F. W. Alt. 1999. Developmental regulation of TCR δ locus accessibility and expression by the TCR δ enhancer. *Immunity* 10: 503–513.
 21. Bassing, C. H., R. E. Tillman, B. B. Woodman, D. Canty, R. J. Monroe, B. P. Sleckman, and F. W. Alt. 2003. T cell receptor (TCR) α/δ locus enhancer identity and position are critical for the assembly of TCR δ and α variable region genes. *Proc. Natl. Acad. Sci. USA* 100: 2598–2603.
 22. Bosc, N., and M. P. Lefranc. 2003. The mouse (*Mus musculus*) T cell receptor α (TRA) and δ (TRD) variable genes. *Dev. Comp. Immunol.* 27: 465–497.
 23. Hawwari, A., and M. S. Krangel. 2005. Regulation of TCR δ and α repertoires by local and long-distance control of variable gene segment chromatin structure. *J. Exp. Med.* 202: 467–472.
 24. Bassing, C. H., F. W. Alt, M. M. Hughes, M. D'Auteuil, T. D. Wehrly, B. B. Woodman, F. Gärtner, J. M. White, L. Davidson, and B. P. Sleckman. 2000. Recombination signal sequences restrict chromosomal V(D)J recombination beyond the 12/23 rule. *Nature* 405: 583–586.
 25. Tillman, R. E., A. L. Wooley, M. M. Hughes, B. Khor, and B. P. Sleckman. 2004. Regulation of T-cell receptor β -chain gene assembly by recombination signals: the beyond 12/23 restriction. *Immunol. Rev.* 200: 36–43.
 26. Drejer-Teel, A. H., S. D. Fugmann, and D. G. Schatz. 2007. The beyond 12/23 restriction is imposed at the nicking and pairing steps of DNA cleavage during V(D)J recombination. *Mol. Cell. Biol.* 27: 6288–6299.
 27. Probst, J., S. G. Blumenthal, S. Tenzer, T. Weinschenk, J. Dittmer, O. Schoor, A. Six, H. G. Rammensee, and S. Pascolo. 2004. A conserved sequence in the mouse variable T cell receptor α recombination signal sequence 23-bp spacer can affect recombination. *Eur. J. Immunol.* 34: 2179–2190.
 28. Xiong, N., C. Kang, and D. H. Raulet. 2004. Positive selection of dendritic epidermal $\gamma\delta$ T cell precursors in the fetal thymus determines expression of skin-homing receptors. *Immunity* 21: 121–131.
 29. Lewis, J. M., M. Girardi, S. J. Roberts, S. D. Barbee, A. C. Hayday, and R. E. Tigelaar. 2006. Selection of the cutaneous intraepithelial $\gamma\delta^+$ T cell repertoire by a thymic stromal determinant. *Nat. Immunol.* 7: 843–850.
 30. Boyden, L. M., J. M. Lewis, S. D. Barbee, A. Bas, M. Girardi, A. C. Hayday, R. E. Tigelaar, and R. P. Lifton. 2008. Skint1, the prototype of a newly identified immunoglobulin superfamily gene cluster, positively selects epidermal $\gamma\delta$ T cells. *Nat. Genet.* 40: 656–662.
 31. Mallick-Wood, C., J. M. Lewis, L. I. Richie, M. J. Owen, R. E. Tigelaar, and A. C. Hayday. 1998. Conservation of T cell receptor conformation in epidermal $\gamma\delta$ cells with disrupted primary V γ gene usage. *Science* 279: 1729–1733.
 32. Hara, H., K. Kishihara, G. Matsuzaki, H. Takimoto, T. Tsukiyama, R. E. Tigelaar, and K. Nomoto. 2000. Development of dendritic epidermal T cells with a skewed diversity of $\gamma\delta$ TCRs in V δ 1-deficient mice. *J. Immunol.* 165: 3695–3705.
 33. Sullivan, S., P. Bergstresser, R. Tigelaar, and J. W. Streilein. 1985. FACS purification of bone marrow-derived epidermal populations in mice: Langerhans cells and Thy-1⁺ dendritic cells. *J. Invest. Derm.* 84: 491–495.
 34. Coligan, J., A. Kruisbeek, D. Margulies, E. Shevach, and W. Strober. 2008. *Current Protocols in Immunology*. Greene Publishing Associates and Wiley & Sons, New York.
 35. Oлару, A., D. N. Patterson, I. Villey, and F. Livak. 2003. DNA-Rag protein interactions in the control of selective D gene utilization in the TCR β locus. *J. Immunol.* 171: 3605–3611.
 36. Ferrero, I., A. Wilson, F. Beermann, W. Held, and H. R. MacDonald. 2001. T cell receptor specificity is critical for the development of epidermal $\gamma\delta$ T cells. *J. Exp. Med.* 194: 1473–1483.
 37. Hughes, M. M., R. E. Tillman, T. D. Wehrly, J. M. White, and B. P. Sleckman. 2003. The B12/23 restriction is critically dependent on recombination signal nonamer and spacer sequences. *J. Immunol.* 171: 6604–6610.
 38. Jung, D., C. H. Bassing, S. D. Fugmann, H. L. Cheng, D. G. Schatz, and F. W. Alt. 2003. Extrachromosomal recombination substrates recapitulate beyond 12/23 restricted VDJ recombination in nonlymphoid cells. *Immunity* 18: 65–74.
 39. Sperling, A. I., D. C. Decker, R. J. DiPaolo, D. A. Stern, A. Shum, and J. A. Bluestone. 1997. Selective expansion of V γ 2-V δ 7 TCR $\gamma\delta$ cells in C57BL/6 mice is postnatal and extrathymic. *J. Immunol.* 159: 86–91.
 40. Feeney, A. J., P. Goebel, and C. R. Espinoza. 2004. Many levels of control of V gene rearrangement frequency. *Immunol. Rev.* 200: 44–56.