TRANSGENIC MICE DEMONSTRATE THAT EPITHELIAL HOMING OF \( \gamma/\delta \) T CELLS IS DETERMINED BY CELL LINEAGES INDEPENDENT OF T CELL RECEPTOR SPECIFICITY

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The search for the genes encoding the TCR-\( \alpha/\beta \) led to the identification of the third rearranging gene, TCR-\( \gamma \) (1-3), which in turn led to the realization of the occurrence of second TCR, TCR-\( \gamma/\delta \) (4-6). One of the most intriguing features of \( \gamma/\delta \) T cells is the compartmentalization of their subsets with different TCR repertoires to various epithelia. Thus, most T cells associated with epidermis, which are referred to as dendritic epidermal cells (DEC)\(^1\) (7, 8), bear TCRs encoded by a particular pair of \( \gamma \) and \( \delta \) genes containing \( V_5\gamma \) and \( V_1\delta \) gene segments (9). Likewise, most of the long-studied intraepithelial lymphocytes (IEL) of the gut (for a review see reference 10) turned out to be composed of \( \gamma/\delta \) T cells bearing TCRs whose \( \gamma \) subunits are preferentially encoded by \( V_7 \)-containing \( \gamma \) genes (11, 12). Yet, another subset of \( \gamma/\delta \) T cells with TCRs encoded by a particular \( V_3 \) \( \gamma \) gene and a particular \( V_1 \) \( \delta \) gene is associated with the mucosal epithelia of uterus, vagina, and tongue (13).

The compartmentalization of these \( \gamma/\delta \) T cell subsets with different TCR repertoires raised the possibility that their homing specificity is determined by a compartmentalization of TCR-\( \gamma/\delta \) ligands in the various epithelia. We, therefore, examined...
the homing properties of γ/δ T cells with predetermined TCRs using the transgenic mouse technique. The results suggest that the homing specificities of these T cells do not depend on their TCR repertoires, but are determined by differentiation programs that may link the rearrangement of particular γ and δ gene segments with the expression of particular homing receptors. In addition, our analysis of the IEL-γ/δ in normal, nude, and transgenic mice using an anti-TCR-γ/δ mAb (14) suggests that Thy-1 is a differentiation marker, while CD8 is an activation marker of this T cell subset.

Materials and Methods

Animals. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Athymic nude nu/nu BALB/c mice and control nu/+ BALB/c mice were obtained from Dr. M. Katsuki (Kawasaki, Japan). γ/δ transgenic (TG) animals carrying the functionally rearranged Vγ1JγC, γVδ2JδC δ (15; Ishida, I., S. Verbeek, M. Bonneville, A. Berns, and S. Tonegawa, manuscript submitted for publication) or Vγ1JγC, γVδ2JδC δ (16) chain genes were constructed after microinjection of DNA from hybridoma KN6 (derived from adult thymocytes) (15, 17) and hybridoma KI129 (derived from day 15.5 fetal thymocytes) (18), respectively, into the pronucleus of fertilized eggs, as described elsewhere (Ishida et al., manuscript submitted for publication).

Antibodies. CD8, CD4, and Thy-1.2 mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA). The anti-CD3 ε mAb 2C11 (19) and the anti-pan TCR a/β mAb H57-597 (20) were kindly provided by J. A. Bluestone and R. Kubo, respectively. F536 mAb directed against the Vγ1JγC γ chain was a kind gift from W. Havran and J. Allison (21). 3A10, 8136, and 5C10 mAbs are directed against a constant region of TCR δ chain, VγVδ-encoded TCR, and a unique epitope of the hybridoma KN6 TCR, respectively (14).

Cells. IEL were isolated according to the technique described by Petit et al. (22). Briefly, small intestine was cut into small pieces and incubated in HBSS without MgCl2, MgSO4, and CaCl2, supplemented with 0.1 mM EDTA. After washes in Hanks + 5% FCS and passage through a nylon wool column, cells were loaded on top of a 30–70% Percoll gradient, and lymphocytes from the 70% gradient were recovered and washed. DEC were prepared according to the technique described by Nixon-Fulton et al. (23). Mice were shaved, dorsal and abdominal skin was isolated, and, after elimination of subcutaneous tissue, cut into 5-mm slices. Skin slices were then incubated (dermal side down) on a 0.3% Trypsin solution in glucose (0.17%), NaCl (0.88%), and KCl (0.04%) at 4°C overnight. Epidermis was gently scraped and incubated 30 min at 37°C in a 0.3% Trypsin, 0.1 mg/ml DNase I solution. Epidermal cells were then washed twice, loaded on a Ficoll solution (Lymphoprep), spun 15 min at 2,000 rpm, and cells from the interface were recovered and analyzed.

Immunofluorescence and FACS Analysis. Cells were stained according to standard procedures using mAbs as hybridoma supernatant (F536, H57-597), purified FITC- (Thy-1.2, CD8, CD3, H57-597, 8D6, 5C10, 3A10), or Phycoerythrin- (CD4) conjugated or biotinylated (CD8, Thy-1.2, 8D6, 5C10, 3A10) antibodies. In the case of noncoupled mAbs, the secondary reagent was streptavidin coupled to phycoerythrin (Becton Dickinson & Co.). After staining, cells were resuspended in PBS-propidium iodide (0.5 μg/ml) solution and subjected to flow cytometric analysis using a FACSCAN apparatus. In all cases, dead cells, mostly composed of residual epithelial cells, were gated out on the FL3 channel.

Immunohistology Procedures. The techniques used for light immunohistochemistry have been described in detail (24).

Results

Surface Phenotype Analysis of IEL. Previous analyses of TCR-γ/δ expression by IEL were carried out by cell surface labeling of purified IEL, followed by immunoprecip-
itation (11, 12). It is difficult to obtain an accurate measurement of the proportion of TCR-α/β- or TCR-γ/δ-bearing IEL by these procedures. The availability of mAbs that recognize all α/β T cells (H57-597) (20) or all γ/δ T cells (3A10) (14) enabled us to address this issue directly and to study the expression of CD8 and Thy-1 antigens on the α/β and γ/δ IEL subsets. Intestinal tissue sections were stained with mAbs H57-597 and with 3A10. γ/δ T cells were detected between columnar epithelial cells much more than in the lamina propria (Fig. 1 A). An opposite pattern of distribution was observed for α/β T cells (Fig. 1 B). To determine the relative abundance of the two types of T cells in the epithelium, the IEL were isolated, stained with various antibodies, and analyzed by flow cytometry. About 50% of the cells expressed TCR-γ/δ, ~30% expressed TCR-α/β, and the remaining cells were CD3- and expressed neither TCR (Fig. 2). The presence of α/β IEL in this abundance was unexpected because the α/β heterodimers could not be detected in the previous immunoprecipitation studies (11, 12). This apparent discrepancy may be explained by the lower TCR density on α/β (CD3 mean fluorescence intensity, 495) than on γ/δ T cells (CD3 mean fluorescence intensity, 566) (Fig. 2).

Virtually all γ/δ and α/β IEL expressed CD8, while Thy-1 was expressed by about two thirds of the γ/δ and α/β IEL (Fig. 3).

**Homing of γ/δ T Cells to Intestinal Epithelia in Nude Mice.** Previous studies have shown that Thy-1- CD8+ IEL are present in the intestines of nude mice (23). We investigated the type of TCR expressed on these IEL using H57-597 and 3A10 mAbs. As shown in Fig. 4 A, almost all IEL from young (6–8 wk old) nu/nu BALB/c mice carry TCR-γ/δ on their surface, while those from nu/+ littermates express TCR-

![Figure 1](image_url)  
**Figure 1.** α/β and γ/δ T cell distribution in the gut mucosa. Staining of jejunum tissue sections using 3A10 (anti-γ/δ) mAb (A) or H57-597 (anti-α/β) mAb (B) was performed as described in Materials and Methods. Indicated are the areas corresponding to the epithelial layer (E) and the lamina propria (LP) of intestinal villi, as well as some intraepithelial (closed arrows) and intralamina propria (open arrows) stained lymphocytes.
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FIGURE 2. TCR-α/β and γδ expression by IEL. IEL from 10-wk-old C57BL/6 mice isolated according to procedures described in Materials and Methods were incubated first with H57-597 (anti-α/β) mAb (biotinylated or FITC conjugated), 2C11 (anti-CD3) mAb (FITC conjugated), or 3A10 (anti-γδ) mAb (biotinylated), followed by an incubation with streptavidin-phycocerythrin. Indicated is the percent of cells in each quadrant.

α/β as well. Both nu/+ and nu/nu TCR-γδ+ IEL are CD8+, but, as reported previously (25), nude IEL are exclusively Thy-1−, while some nu/+ IEL express Thy-1 (Fig. 4 B). Similarly, the rare α/β+ IEL present in gut epithelium of nude mice are CD8+Thy-1− (data not shown). Taken together, these observations demonstrate that Thy-1−CD8+ γδ+ IEL selectively differentiate in the absence of a normal thymic environment and strongly suggest that Thy-1 expression depends on intrathymic differentiation.

The TCR-γδ Does not Determine the Homing Specificity. To test the hypothesis that TCR-γδ determines the homing specificity to a peripheral tissue, we made use of two kinds of γδ double-transgenic mice. Vγ4Vδ5 transgenic mice were produced using productively rearranged Vγ1Cγ1 γ and Vδ1D1J1Cδ genes isolated from a T hybridoma derived from an adult thymocyte (Ishida et al., manuscript submitted for publication). Vγ4Vδ5-encoded TCR are the most frequently used by adult γδ thymocytes (14, 17, 26). We also constructed a second set of transgenic mice (16) using productively rearranged Vγ1Cγ1 γ and Vδ2J2Cδ genes that encode the TCR expressed on most DEC (9). These mice will be referred hereafter to as KN6 (Vγ4Vδ5) and DEC (Vγ5Vδ1) transgenic mice.

Transgene expression on the surface of γδ T cells was investigated by using mAbs that are specifically directed against the transgenic TCR. In the case of KN6 transgenic mice, we used either the 8D6 mAb that recognizes γδ T cells carrying any Vγ4Vδ5-containing TCR, or the 5C10 mAb that specifically reacts with the KN6 clonotype (14). To assess the expression of the transgene-coded TCR in DEC trans-
FIGURE 4. TCR, CD8, and Thy-1 expression by IEL from athymic nude mice and euthymic nu/+ control mice. IEL from 6-wk-old BALB/c nu/nu and nu/+ mice were isolated and stained as described in Figs. 2 and 3. (A) A two-color analysis of nu/nu (top) and nu/+ (bottom) IEL using 2C11 (anti-CD3) mAb (FITC conjugated) and 3A10 (anti-γ/δ) mAb (biotinylated, streptavidin-phycoerythrin). Indicated is the percent of cells in each quadrant. (B) CD8 and Thy-1 expression by nu/nu (top) and nu/+ (bottom) y/b + IEL. IEL were stained with 3A10 mAb (biotinylated, streptavidin-phycoerythrin) and either Thy-1.2 or CD8 (FITC-conjugated) mAbs. For the Thy-1 and CD8 fluorescence histograms, only 3A10+ cells are shown.

FIGURE 5. All the γ/δ IEL from young KN6 and DEC transgenic mice express transgene-encoded TCR. (A) IEL from an 8-wk-old Vγ5Vδ1 transgenic mouse (right) and a nontransgenic littermate (left) were stained using F536 (anti-Vγ5) mAb (followed by an incubation with GAHIg-FITC) and 3A10 (anti-γ/δ) mAb (biotinylated, streptavidin-phycoerythrin). (B) IEL from an 8-wk-old Vγ4Vδ6 transgenic mouse (right) and a nontransgenic littermate (left) were stained using 3A10 mAb (FITC-conjugated) and 8D6 (anti-Vγ4Vδ6) mAb (biotinylated, streptavidin-phycoerythrin).
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Figure 6. TCR phenotype and size of DEC cells from KN6 transgenic mice. (A) DEC cells from two $V_{\gamma4}V_{\delta5}$ transgenic mice (top and middle) and from a nontransgenic littermate (bottom) were isolated according to procedures described in Materials and Methods and stained with 2C11 (anti-CD3) mAb (FITC conjugated) and 3A10 (anti-$\gamma6$) mAb (biotinylated, streptavidin-phycoerythrin) (left), or with 3A10 mAb (FITC conjugated) and 8136 (anti-$V_{\gamma4}V_{\delta5}$) mAb (biotinylated, streptavidin-phycoerythrin) (right). Indicated is the percent of cells in each quadrant. The right panels were obtained after gating out the 3A10$^-$ cells. (B) Size of $\gamma\delta$ (3A10$^+$) DEC cells from two $V_{\gamma4}V_{\delta5}$ transgenic mice (top and middle) and from a control littermate (bottom) was analyzed by forward-angle scatter analysis.

We investigated by immunohistology whether the $\gamma\delta$ T cells in the gut and skin of the transgenic mice are associated with epithelial cells, as are these T cells in normal mice. As shown in Fig. 7, the overall distribution of $\gamma\delta$ T cells in the gut of either KN6 or DEC transgenic mice was not different from that of nontransgenic control mice (Fig. 1). Indeed, unlike $\alpha/\beta$ T cells, which are preferentially localized in the lamina propria, $\gamma\delta$ T cells are mostly observed within the epithelial cell layer. Similarly, the $\gamma\delta$ T cells in the skin of the KN6 transgenic mice are associated with epithelial cells (Fig. 8), although there is clearly a reduction of the T cell density in the epidermis of these mice in comparison with the normal or DEC transgenic mice, in agreement with the results of the immunostaining analysis of DEC populations (Fig. 6 A). In addition, these DEC are on average smaller than the control DEC (Fig. 6 B), which accounts at least in part for the reduced anti-$\gamma\delta$ or anti-CD3 staining (Fig. 6 A).

Taken together, these results demonstrate that $\gamma\delta$ cells bearing TCRs encoded by "wrong" $\gamma$ and $\delta$ genes can home to gut or skin epithelia. This strongly suggests that TCR-$\gamma\delta$ per se are not homing receptors for specific epithelia.

CD8 and Thy-1 Expression on IEL of DEC and KN6 Transgenic Mice. It was previously suggested that expression of Thy-1 and CD8 by at least some $\gamma\delta$ T cells depends on their activation via TCR engagement (27, 28). To obtain an insight into
these hypotheses, we investigated the effect of "wrong" TCRs on the expression of these proteins on the surface of $\gamma/\delta$ IEL.

Most $\gamma/\delta$ IEL in 8-wk-old DEC transgenic mice were Thy-1+ (Fig. 9). TCRs of these IEL are almost exclusively encoded by the transgenes (Fig. 5). On the other hand, about half of the $\gamma/\delta$ IEL of KN6 transgenic mice, like those of nontransgenic littermates, were Thy-1- (Fig. 9).

As to the CD8 expression, the proportion of CD8- cells among $\gamma/\delta$ IEL was much higher than that in nontransgenic mice; ~90% of $\gamma/\delta$ IEL from 8-wk-old nontransgenic mice are CD8+, while no more than 50% of these cells in the age-matched DEC and KN6 transgenic mice expressed CD8 molecules.

Discussion

Previous studies suggested that $\gamma/\delta$ T cells were specifically enriched in gut epithelium (11, 12). By using three mAbs that react with both $\alpha/\beta$ and $\gamma/\delta$ T cells or only with one of the two T cell subsets, we have now determined the proportion of $\alpha/\beta$ and $\gamma/\delta$ T cells among IEL; ~60% of CD3+ cells are TCR-$\gamma/\delta^+$, and the rest are TCR-$\alpha/\beta^+$ (Figs. 1 and 2). The vast majority of these T cells are CD8+ while less than half of the cells in each T cell class are Thy-1+. IEL in the intestine of nude mice are almost all Thy-1^-CD8+ $\gamma/\delta$ T cells; only very few Thy-1^-CD8+
alpha/beta T cells are found. These observations suggest that the homing of gamma/delta T cells to intestinal epithelia is not dependent on thymic education nor on factors produced by intraepithelial alpha/beta T cells.

It was previously shown that gamma/delta T cells with different TCR repertoires are compartmentalized in different epithelia (9, 11, 13). This raised the possibility that the homing specificity of these gamma/delta T cells is determined by a compartmentalization of...
TCR-γ/δ ligands in the various epithelia. The homing behavior of the transgenic γ/δ T cells described here does not support this hypothesis; we found cells expressing "wrong" transgene-encoded TCRs in the intestinal epithelium as well as in the epidermis. This suggests that γ/δ T cells in different epithelia belong to different γ/δ T cell sublineages. Each sublineage has a particular program of differentiation that may link the rearrangements to particular γ and δ gene segments of particular homing receptors. This model predicts that the rearrangement of different γ and δ gene segments depends on different regulatory factors.

While our findings are consistent with this model, we cannot exclude the possibility that the compartmentalization of TCR-γ/δ ligands does play a role in the compartmentalization of γ/δ T cells. In the case of α/β T cells, interactions of the TCR-α/β with MHC-encoded proteins are not only required for the maturation of these cells in the thymus (29), but may also prevent a rapid decay of these cells in the periphery (30). Thus, the interaction between TCR-γ/δ and their ligands differentially expressed in various epithelia could serve a supplementary role in the compartmentalization of these T cells. Indeed, occurrence of such interactions is suggested by a few observations made in this study. First, normal DEC expressing the VγVδ TCR are larger than the DEC expressing the VγVδ transgene-encoded TCR (Fig. 6B). Second, in DEC TG mice, the lack of CD8 expression by many intestinal IEL (Fig. 9) could reflect the failure of transgene-expressing γ/δ T cells to interact with the TCR ligands in this epithelium (see below).

It is noteworthy that while the T cells expressing the KN6 transgenes do find their way to epidermis, their number is 10-40-fold lower than the number of DEC cells in normal mice. Furthermore, γ/δ DEC cells expressing the endogenously rearranged (presumably VγVδ-encoded) γ and δ genes are also detectable in the KN6 TG mice. These observations can be explained as follows.

As discussed elsewhere, the progenitors of DEC cells may proliferate in the fetal thymus as a result of their interaction with a TCR-γ/δ ligand (31,32). Thus, the occurrence in KN6 TG mice may be explained by the expansion of a small number of thymocytes that escaped allelic exclusion. This expansion would not occur in the case of thymocytes expressing the TG-encoded TCR, hence the low number of DEC cells with this TCR in KN6 TG mice.

Interestingly, the proportion of CD8+ cells among γ/δ IEL is much lower in the transgenic mice than in normal mice (Fig. 9). One possible interpretation of this finding is that the introduction of the transgenes and their premature expression in developing T cells affect their differentiation and CD8 expression. An alternative explanation is suggested by the recent findings that TCR-γ/δ ligands can induce CD8 expression in vitro (27, and our unpublished results). Thus, TCR ligands in the intestine may induce CD8 in normal IEL but not in transgenic IEL.

Finally, we would like to discuss our finding that the proportion of Thy-1+ cells among the transgenic γ/δ IEL is at least as high as the proportion of Thy-1+ among γ/δ IEL of normal mice (Fig. 9). We believe that Thy-1 is not an activation marker of IEL. The opposite conclusion was recently drawn by LeFrancois and Goodmann (28) on the basis of their finding that IEL from germ-free mice are mostly Thy-1−, and that acclimation of these mice to nonsterile conditions resulted in the generation of Thy-1 IEL. However, the present study has shown that a substantial amount (40%) of CD3+ IEL bear TCR-α/β (Fig. 2), and our own analysis of IEL in germ-
free and specific pathogen-free mice indicates that the difference in the expression of Thy-1 by IEL of these mice is attributable to the difference in the ratio of \( \gamma/\delta \) and \( \alpha/\beta \) T cells (A. Bandeira, C. Hausser, S. Itohara, S. Tonegawa, and A. Coutinho, unpublished observations). In addition, Thy-1 is not expressed by \( \gamma/\delta \) IEL of nude mice (Fig. 4B). It is therefore likely that Thy-1 is a differentiation marker for \( \gamma/\delta \) IEL as it is for other T cells.

Summary

\( \gamma/\delta \) T cells with different TCR repertoires are compartmentalized in different epithelia. This raises the possibility that the TCR-\( \gamma/\delta \) directs homing of T cells to these epithelia. Alternatively, the signals that induce TCR-\( \gamma/\delta \) expression in developing T cells may also induce homing properties in such cells, presumably in the form of cell surface receptors. We have examined this issue by studying the homing of \( \gamma/\delta \) T cells in transgenic mice constructed with specific pairs of rearranged \( \gamma \) and \( \delta \) genes. In such mice, most \( \gamma/\delta \) T cells express the transgene-encoded TCR. We find that homing to both skin and gut epithelia is a property of T cells and is not determined by the type of \( \gamma \) and \( \delta \) genes used to encode their TCR. We also studied the effect of TCR replacement on the expression of Thy-1 and CD8 proteins on the \( \gamma/\delta \) T cells associated with gut epithelia. Our results show that the expression of the appropriate type of TCR-\( \gamma/\delta \) is not required for the Thy-1 expression by these T cells, suggesting that Thy-1 is not an activation marker. In contrast, CD8 expression by gut \( \gamma/\delta \) T cells seems to depend on the expression of the appropriate type of TCR.

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