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Diversity, Development, Ligands, and Probable Functions of γδ T Cells

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The most critical step in the vertebrate immune response is the recognition of antigens by lymphocytes. This task is accomplished by two sets of glycoproteins, immunoglobulins (Igs), and T-cell antigen receptors (TCRs). The most extraordinary feature of these proteins is their structural variability, much of which originates from the ability of the encoding gene segments to undergo somatic rearrangement (Tonegawa 1983). All TCRs were initially thought to be composed of a heterodimeric protein composed of α and β subunits. However, the search for the genes encoding these polypeptides led to the identification of a third rearranging gene (Saito et al. 1984; Hayday et al. 1985) that was later shown to code for one of the two subunits of another heterodimeric, TCR$\gamma\delta$ (Bank et al. 1986; Brenner et al. 1986; Weiss et al. 1986).

Despite the striking similarities in the overall structure of their genes and polypeptide chains, TCR$\gamma\delta$ and the T cells that express it are significantly different from their $\alpha\beta$ counterparts. For instance, $\gamma\delta$ T cells are detected in both the thymus (Bank et al. 1986; Lew et al. 1986; Nakanishi et al. 1987) and peripheral lymphoid organs (Brenner et al. 1986; Maeda et al. 1987) in relatively low numbers ($<5\%$ of T cells) but predominate (50–100%) within epithelia such as epidermis (Kuziel et al. 1987; Stingl et al. 1987) and the small intestine (Bonneville et al. 1988; Goodman and Lefrancois 1988). In contrast with most $\alpha\beta$ T cells, the majority of $\gamma\delta$ T cells in the thymus and spleen do not express either CD4 or CD8 (Bank et al. 1986; Brenner et al. 1986; Lew et al. 1986; Maeda et al. 1987; Nakanishi et al. 1987). Neither the specificity of TCR$\gamma\delta$ recognition nor $\gamma\delta$ T-cell function in immune defense is understood. In this paper, we summarize results of our recent studies on the diversity, development, and specificity of $\gamma\delta$ T cells, and we discuss possible functions of these cells.

RESULTS

Anti-mouse TCRγδ MAbs

To study the nature of the TCR$\gamma\delta$ and the role of $\gamma\delta$ T cells, monoclonal antibodies (MAbs) directed against the native receptor are useful. To generate such antibodies, we immunized Armenian hamsters with the anti-CD3 immunoprecipitate of a lysate of $\gamma\delta$ T hybridoma KN6 (Ito et al. 1989) and prepared three anti-$\gamma\delta$-TCR MAbs (Itohara et al. 1989): MAb 3A10, specific for a C_δ (constant) region determinant, MAb 8D6, specific for (variable) V_γ4- and V_δ5-encoded TCR$\gamma\delta$, and MAb 5C10, specific for a KN6 TCR idiotope.

Appearance of αβ and γδ T Cells in the Developing and Mature Thymus

Using appropriate MAbs, we determined the number of thymocytes bearing TCR$\gamma\delta$ (abbreviated hereafter as $\gamma\delta$ thymocytes) or TCR$\alpha\beta$ ($\alpha\beta$ thymocytes) as a function of age (Fig. 1). $\gamma\delta$ Thymocytes were detected at day 14.5 of gestation (E14.5), the earliest time point analyzed. Their number increased until E17.5, remained at this level until birth, dipped, and then gradually increased to the adult level of 1 million cells per thymus. In relative terms, only 0.4–0.6% of total thymocytes at E14.5 are $\gamma\delta$ thymocytes, increasing rapidly to its highest value (5%) at E16.5, then gradually decreasing through embryonic life and the first postnatal week until it reaches a stationary adult level of 0.3–0.5% at about 10 days after birth. $\alpha\beta$ Thymocytes are rare (<100 cells/thymus) at E14.5 but outnumber $\gamma\delta$ thymocytes after E16.5. By E18.5, they are the dominant thymocyte population.

Two-color analysis of thymocytes from E16, E18.5, and adult mice showed that TCR$\alpha\beta$ and TCR$\gamma\delta$

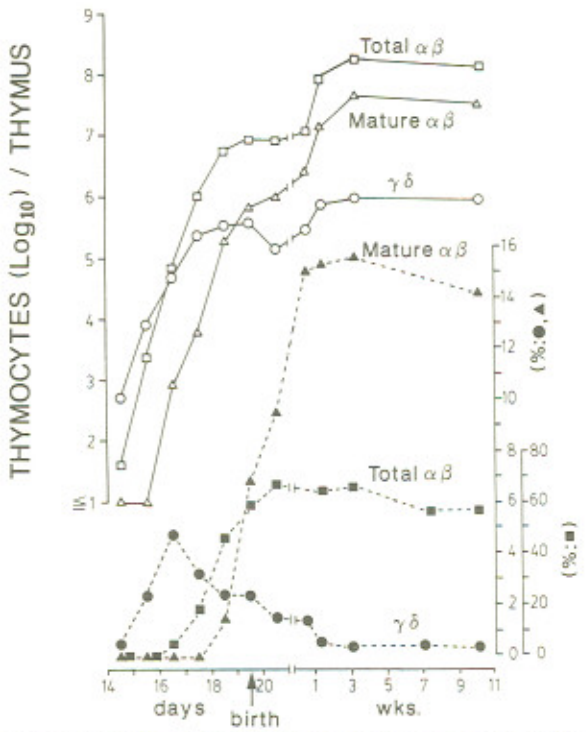

Figure 1. Ontogeny of TCR $\gamma\delta$ and $\alpha\beta$ thymocytes in C57BL/6 mice. Thymocytes of mice at different ages were stained with anti-$\gamma\delta$ (3A10) or anti-$\alpha\beta$ (H57-597) biotin conjugates followed by a streptavidin phycoerythrin conjugate together with an anti-CD3 (2C11) fluorescein isothiocyanate conjugate. The samples were analyzed with FACScan (Becton-Dickinson) using FACScan software.

double-positive cells must be extremely rare or do not exist at all. Thus, a mechanism appears to exist that restricts the surface expression of TCRs to only one of the two types.

$\gamma\delta$ T Cells Compose Minor T-cell Subpopulations in Peripheral Lymphoid Organs

We determined the frequency of $\gamma\delta$ T cells in peripheral lymphoid organs (i.e., spleen, lymph node, and blood) as well as their CD4 CD8 phenotype. The results are summarized in Table 1. In adult mice (7–13 weeks old), the fraction of $\gamma\delta$ T cells among $CD3^+$ T cells is no more than 3% in any of the three sites studied although it is as high as 10% in the spleen of 4-day-old mice. This $\gamma\delta^+/CD3^+$ ratio is much lower than that reported for the chicken (Sowder et al. 1988) but is similar to that in humans (1–15%) (Borst et al. 1988; Chen et al. 1989). As suggested in the original report on $\gamma\delta$ T cells (Brenner et al. 1986), the majority of peripheral $\gamma\delta$ T cells do not express CD4 or CD8 (Table 1). However, a significant fraction of $\gamma\delta$ T cells is either $CD4^+$ or $CD8^+$ in all three peripheral lymphoid organs studied, and high CD8 expression can be induced in vitro in peripheral $\gamma\delta$ T cells with the MAb 3A10 (Y. Utsunomiya et al., unpubl.).

Intrathymic Distribution of $\gamma\delta$ T Cells Alters Drastically during the First Postnatal Week

To relate the spatial distribution of $\gamma\delta$ thymocytes to the organization of the thymic environment, we analyzed sections of fetal and adult thymuses using immunohistochemical technique. Adjacent sections of thymus tissue were alternatively labeled with the MAb 3A10 or with the *Ulex europeus* agglutinin (UEA), which we have previously shown to be a reliable marker for medullary thymic epithelial cells in the adult murine thymus (Farr and Anderson 1985). We observed a striking colocalization of $\gamma\delta^+$ thymocytes and UEA^+ medullary epithelial cells during late fetal and neonatal periods of development (Fig. 2a,b). In contrast, $\gamma\delta$ thymocytes were scattered throughout cortical and medullary areas of the thymus (Fig. 2c,d) and most concentrated in the subcapsular areas of the thymus in the thymuses of adult mice. Results of ultrastructural immunohistochemistry confirmed the close association between medullary thymic epithelial cells and $\gamma\delta$ thymocytes in the neonatal thymus and also showed that some TCR$\gamma\delta$ molecules were patched to areas of contact with medullary epithelial cells. As shown below, the pattern of TCR$\gamma\delta$ expression in the thymus is limited in diversity and is developmentally ordered. Thus, these histochemical results indicate that changes in receptor repertoire correlate with the changes in the intrathymic distribution of $\gamma\delta$ thymocytes.

$\gamma\delta$ T Cells Are Present in Many Organs Containing Epithelia

Since $\gamma\delta$ T cells are relatively scant in peripheral lymphoid organs (Table 1), we searched for these cells in sections of various organs and tissues. The results are summarized in Table 2. The $\gamma\delta$ T cells were most abundant in the small intestine, moderately abundant in the tongue, stomach, and large intestine, and very scarce in the esophagus. In the tongue and stomach, the vast majority of $\gamma\delta$ T cells were associated with the basal layer of stratified squamous epithelium (Fig. 3a,b). Consistent with previous studies of human and chicken tissues (Bucy et al. 1988; Groh et al. 1989), $\gamma\delta$ T cells were observed in mouse intestines within the columnar epithelium of the villi (Fig. 3c) but not in intestinal crypts nor in the lamina propria.

A substantial number of $\gamma\delta$ T cells was also found to be associated with the columnar luminal epithelium of the uterus (Fig. 3d), the stratified squamous epithelium of the vagina (Fig. 3e), and the transitional epithelium of the bladder. In contrast, no $\gamma\delta$ T cells were found in the liver, pancreas, kidney, or brain.

Unlike the human and chicken epidermis (Bucy et al. 1988; Groh et al. 1989), the mouse epidermis is the site of a major $\gamma\delta$ T-cell subset. About 5×10^6 $\gamma\delta$ T cells are present within epidermis and hair follicles of each mouse (Fig. 3f) (Bergstresser et al. 1983; Tschachler et

Table 1. Frequency and Surface Phenotype of γδ Cells in Periphery

Organ	Age of animals	No. of animals examined	No. of cells bearing[c] (10^{-5}/animal)			γδ Cells among $CD3^+$ (%)	γδ Cells bearing[c] (%)		
			CD3	TCRγδ	TCRαβ		$CD4^+$	$CD8^+$	$CD4^-$ $CD8^-$
Spleen	4 days	7[a]	0.74	0.07	0.67	10	5.5	63	32
	7–13 wk	12	300 ± 73	8.2 ± 2.0	279 ± 70	2.8 ± 0.5	10 ± 0.6	15 ± 4.8	75 ± 5.4
Mesenteric lymph node	7–13 wk	12	51 ± 23	1.2 ± 0.5	50 ± 22	2.3 ± 0.3	7.6 ± 2.6	32 ± 14	60 ± 16
Blood	7–13 wk	17[a]	25[b]	0.6	24	2.4	10	18	72

[a] Cells from the indicated number of animals were pooled.
[b] Estimated from the data of Green (1966).
[c] Mean ± S.D.

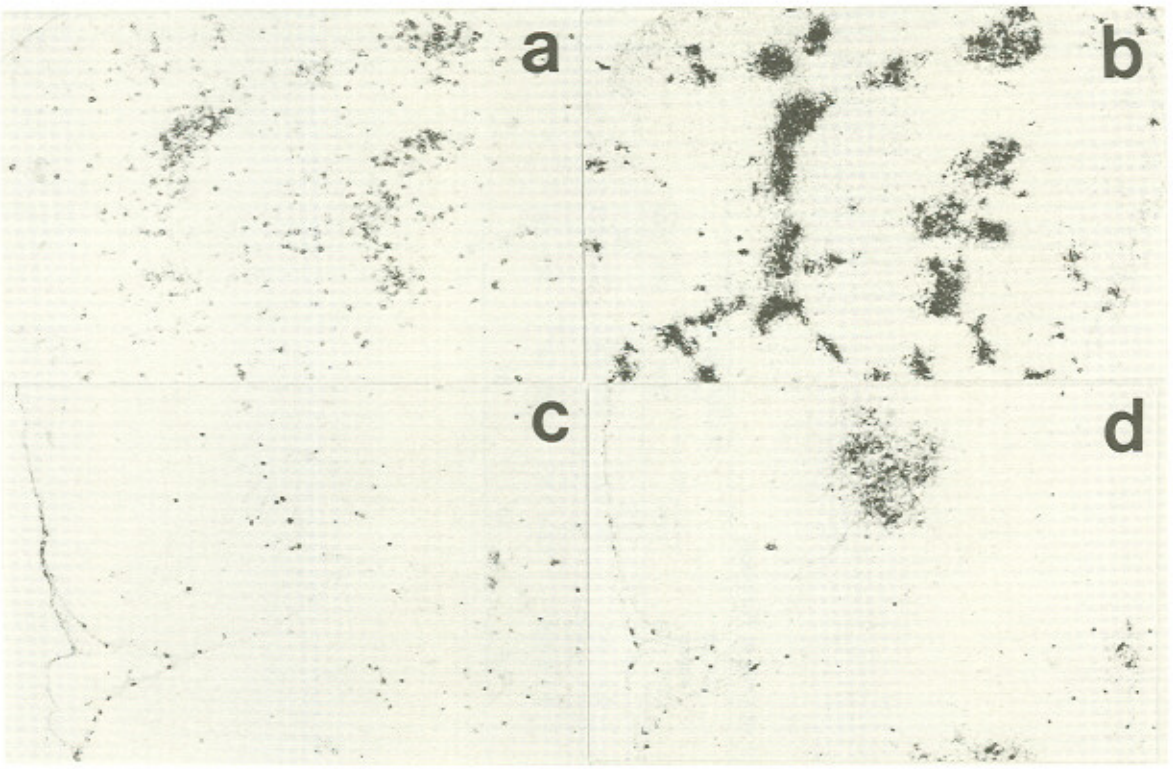

Figure 2. Intrathymic distribution of γδ cells in fetal and adult thymus tissues. Adjacent serial sections of thymus tissue were labeled with anti-γδ MAb 3A10 (*a* and *c*) or with UEA (*b* and *d*). (*a* and *b*) Day 18; (*c* and *d*) adult.

Table 2. Distribution of γδ T Cells in Mice

Organs	Presence of γδ T cells in mice		Contact with epithelial cells	No. of cells per animal	Major V_γ gene segments used	Diversity of TCRγδ
	normal	nude				
Digestive system						
tongue	+		+		6	−
esophagus	+/−		+			
stomach	+		+			
small intestine	+	+	+	1×10^6	7	+
large intestine	+	+	+	1×10^5	7, 4	
liver	−					
pancreas	−					
Reproductive system						
ovary	+/−					
uterus	+	−	+	1×10^5	6	−
vagina	+	−	+	1×10^5	6	−
testis	−					
epididymis	+/−		−			
seminal vesicle					7, 5	
Urinary system						
kidney	−					
bladder	+	−	+			
Others						
skin	+	−	+	5×10^6	5	−
lung					7, 4, 6	
brain	−					
heart	−					
Lymphoid organs						
thymus	+			1×10^6	4, 7	+
spleen	+	−		8×10^5	4, 7, 6	+
lymph node	+	−		1×10^5	4, 7, 6	+
blood	+			6×10^4	4, 7, 6	+

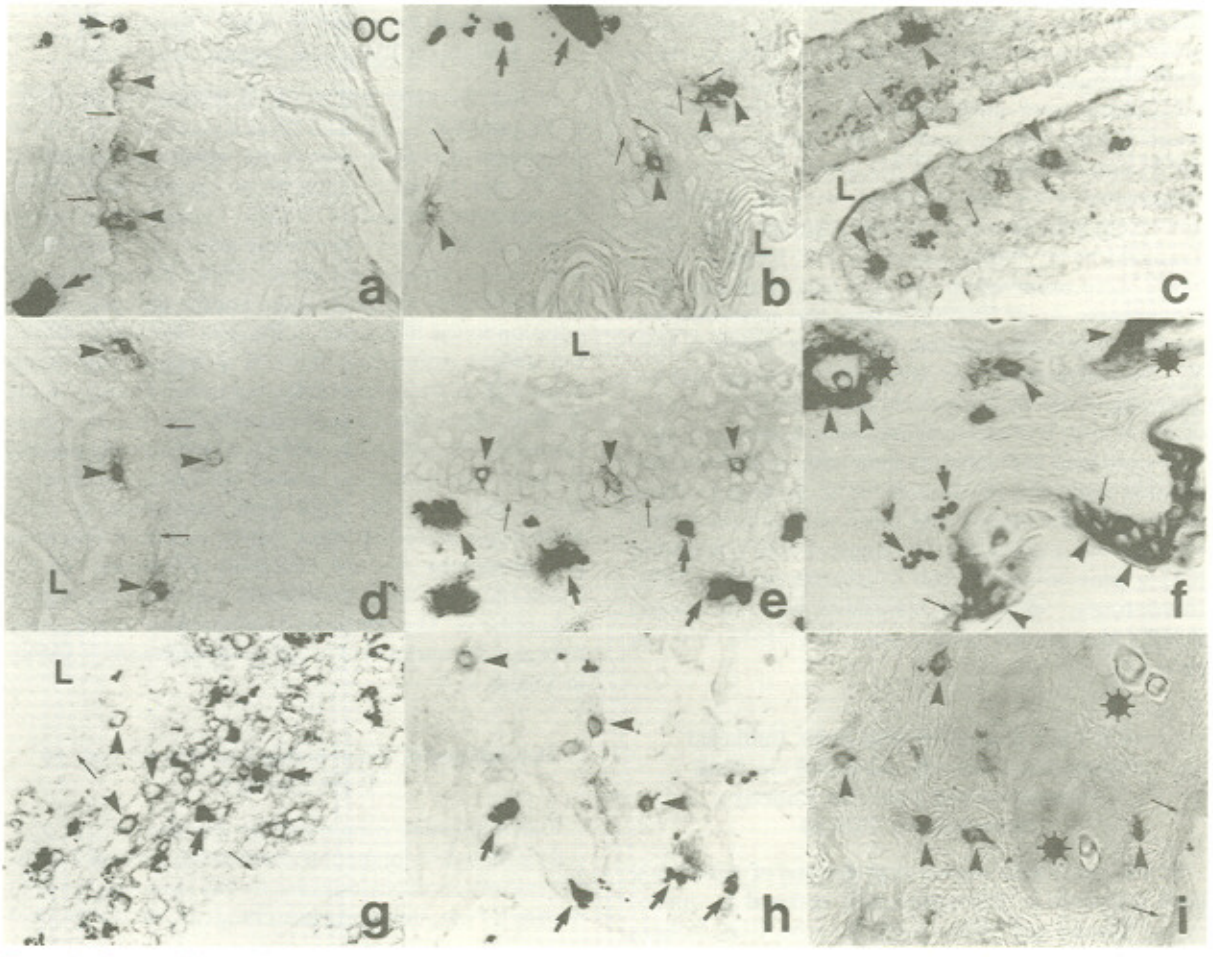

Figure 3. Immunohistostaining of various epithelium-containing organs with anti-γδ and anti-αβ MAbs. Fresh tissues from 8- to 10-week-old BALB/c mice were snap frozen, and 6 μm sections were fixed with cold acetone, stained with a MAb anti-γδ (3A10) (*a–f*) or anti-αβ (H57-597) (*g–i*) followed by affinity-purified goat anti-hamster IgG biotin (Caltag Lab., California) and avidin-horseradish peroxidase conjugates. (*a*) Tongue; (*b*) stomach; (*c* and *g*) small intestine; (*d* and *h*) uterus; (*e*) vagina; and (*f* and *i*) skin. Arrowheads indicate some of the stained TCR cells. Endogenous peroxidase activity is indicated by large arrows. Small arrows indicate basal lamina. Asterisks indicate hair follicles. (OC) Oral cavity; (L) lumen. Magnification, 220×.

al. 1983). In some areas, these cells tended to form clusters (Fig. 3f).

For comparison, adjacent sections were stained with the MAb against TCRαβ (Fig. 3g,h,i). These T cells were preferentially localized in the lamina propria of the intestines (Fig. 3g), the endometrium and myometrium of the uterus (Fig. 3h), the dermis of the skin (Fig. 3i), and the connective tissue of the vagina. Occasionally, some αβ T cells were observed in association with the epithelia of the intestine, vagina, and uterus. However, the majority of $CD3^+$ intraepithelial lymphocytes are clearly γδ T cells. In light of these observations, we propose to refer to these T cells as intraepithelial lymphocytes (IEL) as a generic term and to attach the initial of each organ to designate various IEL subpopulations such as i-IEL, s-IEL, r-IEL, and t-IEL for the IELs in the intestine, skin, reproductive organs, and tongue, respectively.

We extended our immunohistological analyses to 8-week-old athymic nude mice. As expected, no αβ or γδ T cells were detectable in most organs analyzed, suggesting a thymus dependency of these T cells. However, γδ T cells were observed in the small intestines of these nude mice in about half the number present in the strain- and age-matched normal mice (Table 2). These γδ T cells were, as were those of normal mice, IELs.

γδ T Cells of Different Peripheral Sites Use Different V_γ Gene Segments to Encode Their TCR

Previous studies have demonstrated that s-IEL (DEC) and i-IEL use distinct V_γ gene segments, V5 (Asarnow et al. 1988) and V7 (Takagaki et al. 1989a), respectively, to encode the γ subunits of their TCR. To determine whether there was a similar preferential usage of specific V_γ gene segments by γδ T cells present in other sites, DNA extracted from crude lymphocyte preparations from these sites was amplified by the polymerase chain reaction (PCR) technique (Saiki et al. 1988) using a V_γ ($V_\gamma4$, $V_\gamma5$, $V_\gamma6$, or $V_\gamma7$)-specific primer in combination with a (joining) $J_\gamma1$-specific primer. The PCR products were then analyzed by

Southern blot analysis using a $J_\gamma 1$ oligonucleotide probe. The results are summarized in Table 2. As in adult thymuses (Ito et al. 1989), the $V_\gamma 4/J_\gamma 1$ rearrangements were most abundant in peripheral lymphoid organs (blood, spleen, and lymph nodes) but bands corresponding to $V_\gamma 6/J_\gamma 1$ and $V_\gamma 7/J_\gamma 1$ rearrangements were also detected in these organs. In agreement with previous studies (Asarnow et al. 1988; Takagaki et al. 1989a), strong $V_\gamma 5/J_\gamma 1$ and $V_\gamma 7/J_\gamma 1$ bands were observed in the skin and small intestine, respectively. Apart from these sites, the most conspicuous were the vagina, uterus, and tongue in which $V_\gamma 6/V_\gamma 1$ rearrangement was abundant. Cloning and sequencing of the PCR products indicated that most (12 of 14) $V_\gamma 6/J_\gamma 1$ clones isolated from vaginas and uteruses contained in-frame junctions with an identical nucleotide sequence (S. Itohara et al. in prep.). These results strongly suggest that most γδ T cells in female reproductive organs use the single V6J1C1 (constant [C]) γ gene to encode the γ subunits of their TCR. Interestingly, γδ T cells associated with the tongue (t-IEL) also use the same γ gene (S. Itohara et al., in prep.).

γδ T Cells Associated with Some Epithelial Organs Are Primarily Derived from Fetal Thymocytes and Carry an Entirely Homogeneous TCRγδ

It was previously shown that the use of γ and δ V gene segments is developmentally ordered in thymocytes (Havran and Allison 1988; Ito et al. 1989; Itohara et al. 1989). γδ Thymocytes from early fetuses (i.e., at around day 15 of the embryonic life) preferentially express TCR encoded by V5J1C1 γ and V1D2J2C δ genes (Havran and Allison 1988; Ito et al. 1989). We found that these TCRs are entirely homogeneous (J.J. Lafaille et al., in prep.) and that they are identical to the TCRγδ expressed on s-IEL (Asarnow et al. 1988). Thus, s-IEL probably originates from this first wave of fetal γδ thymocytes. At late fetal and newborn stages, most γδ thymocytes bear TCR encoded by another pair of γ and δ genes, V6J1C1 γ and V1D2J2C δ (Ito et al. 1989), which are also structurally homogeneous (J.J. Lafaille et al., in prep.). Since the nucleotide sequence of this V6J1C1 γ gene is identical to the sequence of the sole in-frame joined V6J1C1 γ gene present in r-IEL and t-IEL (S. Itohara et al., in prep.), we suspected that these cells are derived from the second wave of fetal γδ thymocytes. If this were the case, the δ chains of these T-cell populations also should be identical. We therefore cloned and sequenced the PCR products of V1DJ2C δ genes from r-IEL preparations and found indeed that these genes are also homogeneous in their junctional sequences (S. Itohara et al., in prep.) and that the r-IEL δ gene sequence corresponds exactly to the sequence of the δ gene expressed on the surface of the late fetal thymocyte population (J.J. Lafaille et al., in prep.). We conclude from these results that r-IELs are derived from the late fetal wave of thymocytes.

TCRγδ Expressed in Adult Thymus and Peripheral Lymphoid Organs Are Diverse

Unlike the early and late fetal thymocytes or s-IEL, r-IEL, and t-IEL, adult γδ thymocytes and γδ T cells of peripheral lymphoid organs use various combinations of V_γ and V_δ gene segments to encode their TCR. Furthermore, abundant V-(D)-J junctional diversity occurs among the TCR of these adult γδ T cells (Korman et al. 1988; Lacy et al. 1989; Takagaki et al. 1989b). Taken together, these results indicate that in addition to the difference in the gene segments utilized for surface expression, there is a drastic fetal versus adult shift in the extent of the junctional diversity in the TCRγδ. Since i-IELs preferentially use the $V_\gamma 7$ gene segment (Takagaki et al. 1989a), which is rarely utilized by fetal thymocytes but is used by some adult thymocytes, and since the TCR gene assembled in adult thymocytes and i-IEL show similar extensive deletions and insertions in the junctions (Takagaki et al. 1989a,b), some adult thymocytes may home to intestinal epithelia. The probable developmental relationship between the various γδ T cell subpopulations is shown in Figure 4.

Developmental Relationship between αβ and γδ T-cell Lineages

One interesting issue raised by the discovery of γδ T cells is their developmental relationship with αβ T cells. Several observations relevant to this issue can be summarized. First, during thymic ontogeny, rearrangement

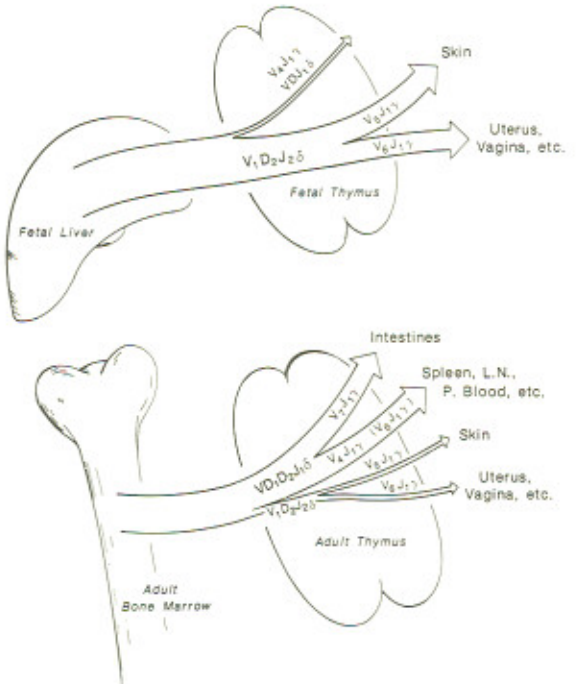

Figure 4. Development of various γδ T-cell subsets. The arrows indicate the proposed pathways followed by γδ T lymphocytes expressing the $C_\gamma 1$ region in fetal and adult mice.

and cell-surface expression of at least some γ and δ genes precede that of α and β genes (Raulet et al. 1985; Pardoll et al. 1987; Havran and Allison 1988; Itohara et al. 1989). Second, in peripheral αβ T cells, some γ genes are almost always rearranged, often in a nonfunctional (i.e., out-of-frame) form (Reilly et al. 1986; Rupp et al. 1986; Heilig and Tonegawa 1987). In contrast, in γδ thymocytes or peripheral γδ T cells, β genes are almost always rearranged only incompletely with a diversity (D) gene segment joined to a J gene segment and no V gene segment attached, whereas α genes are never rearranged (Korman et al. 1988; Ito et al. 1989; Takagaki et al. 1989b). Finally, cells bearing both αβ and γδ TCRs or "hybrid" TCRs such as βδ or αγ heterodimers do not seem to exist (Pardoll et al. 1987; and see above). On the basis of these observations, Pardoll et al. (1987) and Allison and Lanier (1987) proposed that if γ and δ genes rearrange productively (i.e., by in-frame joining), the cell proceeds to surface expression of TCRγδ, which in analogy with the Ig system (Alt et al. 1987) inhibits further rearrangement of any other TCR gene. αβ T cells are generated only from those cells that failed to rearrange productively both γ and δ genes. We tested this model by constructing and analyzing three different sets of transgenic mice all carrying in their germ line productively rearranged TCRγδ or TCRγ genes.

Normal Numbers of αβ T Cells Are Generated in TCR γ and δ Double Transgenic Mice

The first set of transgenic mice (called KN6 transgenic mice) were made by injecting into oocytes a mixture of relatively long γ (40 kb) and δ (43 kb) genomic DNA fragments cloned from the γδ T-cell hybridoma KN6 (Fig. 5). According to the Pardoll-Allison model, generation of αβ T cells will be disrupted because all T-cell precursors in these mice should have productively rearranged transgenic γ and δ genes. However, contrary to this prediction neither the absolute number nor the proportion of αβ T cells is significantly altered in the thymus and spleen of the transgenic mice (Table 3). Furthermore, the thymocytes and splenocytes of these mice exhibit a normal expression pattern of CD4 and CD8 glycoproteins, suggesting that their maturation is normal. The apparently normal generation of αβ T cells is not due to a failure of the expression of the transgenes in these mice: These mice do contain an elevated level of γδ T cells, and nearly all of their TCRs are encoded by the transgenes (Table 3).

As in normal mice (see above), γδ/αβ double producers are not detectable in these transgenic mice (I. Ishida et al., in prep.). We conclude that there must be a mechanism restricting the expression of TCR to one

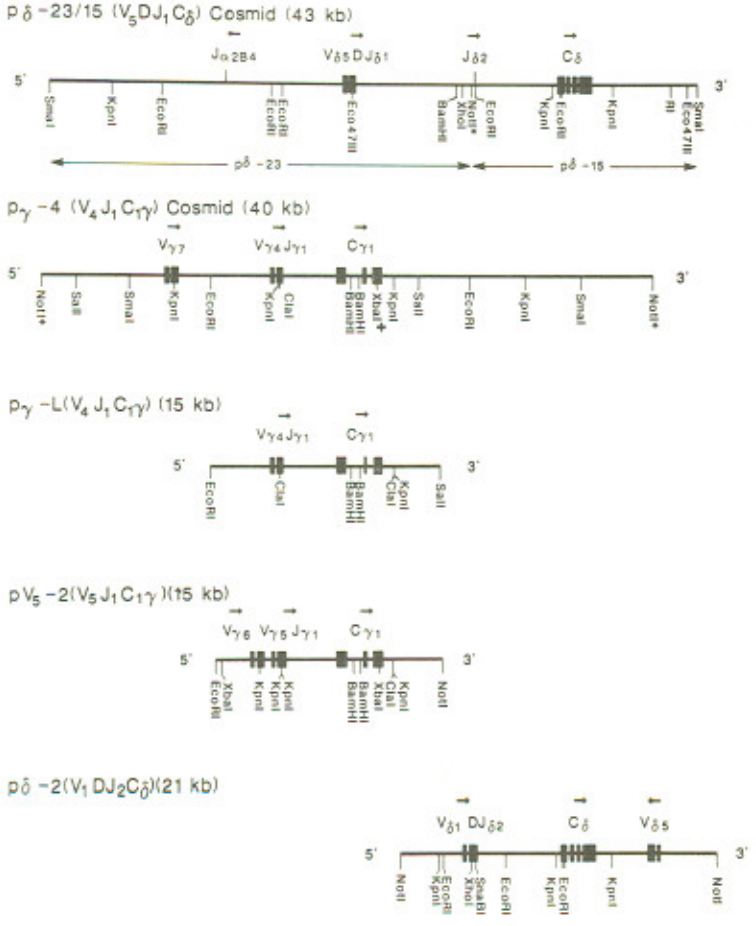

Figure 5. Restriction maps of γ and δ genomic DNA clones used for the construction of transgenic mice. The cosmid clones pδ-23/15 and pγ-4 were cloned from a γδ T hybridoma KN6 (C57BL/6) and were used to prepare KN6 transgenic mice. Phage λ clone pγ-L was cloned from a T-cell hybridoma 3D10 (C3H, Nakauchi et al. 1984) and was used to prepare the γ-only transgenic mice. The cosmid clones pV5-2 and pδ-2 were cloned from a γδ T hybridoma KI-129 (C57BL/6) (Ito et al. 1989) and were used to prepare the DEC transgenic mice. (■) Exons or gene segments; (→) their transcriptional orientation. The *Xba*I (+) site was destroyed deliberately in clone pγ-L.

Table 3. Surface Expression of TCRγδ and TCRαβ on Thymocytes and Splenocytes of γδ Transgenic Mice

Mice	Age (wk)	Total cells	Thymocytes ($\times 10^{-6}$) stained with anti-γδ	anti-KN6	anti-αβ	Total cells	Splenocytes ($\times 10^{-6}$) stained with anti-γδ	anti-KN6	anti-αβ
γδ 1313	9	79	1.26 (1.6)	1.02 (1.3)	56.8 (71.9)	153	6.32 (4.1)	6.27 (4.1)	43.0 (28.1)
LM	9	78	0.16 (0.2)	0.00 (0.0)	55.9 (71.7)	167	0.66 (0.4)	0.00 (0.0)	43.6 (26.1)
γδ 1355	8	100	2.66 (2.7)	2.66 (2.7)	72.7 (72.7)	161	4.96 (3.1)	4.83 (3.0)	36.1 (22.4)
LM	8	99	0.10 (0.1)	0.00 (0.0)	74.2 (74.9)	152	0.89 (0.6)	0.00 (0.0)	39.7 (26.1)

Thymocytes and splenocytes were stained with anti-γδ, anti-KN6, or anti-αβ MAb. Proportion (%) of TCRγδ- or TCRαβ-bearing T cells among total thymocytes or splenocytes is also shown in parentheses. LM designates nontransgenic littermates.

of the two types in a given T-cell precursor that is active before either of the two types of TCR is expressed on the cell surface.

A Transcriptional Silencer in the Flanking Region of the γ Gene

We investigated the reason why the γδ transgene products are not expressed on the surface of the αβ T cells in the KN6 transgenic mice by analyzing DNA and RNA of these cells. First, Southern blot analysis of DNA isolated from a T-cell population enriched with TCRαβ$^+$ cells as well as from T-cell hybridomas prepared from TCRαβ$^+$ cells showed that both the γ and δ transgenes are retained by the αβ T cells. Second, the analysis of the RNA extracted from the αβ T-cell population or from the αβ T hybridomas demonstrated that no transcripts derived from the transgenes accumulate in the αβ T cells. These results suggest that there exists a *cis*-acting DNA element(s) that mediates the repression of γ and δ RNA accumulation in αβ T cells. This putative element(s) must be contained within the 40-kb γ and/or 43-kb δ genomic DNA fragments used for the construction of the transgenic mice.

To test this possibility, we constructed a second set of transgenic mice (called "short γ" transgenic mice) using a γ gene clone containing very limited flanking sequences (Fig. 5, clone pγ-L.). We found that αβ T cells in this set of transgenic mice, in contrast with those in the KN6 transgenic mice, did harbor abundant RNA transcribed from the γ transgene but not from the endogenous γ genes (I. Ishida et al., in prep.). Since these results were obtained with three transgenic founder mice, it is very unlikely that the differential expression of the transgenes and endogenous γ genes is caused by the specific sequence context in which transgenes are integrated. We conclude that the γ gene carries in its flanking regions a *cis*-acting DNA element that down-regulates γ transcription in αβ T cells. This "silencer" element must be in the 5′- or 3′-flanking regions present on the pγ-4 clone but absent on the pγ-L clone.

Silencer Model of αβ and γδ T-cell Lineages

In view of these findings, we propose a model for the differentiation of αβ and γδ T-cell lineages (Fig. 6). γ and δ gene rearrangements occur prior to the completion (i.e., V to DJ joining) of β gene rearrangement and the initiation of α gene rearrangement. In-frame rearrangements of both γ and δ genes are, of course, a prerequisite for the generation of γδ thymocytes. However, we propose on the basis of the normal appearance of αβ T cells in the γδ transgenic mice that the failure to rearrange both γ and δ genes in-frame is not a requirement for the generation of αβ thymocytes. This implies that some αβ thymocytes could be generated from cells that harbor both in-frame rearranged γ and δ genes, and therefore precursors of some αβ T cells may express TCRγδ on their surface. Although this possibility cannot be ruled out, any TCRγδ expression in these cells is likely to be transient because we detected no thymocytes coexpressing both TCRαβ and TCRγδ either in the γδ transgenic mice (I. Ishida et al., in prep.) or normal mice (Itohara et al. 1989).

We propose that the putative machinery acting upon the γ silencer is activated in a fraction of immature thymocytes and that it is from these cells that αβ thymocytes are generated. The activation of the silencer machinery could occur prior to γ and δ gene rearrangements. In this case, αβ and γδ T-cell lineages are completely separate, and no αβ thymocyte precursors will ever express TCRγδ. The activation of the machinery, however, could occur between the completion of γ and δ gene rearrangements and that of α and β gene rearrangements. In this case, precursors of some αβ T cells may express TCRγδ transiently.

One may argue, in support of the Pardoll-Allison model, that the expression of the transgenes in our γδ transgenic mice is abnormally delayed for an unknown reason during the thymocyte differentiation and that this is the reason why rearrangements of the endogenous α and β genes and subsequent generation of αβ T cells are not disrupted in these mice. We argue that this is unlikely for the following two reasons. First, when we examined the cell-surface expression of the transgene-

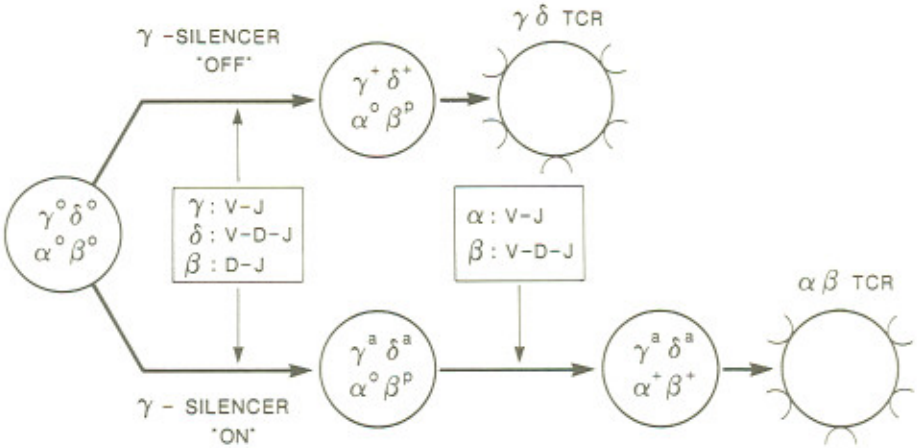

Figure 6. The silencer model of $\alpha\beta$ and $\gamma\delta$ T-cell differentiation. The various states of rearrangement of four TCR genes are indicated by superscripts. (0 and +) The gene is in the germ-line configuration and productively rearranged, respectively; (a and p) gene is in any state (i.e., in germ-line configuration, productively or nonproductively rearranged or deleted); (p) partially rearranged (i.e., D-J joined). The γ gene associated silencer is inactive in the $\gamma\delta$ T-cell lineage (*top*) and is activated in the $\alpha\beta$ T-cell lineage (*bottom*). The activation may occur either before any TCR gene is rearranged or after γ and δ genes are rearranged but before α and β gene rearrangements are completed. See text for more details.

coded TCR$\gamma\delta$ on fetal thymocytes, we found that the transgene-coded TCRs are expressed earlier than TCR$\alpha\beta$ (data not shown). Second, in the $\gamma\delta$1355 transgenic mice, we observed a near complete inhibition of rearrangement of the endogenous γ and δ genes. This inhibition most probably occurred by a mechanism analogous to the one known to form the basis for "allelic exclusion" of Ig and TCR$\alpha\beta$ genes. According to the widely accepted model of allelic exclusion (Alt et al. 1987), Ig or TCR feed back as soon as they are expressed on the cell surface to corresponding genes and inhibit their rearrangement. Thus, if the surface expression of the $\gamma\delta$-transgene-coded TCR$\gamma\delta$ had been abnormally delayed in the $\gamma\delta$ transgenic mice, we would have expected rearrangement of endogenous γ and δ genes and the surface expression of their products. Contrary to this prediction, we detected virtually no rearrangement and no cell-surface expression (Table 3) of the endogenous γ or δ genes in the transgenic mouse $\gamma\delta$1355. We therefore conclude that the transgene expression is not abnormally delayed in this mouse.

A Third Set of Transgenic Mice Support the Silencer Model

One prediction that could be made by the silencer model of T-cell development is that the generation of $\alpha\beta$ T cells would be hampered in a transgenic mouse constructed with γ and δ genes carrying no silencer element. We tested this prediction by constructing the third set of γ/δ double transgenic mice using "short" γ and δ gene clones (Fig. 5, clones pV5-2 and pδ2). As in the KN6 transgenic mice constructed with the "long" γ and δ gene clones, an overwhelming majority of the TCR$\gamma\delta$ in these transgenic mice (called DEC transgenic mice) is encoded by the transgenes (Fig. 7A).

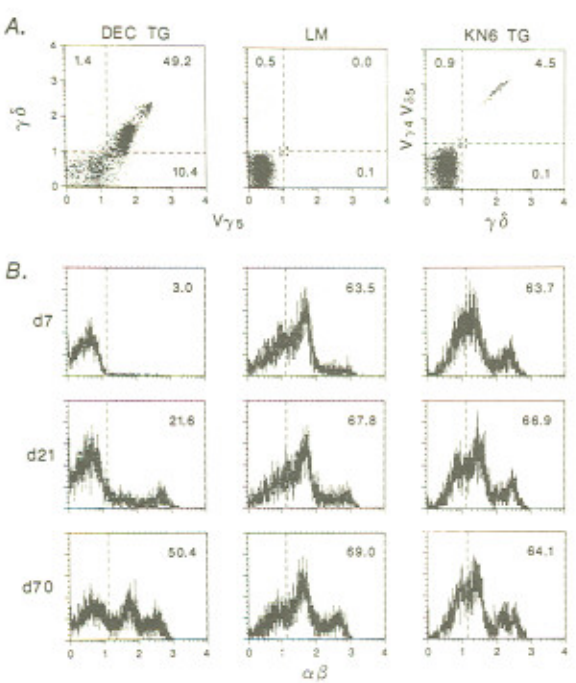

Figure 7. TCR$\gamma\delta$ and TCR$\alpha\beta$ surface expression by thymocytes from DEC transgenic, KN6 transgenic, and nontransgenic mice. (*A*) Thymocytes from 10-week-old DEC transgenic (*left*) and nontransgenic (*middle*) littermate and KN6 transgenic mice (*right*) were analyzed by two-color flow cytometry using anti-V_γ5 MAb F536, anti-$\gamma\delta$ MAb 3A10, and anti-V_γ4V_δ5 MAb 8D6. The number in each quadrant is the percentage of cells stained by one or both MAbs. Fluorescence intensity is expressed in $\log_{10}$ units. For comparison, an analysis of age-matched KNG transgenic mice is shown. (*B*) Thymocytes from 7-, 21-, and 70-day-old DEC transgenic (*left*), nontransgenic (*middle*), and KN6 transgenic (*right*) mice were stained with anti-$\alpha\beta$ MAb H57-597. The percentages of positive cells are indicated to the right of each histogram. Fluorescence intensity is expressed in $\log_{10}$ units.

Although the number of TCR$\gamma\delta^+$ thymocytes is 10- to 100-fold higher in the transgenic mice than in the non-transgenic littermates, the total number of thymocytes is greatly reduced because the development of $\alpha\beta$ T cells is severely hampered (Fig. 7B). In older transgenic mice, some $\alpha\beta$ T cells become detectable, most of which coexpress TCR$\gamma\delta$. Southern blot analysis of DNA extracted from the total thymocytes indicated that β gene rearrangement is blocked in these mice (M. Bonneville et al., in prep.).

Recognition of a Self Histocompatibility Complex TL Region Product by TCR$\gamma\delta$

To understand the function of $\gamma\delta$ T cells, it is essential to identify the putative ligand of the TCR. For this purpose, we prepared a number of $\gamma\delta$ T-cell hybridomas from fetal and adult thymocytes and screened them for specificity using a growth-inhibition assay. It was previously shown that cross-linking the TCR$\alpha\beta$, which usually promotes growth of normal T cells, results in growth inhibition of T-cell hybridomas (Ashwell et al. 1987). Assuming a similar effect of TCR$\gamma\delta$ cross-linking by a ligand, we cocultivated the $\gamma\delta$ T-cell hybridomas with a variety of irradiated (6000 rads) test cells, and we measured the effect of these cells on the incorporation of [^{3}H]thymidine into the DNA of the hybridomas. We identified one $\gamma\delta$ T hybridoma, KN6, whose growth was inhibited by syngeneic (C57BL/6) but not allogeneic (BALB/c, CBA/J, and AKR/J) spleen cells (Bonneville et al. 1989). KN6 hybridoma growth was inhibited efficiently by thymocytes, peritoneal macrophages, splenocytes, cells from the Abelson transformed B6 T-cell line 2052C, and by PCC3 embryonal carcinoma cells. The response to syngeneic spleen cells was observed in three different CD3$^+$ subclones of the KN6 hybridoma (KN6-7, KN6-12, and KN6-10) but not with TCR$^-$ variants of KN6 (KN6-19 and KN6-2), which indicated that growth inhibition was mediated by the TCR. This conclusion was further supported by the finding that the KN6 growth inhibition was blocked in a dose-dependent fashion by the anti-TCR$\gamma\delta$ MAb 3A10 as well as by the anti-KN6 TCR clonotypic MAb 5C10 (Bonneville et al. 1989).

Since the KN6 hybridoma responds to syngeneic but not to allogeneic cells, the gene encoding or controlling the KN6 ligand must be polymorphic. To confirm this and to map the gene, the KN6 hybridoma was screened for reactivity with a panel of spleen cells from various mouse strains (Table 4). Linkage to the major histocompatibility complex (MHC) was demonstrated by results with congenic strains that differ only at H-2. Thus, KN6 cell proliferation was strongly inhibited by cells from H-2^b strains, partially inhibited by cells from H-2^f strains, and not affected by cells from H-2^d, H-2^k, H-2^p, or H-2^s mice. Most importantly, the analysis of recombinant strains showed that the gene controlling the KN6 ligand is located in or distal to the TL region. This conclusion is based on the observation that the hybridoma responds to B6/Boy (K^{b}D^{b}Qa^{b}Tl^b) and A.Tla^b/Boy cells (K^{a}D^{a}Qa^a/TL^b) but not to A/Boy (K^{a}D^{a}Qa^{a}TL^a) or B6.Tla^a/Boy (K^{b}D^{b}Qa^b/TL^a) cells (Table 4).

Table 4. The Ligand Recognized by KN6 is Controlled by MHC Genes Telomeric to the Q Region

Strain[a]	Region[b] K	D	Q	TL	Percentage phenotype[c]	proliferation[d]
A.BY/SnJ	b	b	b	b	+	−75
C57BL/6J (B6)	b	b	b	b	+	−76 ± 12
C57BL/10SnJ (B10)	b	b	b	b	+	−89 ± 6
A.CA/Sn	f	f	f	f	±?	−37 ± 19
B10.M/Sn	f	f	f	f	±?	−36 ± 29
B10.D2/nSnJ	d	d	d	d	−	+30 ± 17
B10.BR/SgSnJ	k	k	k	k	−	−5 ± 12
P/J	p	p	p	p	−	+31 ± 27
B10.G/Sg	q	q	q	q	−	+8 ± 6
B10.RIII(7INS)/SnJ	r	r	r	r	−	+11 ± 2
B10.S/Sg	s	s	s	s	−	+8 ± 23
A/Boy	a	a	a	a	−	+4 ± 8
B6/Boy	b	b	b	b	+	−85 ± 4
A-Tla^b/Boy	a	a	a	b	+	−66 ± 14
B6-Tla^a/Boy	b	b	b	a	−	−2 ± 13

[a] Other + strains include BALB.B/Li, B10.A(2R)SgSnJ, B10.A(R149)-Tla^b/Mrp, B10.A(R410)-Tla^b/Mrp, B10.P(13R)/Sg, B10.SM(70NS)/Sn, and TBR2. Other − strains include AKR/J, A/WySnJ, BALB/c, B6-H-2^k/Boy, B6.K1/Fla, B6.K2/Fla, B10.A/SgSnJ, B10.PL(73NS)/Sn, B10(R297)-Tla^a/Mrp, B10(R310)-Tla^a/Mrp, C3H/HeJ, and MA/MyJ.

[b] Haplotype origin of region.

[c] + indicates strong inhibition. ± indicates possible intermediate inhibition. Results variable and not consistent. − indicated no inhibition.

[d] Data shown are with a single clone, KN6-7. All experiments done at least twice, except with A.BY, which also tested + with the original KN6 line.

Table 5. Percentage of Homology of the cDNAs Encoding the Putative KN6 Ligand with Known Class I Molecules

		Exon					
Region	Gene	I	II	III	IV	V	VI
H-2	H2-K^d	55	71	62	92	43	52
Qa-2, 3	Q10	52	72	60	95	36	49
Tla	T3	48	66	56	92	41	46
	37	52	71	65	95	39	39

TL Gene That May Encode the Class I Molecule Recognized by KN6 TCRγδ

The embryonic carcinoma line PCC3 does not express class I H-2K, D, or L or class II I-A or I-E MHC molecules on their surface (Maher and Dove 1984; Stern et al. 1986), and yet it is specifically recognized by KN6 TCR. To determine the structure of the molecule recognized by KN6 TCRγδ, we made a cDNA library from this cell line and screened the library with a probe composed of the exon 4 region (Weiss et al. 1984), which is conserved among all MHC class I genes known to date. This led to the isolation of 12 cDNA clones that fell into 7 kinds on the basis of their nucleotide sequences. The gene represented by one and only one of these 7 kinds of clones was distinct from all known MHC class I genes and appears to belong to the TL gene family on the basis of the pattern of sequence homology in the various exons (Table 5). Thus, the product of this gene is a good candidate for the TL-mapped ligand recognized by the KN6 TCRγδ.

Fate of Self-reactive γδ Thymocytes in the KN6 Transgenic Mice

Recent studies suggest that T-cell tolerance is accomplished, at least in part, by intrathymic deletion of self-reactive αβ T cells (Kappler et al. 1987; Kisielow et al. 1988; MacDonald et al. 1988). We have begun studies to determine whether or not a similar deletion of self-reactive γδ T cells occurs. Since the KN6 TCRγδ recognizes syngeneic (TL^b) thymocytes and splenocytes, we analyzed thymocytes and splenic T cells from the KN6 γδ transgenic mice expressing MHC^b or MHC$^{k/d}$ haplotypes. As shown in Table 6, nearly all γδ T cells bear TCR encoded by the transgenes regardless of the MHC haplotype of the host (i.e., MHC^b or MHC$^{k/d}$). Furthermore, the number of γδ T cells present in either thymus or spleen is not lower in the MHC^b mice than in MHC$^{k/d}$ mice. Thus, there was no obvious deletion of self-reactive γδ T cells. γδ Thymocytes isolated from MHC^b hosts but not from MHC$^{k/d}$ hosts propagate rapidly in vitro in the presence of concanavalin A supernatant or recombinant interleukin-2. γδ Thymocytes derived from MHC$^{k/d}$ transgenic mice require, in addition to the lymphokine, stimulation by TL^b ligand-bearing cells. The average density of TCRγδ is lower in the MHC^b thymocytes than in MHC$^{k/d}$ thymocytes, suggesting the occurrence of receptor modulation in the former transgenic mice. These results indicate that the γδ thymocytes recognize and respond to the putative TL^b ligand in vivo in the thymus of the KN6 transgenic mice.

DISCUSSION

Our study sheds considerable light on the characteristics of TCRγδ and T cells bearing this receptor. Except for some γδ T cells associated with intestinal epithelia, the majority of γδ T cells develops in the thymus as αβ T cells do. However, there are a number of features that distinguish the two types of T cells. First, in mouse, γδ T cells constitute a relatively minor T-cell subpopulation in peripheral lymphoid organs, and they preferentially home to a variety of epithelial tissues. Second, whereas the TCRγδ expressed in the peripheral lymphoid organs are diverse, those expressed in some epithelia such as those of the epidermis (DEC or s-IEL) and reproductive organs (r-IEL) are encoded by specific γ and δ genes and exhibit no structural diversity. Third, γδ thymocyte subsets having the same TCR as those of various peripheral T-cell subsets appear in ontogeny in ordered waves: Thymocytes corresponding to s-IEL and r-IEL appear in early and late

Table 6. Surface Expression of TCRγδ in KN6 γδ Transgenic Mice

		Thymocytes ($\times 10^{-6}$)			Splenocytes ($\times 10^{-6}$)		
Mice	H-2	total cells	γδ$^+$	KN6$^+$	total cells	γδ$^+$	KN6$^+$
LM	b/k	99	0.1	0	152	0.89	0
γδ	k/d	100	2.66	2.66	161	4.96	4.83
γδ	b/k	44	3.35	3.13	148	4.18	4.02
γδ	b/b	24	1.83	1.79	106	4.16	4.18

Thymus and spleen cell suspensions were stained with anti-δ (3A10) or anticlonotypic KN6 (5C10) MAbs. The H-2 haplotypes of nontransgenic and transgenic mice were determined by using anti-H-2^K MAbs.

embryos, respectively, and thymocytes corresponding to spleen, lymph node, and peripheral blood T cells appear in postnatal animals. Fourth, there is a drastic shift in the diversity of TCR$\gamma\delta$ expressed in fetal and postnatal thymuses: Fetal TCR$\gamma\delta$ are virtually of two primary structures (those for s-IEL and those for r-IEL), whereas postnatal TCR$\gamma\delta$ exhibit both combinatorial and junctional diversity. Fifth, in accordance with the fetal versus postnatal shift in the usage of γ and δ gene segments and the receptor diversity, the intrathymic localization of $\gamma\delta$ T cells changes drastically during the first few postnatal days: Fetal and neonatal $\gamma\delta$ thymocytes are closely associated with medullary epithelial cells, whereas adult $\gamma\delta$ thymocytes are scattered throughout the thymus and are most concentrated in the subcapsular areas. Sixth, although $\gamma\delta$ and $\alpha\beta$ T cells share common precursor cells, the two cell lineages seem to separate from each other early in differentiation with no coexpression stage. A transcriptional silencer element associated with the γ gene seems to play a critical role in the branching of the two cell lineages. Seventh, at least some $\gamma\delta$ T cells recognize a determinant encoded by a self-MHC gene mapped in or distal to the TL region. Preliminary data indicate that this gene encodes a new MHC class I molecule that is not known to present peptides to $\alpha\beta$ T cells. Finally, at least some self-reactive $\gamma\delta$ T cells, although activated in the thymus, are not deleted in the syngeneic thymus or spleen in contrast with the intrathymic deletion of some self-reactive $\alpha\beta$ T cells.

Despite the considerable information that has accumulated during the past few years on TCR$\gamma\delta$ and $\gamma\delta$ T cells, the biological functions of these cells remain to be determined. Below, we wish to present some thoughts on this issue.

There is considerable evidence indicating that $\gamma\delta$ T cells recognize molecules similar to but distinct from the MHC class I molecules that are used to present antigen-derived peptides to $\alpha\beta$ T cells (this paper; Bluestone et al. 1988; Bonneville et al. 1989). Although there also are reports that some $\gamma\delta$ T cells recognize allogeneic K or D region class I molecules or the I region class II molecules, these specificities may have resulted from cross-reactivity, since strong selective pressure was applied to detect them (Matis et al. 1987; Bluestone et al. 1988). Indeed, the recent finding (Y. Utsunomiya et al., unpubl.) that the subunit structure of CD8 molecules expressed on activated i-IEL and splenic $\gamma\delta$ T cells is different from that expressed on $\alpha\beta$ T cells supports the hypothesis that $\gamma\delta$ and $\alpha\beta$ T cells recognize distinct sets of class I (or class-I-like) molecules (Fig. 8).

If $\gamma\delta$ T cells generally recognize distinct class I (or class-I-like) molecules, the next question is whether or not these class I molecules present peptides to TCR$\gamma\delta$, as do the MHC class I, K, D, and L molecules to TCR$\alpha\beta$. Although no direct evidence is available at the moment, the structural similarities both between the TCR$\alpha\beta$ and TCR$\gamma\delta$ and between the class I (or class-I-like) molecules recognized by the two types of TCR suggest that the ligands for TCR$\gamma\delta$ generally include antigen-derived peptides (Fig. 8).

Are $\gamma\delta$ T cells, then, specific to a variety of antigens as are $\alpha\beta$ T cells and is the repertoire of the antigens recognized by TCR$\gamma\delta$ more or less identical with that of the antigens recognized by TCR$\alpha\beta$? Here, it would be useful to consider separately the two types of $\gamma\delta$ T-cell subsets: one with a high degree of TCR diversity such as i-IEL and splenic $\gamma\delta$ T cells and the other with invariant TCR such as s-IEL and r-IEL. In the former case, it is likely that the antigens (hence, the peptides derived from them) recognized by $\gamma\delta$ T cells are structurally diverse. However, the antigenic repertoire for $\gamma\delta$ T cells may be different from that for $\alpha\beta$ T cells. Perhaps the class I molecules recognized by TCR$\gamma\delta$ (hereafter, abbreviated as RE$\gamma\delta$, antigen-restriction elements for TCR$\gamma\delta$) have evolved to present a special set of antigens to the immune system. The selectivity of RE$\gamma\delta$ for a particular set of antigens might be explained by postulating a special intracellular pathway of peptide loading for RE$\gamma\delta$ and/or common structural features of the presented peptides. A hint as to which set of proteins may be presented efficiently by RE$\gamma\delta$ has come from recent experiments that have shown the recognition of mycobacterial heat-shock-like proteins by some $\gamma\delta$ T cells (Holoshitz et al. 1989; Janis et al. 1989; Modlin et al. 1989; O'Brien et al. 1989). It may be that $\gamma\delta$ T cells with diverse TCR are primarily directed to a variety of mycobacteria and parasitical protozoa known to produce constitutively structurally

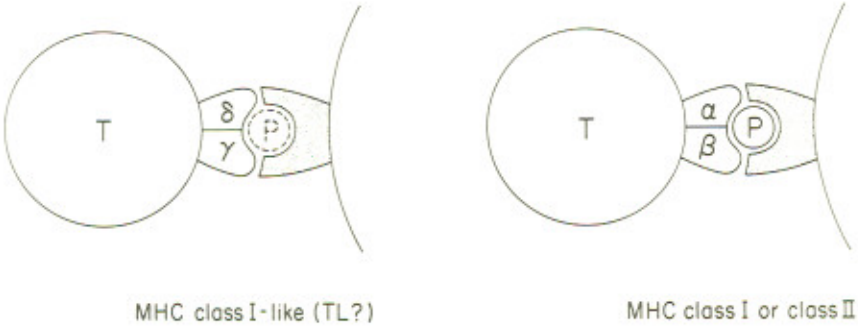

Figure 8. Comparison of the proposed recognition by a $\gamma\delta$ T cell with the established recognition by a $\alpha\beta$ T cell.

related but distinct heat-shock-like proteins. The effector function of the γδ T cells is unknown, but one might speculate that these cells play a role in the initiation or regulation of defense reactions by lymphokine secretion or elimination of undesirable cells such as persistent antigen-presenting cells.

In contrast with a γδ T-cell subset capable of recognizing structurally diverse ligands, a γδ T-cell subset with an invariable TCR must recognize an equally invariable ligand. Despite this important difference in the diversity of the ligands, it is likely that the basic composition (i.e., a peptide/RE complex) of the ligand recognized by the invariant TCRγδ is the same as that of the ligands recognized by diverse TCRγδ. Assuming that this is indeed the case, the key issue is again the origin of the peptide. Because it does not make sense for an organism to secure an entire subset of T cells localized in an organ for the protection against just a single foreign antigen, we should consider an alternative role and/or mechanism for the undiversified γδ T cells. Perhaps these γδ T cells recognize a tissue-specific host antigen whose synthesis may be induced in the epithelial cells by a variety of unfavorable stimuli, such as viral infections, toxic chemicals, radiation, heat shock, and malignancy (Janeway et al. 1988). One candidate of such a host antigen is a stress protein whose synthesis may be induced in tissue-specific fashion. Since at least some of these proteins are known to be structurally related to mycobacterial heat-shock-like proteins, it is conceivable that the peptides derived from them could also effectively bind to and be presented by REγδ. Since the postulated antigen is induced only under specific conditions, autoreactivity of these γδ T cells may not have any hazardous consequences but rather play an important regulatory and/or effector role. The peculiar specificity of γδ T cells envisaged in our model is somewhat reminiscent of the specificity of another class of lymphocytes, those called CD5 B cells. These B cells also appear to preferentially recognize common bacterial antigens as well as self-antigens.

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