

## Mechanism of Self-Tolerance of $\gamma/\delta$ T Cells in Epithelial Tissue

By Terrence A. Barrett,\* Michelle L. Delvy,\* David M. Kennedy,\*  
Leo Lefrancois,† Louis A. Matis,§ Alexander L. Dent,||  
Stephen M. Hedrick,|| and Jeffrey A. Bluestone\*

From the \*Ben May Institute, University of Chicago, Chicago, Illinois 60637; the †Division of Cell Biology, The Upjohn Company, Kalamazoo, Michigan 49001; the §Division of Cytokine Biology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland 20892; and the ||Department of Biology and Cancer Center, University of California, San Diego, La Jolla, California 92093

### Summary

The present study examined mechanisms of tolerance for T cell receptor  $\gamma/\delta$  (TCR- $\gamma/\delta$ ) cells. Using a transgenic (Tg) model, we demonstrate that although alloantigen (Ag)-specific TCR- $\gamma/\delta$  cells are deleted in the thymus and spleen of Ag-bearing mice, intraepithelial lymphocytes (IELs) expressing normal levels of the Tg TCR were present. However, Tg<sup>+</sup> IELs from Ag-bearing mice were unresponsive to activation. Furthermore, self-reactive Tg<sup>+</sup> IELs decreased in number over time. Thus, in epithelial tissue, Tg TCR- $\gamma/\delta$  cells are eliminated subsequent to and most likely as a result of the induction of clonal anergy.

Clonal deletion of immature self-reactive TCR- $\alpha/\beta$  cells is the major intrathymic mechanism for the maintenance of T cell tolerance (1–7). Self-reactive peripheral TCR- $\alpha/\beta$  cells that escape thymic clonal deletion or encounter antigen in the periphery can be rendered unresponsive by clonal anergy (8–15) or be eliminated in the periphery (16, 17). However, less is known about the maintenance of self-tolerance among TCR- $\gamma/\delta$  cells. These T cells, which comprise a small subset of T cells in peripheral lymphoid tissue, predominate in epithelial linings of the skin, intestine, lung, and reproductive organs (18). To examine self-tolerance in TCR- $\gamma/\delta$  cells, we generated transgenic (Tg)<sup>1</sup> mice expressing an alloreactive TCR- $\gamma/\delta$ . Earlier studies demonstrated that Tg TCR- $\gamma/\delta$  cells localizing to peripheral lymphoid tissue in alloantigen (Ag)-bearing mice were deleted in the thymus (19). In the present study, we examined tolerance of Tg TCR- $\gamma/\delta$  intraepithelial lymphocytes (IELs) in Ag-bearing H-2<sup>b</sup> mice.

### Materials and Methods

**Animals.** Adult H-2<sup>d/d</sup> and H-2<sup>b/d</sup> normal and transgenic mice (Tg<sup>d/d</sup> and Tg<sup>b/d</sup>) were generated by the cross of a Tg<sup>d/d</sup> (founder no. 75  $\times$  BALB/c)F<sub>1</sub> male (19) to either C57BL/10 or BALB/c females. Mice were raised in conventional conditions in the University of Chicago Carlson animal facility.

**Immunohistochemistry.** Tissue sections using fresh tissue from

3–5-wk-old mice were snap-frozen, and 6- $\mu$ m sections were fixed with cold acetone, and stained with mAbs: anti-V $\gamma$ 3 (F536) (20), anti-pan-TCR- $\gamma/\delta$  (GL3) (21), or anti-V $\gamma$ 2 (UC3-10A6) (19); affinity-purified goat anti-hamster IgG-biotin and avidin-horseradish peroxidase conjugates (Vector, Burlingame, CA). The specificity of the staining was confirmed by control staining of adjacent sections with an irrelevant hamster mAb.

**Cell Isolation and Immunofluorescence Analysis.** Intestines were removed from adult mice, and intestinal IELs (iIELs) were isolated as previously described (21). The following mAbs coupled to FITC, PE, or biotin (followed by streptavidin-PE; Southern Biotechnology, Birmingham, AL) were used: anti-TCR- $\gamma/\delta$ -FITC, anti-V $\gamma$ 2-FITC, anti-TCR- $\alpha/\beta$  (H57-597)-FITC (22), anti-IL-2R-biotin (PC61) (23), and anti-Lyt-2 (CD8 $\alpha$ ) (53-6.7) (24)-PE (all mAbs except for PC61 were purchased from PharMingen, San Diego, CA). Dead cells were excluded from analysis on the basis of propidium iodide staining. Flow cytometric analysis was performed on a FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA), and data were analyzed on the Consort 30 program.

**Proliferation of iIELs.** Isolated iIELs were cultured with splenic APC or immobilized anti-V $\gamma$ 2 mAb. The proliferative activity was assessed by [<sup>3</sup>H]thymidine uptake.  $2 \times 10^5$  irradiated anti-Thy-1 mAbs (AT83A) (a gift from F. Fitch, University of Chicago) and complement-treated splenic APC from H-2<sup>b</sup> or H-2<sup>d</sup> mice were co-cultured with  $2 \times 10^5$  responder iIELs in 96-well flat-bottomed microtiter plates. In some experimental groups, wells were coated overnight at 4°C with 50  $\mu$ l of purified anti-V $\gamma$ 2 mAb (30  $\mu$ g/ml). Exogenous human rIL-2 (60 U/ml) (Cetus Corp., Emeryville, CA) was added when indicated. Irradiated syngeneic H-2<sup>d</sup> splenic APC were added to all anti-V $\gamma$ 2-coated wells at  $2 \times 10^5$  cells/well. Responder iIELs were isolated as described and sorted from non-iIELs on the basis of forward angle and 90° light scatter. Sorting was

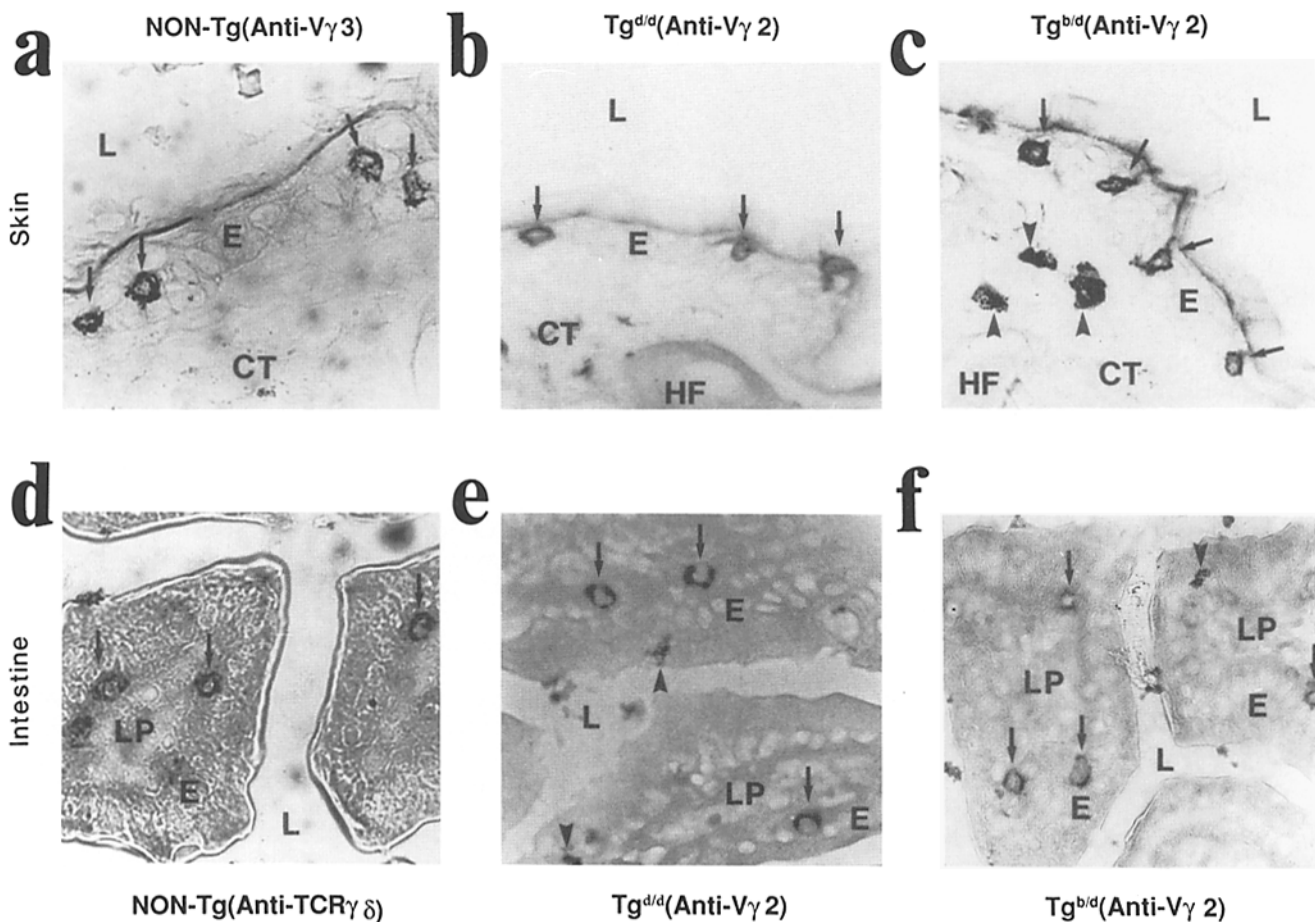
<sup>1</sup> Abbreviations used in this paper: Ag, alloantigen; IEL, intraepithelial lymphocyte; iIEL, intestinal IEL; sIEL, skin IEL; Tg, transgenic.

performed on an Epics 753 flow cytometer (Coulter Electronics, Hialeah, FL). At 36 h, cultures were pulsed for 18 h with [<sup>3</sup>H]thymidine (1 μCi/well). Cells were collected and analyzed in a scintillation counter.

**Lymphokine Production by iIELs.** iIELs were isolated and sorted as described and co-cultured in 24-well plates (4 × 10<sup>6</sup> cells/well) with irradiated H-2<sup>d/d</sup> or H-2<sup>b/b</sup> APC (4 × 10<sup>6</sup> cells/well) for 10, 27, or 60 h. Supernatant was analyzed for IFN-γ, IL-3, and IL-2 as previously described (25), and the maximal concentrations were reported. Briefly, IFN-γ concentrations were determined using an ELISA and compared to a known standard. IL-3 activity was assessed using a subclone of the FDCP1 cell line that responded to IL-3 and not granulocyte/macrophage (GM)-CSF. IL-2 activity was assessed using a subclone of the HT-2 cell line that did not respond to GM-CSF. An anti-IL-2 mAb (S4B6) was used for specifically blocking HT-2-stimulating activity in each supernatant. The responses of the FDCP1 and HT-2 cell lines were determined using a colorimetric MTT (3-[4,5-demethylthiazol-2-yl]-2,5-dephenyl-tetrazolium bromide) assay and the titer compared to a known concentration of purified IL-3 (Genzyme, Boston, MA) or murine rIL-2 (DNAX, Palo Alto, CA).

## Results and Discussion

Tg mice were generated by inserting the productively rearranged TCR γ (Vγ2/Jγ1/Cγ1) and δ (Vα11/Dδ2/Jδ1/Cδ1) genes isolated from an alloreactive BALB/c-derived H-2<sup>d</sup> T cell clone (G8) specific for an MHC class I gene product encoded in the TL region of the MHC of H-2<sup>k</sup> and H-2<sup>b</sup> strains of mice (26). In syngeneic Tg<sup>d/d</sup> mice, functional Tg TCR-γ/δ cells dominated the thymus as well as peripheral lymphoid organs (19). Since the TCR γ and δ genes expressed in this transgenic mouse were not normally found in epithelial tissues, both skin IELs (sIELs) and iIELs were examined by immunohistochemistry to determine whether endogenous TCR-γ/δ cells were replaced by Tg TCR-γ/δ cells. In normal Tg littermates, sIELs exclusively expressed the Vγ3 gene element (Fig. 1 a), consistent with previous observations (20). In contrast, all sIELs in Tg<sup>d/d</sup> mice expressed the Vγ2 gene segment encoded by the transgene (Fig. 1 b). Similar results were found in the intestine where iIELs from Tg<sup>d/d</sup> mice ex-



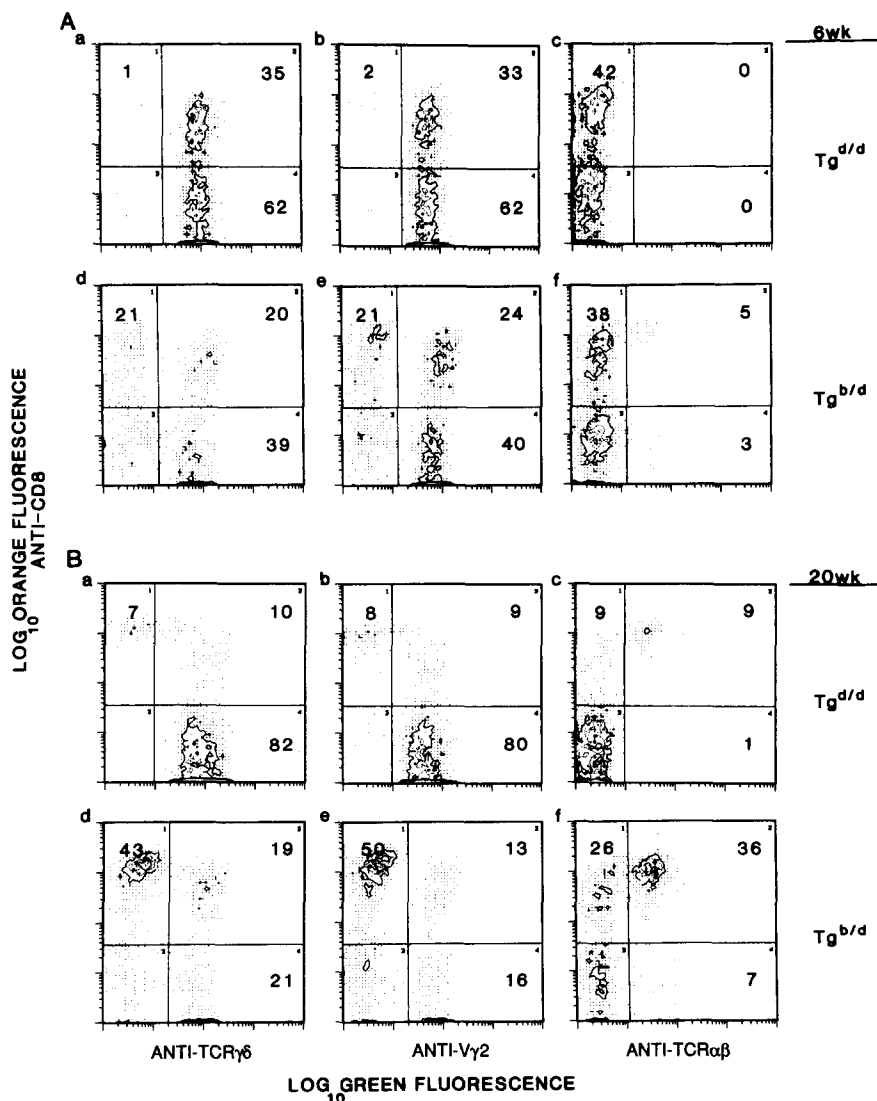
**Figure 1.** Potentially self-reactive IELs are present in skin and intestine of TCR-γ/δ transgenic mice. No staining of sIELs was observed using anti-Vγ3 in Tg mice and only rare staining (<1%) of iIELs was observed with anti-Vγ2 in non-Tg mice. Arrows denote some of the stained TCR-positive cells. Arrowheads indicate endogenous or nonspecific peroxidase activity. E, epithelium; L, lumen or exterior; CT, connective tissue; HF, hair follicle; LP, lamina propria.

clusively expressed V $\gamma$ 2 (Fig. 1 *e*) rather than the usually predominant V $\gamma$ 5 gene element (data not shown) (21). Taken together, these results confirm previous reports suggesting that localization of TCR- $\gamma/\delta$  cells to intraepithelial tissue does not depend on TCR usage (27).

Given the expression of the Tg TCR in Tg<sup>d/d</sup> mice, we examined the effect of introducing the Ag into the transgenic environment. In previous studies, we demonstrated that alloreactive Tg TCR- $\gamma/\delta$  cells were largely deleted from the thymus and totally absent from the spleen of Ag-expressing Tg<sup>b/d</sup> mice (19). However, in the epithelial lining of the skin or intestine there is no qualitative difference in the appearance of Tg TCR- $\gamma/\delta$  cells of Tg<sup>d/d</sup> or Tg<sup>b/d</sup> mice (Fig. 1, *b* and *c*, and *e* and *f*, respectively), all of which express the Tg TCR. To examine Tg TCR- $\gamma/\delta$  cells in Ag-bearing animals in detail, iIELs were purified and analyzed by flow cytometry. Several similarities existed among the Tg<sup>+</sup> iIELs from Tg<sup>d/d</sup> and Ag-bearing Tg<sup>b/d</sup> mice. First, cell surface TCR density was equivalent in Tg<sup>d/d</sup>, Tg<sup>b/d</sup> (Fig. 2 *A*, *a*, *b*, *d*, and

*e*) and non-Tg iIELs (data not shown). Second, unlike the Tg cells found in lymphoid tissues, a significant percentage of the Tg iIELs were CD8 $\alpha$ <sup>+</sup> (Fig. 2 *A*, *b* and *e*). However, the percentage of CD8 $\alpha$ <sup>+</sup> iIELs was significantly less than among normal iIELs, which were typically >85% CD8 $\alpha$ <sup>+</sup> (data not shown). Therefore, CD8 $\alpha$  expression on Tg iIELs is not necessary for homing or localization. Third, the CD8 $\alpha$ <sup>+</sup> subset of Tg<sup>+</sup> iIELs preferentially decreased over time in Tg<sup>d/d</sup> and Tg<sup>b/d</sup> mice (Fig. 2 *A*, *b* and *e* vs. Fig. 2 *B*, *b* and *e*). The preferential depletion of CD8 $\alpha$ <sup>+</sup> cells may reflect a loss of the CD8 $\alpha$  protein on the cell surface of resident iIELs or the influx of new Tg TCR- $\gamma/\delta$  cells that derive from a CD4<sup>-</sup>CD8<sup>-</sup> population. Therefore, in contrast to previous observations in peripheral lymphoid tissue (19), significant numbers of potentially self-reactive iIELs with normal surface density of TCR- $\gamma/\delta$  were present in H-2<sup>b/d</sup> mice.

To examine the function of Tg TCR- $\gamma/\delta$  cells, the proliferative responses of isolated iIELs from Tg<sup>d/d</sup>, Tg<sup>b/d</sup>, and non-



**Figure 2.** Deletion occurs in epithelial tissues of Ag-bearing mice. Flow cytometric analysis of iIELs from Tg<sup>d/d</sup> and Tg<sup>b/d</sup> mice at (A) 6 wk and (B) 20 wk. Quadrants were determined on the basis of control staining with irrelevant hamster-FITC and rat-PE mAbs. The percentage of positive cells in each quadrant is shown.

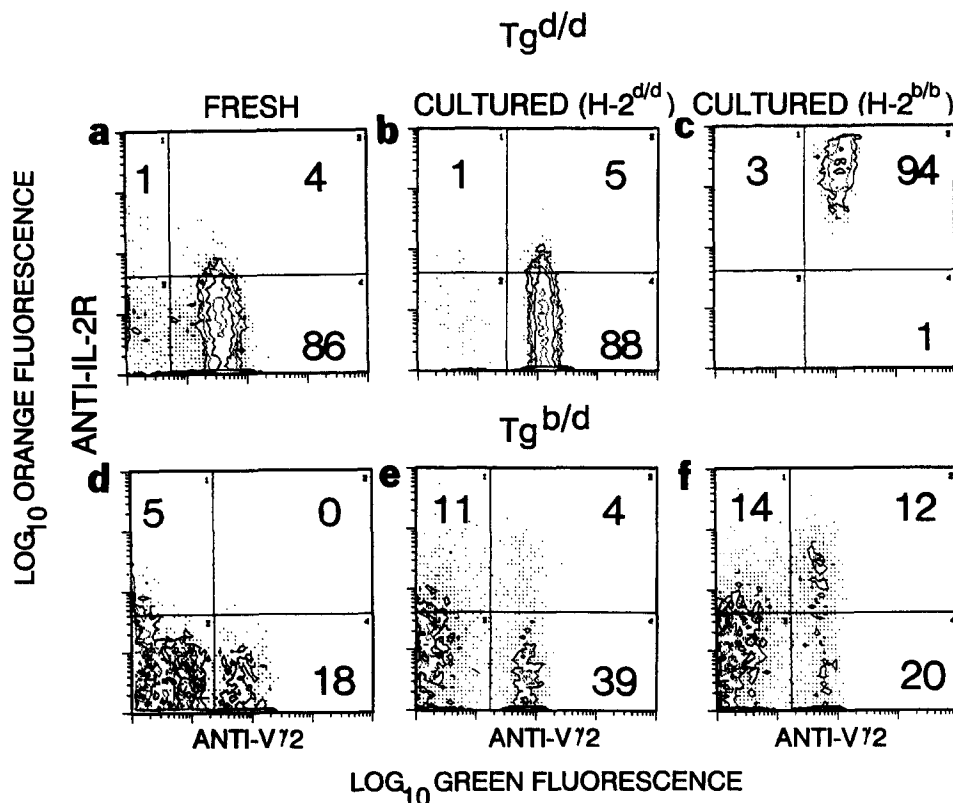
**Table 1. Unresponsiveness of Intestinal IELs from Alloantigen-expressing Transgenic Mice as Measured by Proliferation**

Stimulus	rIL-2	Proliferative response		
		Tg <sup>d/d</sup>	Tg <sup>b/d</sup>	Non-Tg
<i>cpm × 10<sup>-3</sup></i>				
H-2 <sup>b</sup> APC	-	177.5	3.3	0.8
	+	149.1	17.1	5.4
H-2 <sup>d</sup> APC	-	0.3	0.6	1.1
	+	1.8	6.9	4.9
Anti-Vγ2 mAb	-	35.1	0.5	0.6
	+	79.9	3.7	2.7

Measurements were made in triplicate and expressed as geometric mean. The SEM was <5% in all groups. Results are representative of three separate experiments.

Tg mice were assessed after stimulation by syngeneic H-2<sup>d</sup> and allogeneic H-2<sup>b</sup> splenic APCs. As seen in Table 1, Tg<sup>d/d</sup> iIELs proliferated vigorously in response to H-2<sup>b</sup> but not H-2<sup>d</sup> APCs (stimulation index [SI] = 562). There was no significant allogeneic MLR detected in non-Tg iIEL cultures at 36 h. In contrast to Tg<sup>d/d</sup> iIELs, purified iIELs from Tg<sup>b/d</sup> mice proliferated 100-fold less well to H-2<sup>b</sup> splenic APCs (SI = 5.4) indicative of an unresponsive or anergic state (14).

In addition, Tg H-2<sup>b/d</sup> iIELs could not be activated by immobilized anti-Vγ2 mAb (Table 1). Thus, Tg iIELs were unresponsive as measured both by Ag reactivity as well as TCR crosslinking by anti-TCR mAb. Since the anti-Vγ2 mAb activates independently of δ usage, the nonresponsiveness of the Tg<sup>b/d</sup> iIELs is not due to their inability to recognize antigen. In several examples of tolerance, including another TCR-γ/δ Tg model, exogenous IL-2 will reconstitute the aborted proliferative response of antigen-stimulated T cells (28, 29). Therefore, rIL-2 (60 u/ml) was added to the cultures of H-2<sup>b</sup>-stimulated Tg<sup>b/d</sup> iIELs. The addition of rIL-2 to these cultures did not reconstitute the alloresponse (SI = 2.5). These results suggested that the lack of proliferation exhibited by Tg<sup>b/d</sup> iIELs was due, in part, to deficient IL-2R upregulation. Stimulation of Tg<sup>d/d</sup> iIELs with alloantigen led to a dramatic increase in IL-2R α chain expression on all Tg TCR-γ/δ cells (Fig. 3 c; mean fluorescence index [MFI] = 236). In comparison, IL-2R was induced on only a small subset of Tg<sup>b/d</sup> iIELs and the level of IL-2R was <2% of that observed in activated Tg<sup>d/d</sup> iIELs (Fig. 3 f). Thus, the state of unresponsiveness in Tg<sup>b/d</sup> iIELs was characterized by a limited upregulation of IL-2R in response to alloantigen. Finally, lymphokine production by Tg<sup>d/d</sup> and Tg<sup>b/d</sup> iIELs was examined (Table 2). IL-2, IL-3, and IFN-γ were induced in alloantigen-stimulated cultures of Tg<sup>d/d</sup> iIELs, whereas no IL-2, IL-3, or IFN-γ was detected in Tg<sup>b/d</sup> iIELs cultured with allogeneic APCs. Thus, functional analysis of potentially self-reactive Tg<sup>b/d</sup> iIELs demonstrated a global level of unresponsiveness after Ag stimulation, including the lack of proliferation, IL-2R induction, and lymphokine production.



**Figure 3.** Upregulation of IL-2R α chain expression is deficient in Tg IELs from alloantigen-bearing H-2<sup>b/d</sup> mice. Flow cytometric analysis of iIELs were performed immediately after isolation (FRESH) or after 18 h of culture (CULTURED) with syngeneic H-2<sup>d/d</sup> APCs or allogeneic H-2<sup>b/b</sup> APCs (see Materials and Methods). The percentage of positive cells in each quadrant is shown.

**Table 2.** *Lymphokine Production from Purified iIELs*

Mice	Stimulus	Maximal lymphokine production		
		IL-2	IL-3	IFN- $\gamma$
			<i>U/ml</i>	
Tg <sup>d/d</sup>	H-2 <sup>b</sup>	200	1,800	250
	H-2 <sup>d</sup>	<10	<30	<10
Tg <sup>b/d</sup>	H-2 <sup>b</sup>	<10	<30	<10
Non-Tg	H-2 <sup>b</sup>	<10	<30	<10

These in vitro findings are consistent with the absence of any detectable autoimmune or inflammatory response in vivo.

One difference between Tg<sup>d/d</sup> and Tg<sup>b/d</sup> mice was the observation that the percentage of Tg TCR- $\gamma/\delta$  iIELs in Ag-bearing Tg<sup>b/d</sup> mice was consistently less than in Tg<sup>d/d</sup> mice (Fig. 2 A, a and b vs. d and e). This was especially apparent in 20-wk-old Tg<sup>b/d</sup> mice (Fig. 2 B, a and b vs. d and e) in which Tg<sup>+</sup> iIELs decreased to 35%, whereas in Tg<sup>d/d</sup> mice, 90% of iIELs were Tg TCR- $\gamma/\delta$  cells (Fig. 2 B, b vs. e). Conversely, the corresponding percentages of TCR- $\alpha/\beta$  iIELs increased substantially in 20-wk-old Tg<sup>b/d</sup> mice compared with Tg<sup>d/d</sup> or 6-wk-old Tg<sup>b/d</sup> mice (Fig. 2 B, f vs. c; or Fig. 2 A, f; 50% vs. 8% or 12% of T cells, respectively). Also, in 20-wk-old Tg<sup>b/d</sup> mice a distinct population of non-Tg TCR- $\gamma/\delta$  iIELs was detected based on the percentage of total TCR- $\gamma/\delta$  cells that expressed V $\gamma$ 2 (Fig. 2 B, d and e; 40% and 29%, respectively). These data suggested that substantial deletion or turnover was occurring in epithelial tissues of Ag-bearing animals.

In summary, these results demonstrate that  $\gamma/\delta$  T cells expressing the same self-reactive transgenic TCR were tolerated by either intrathymic deletion or anergy depending on

the anatomic site of localization. The initial presence of TCR- $\gamma/\delta$  cells in epithelial tissues may be due to one of several alternative possibilities. First, TCR- $\gamma/\delta$  cells that home to the intraepithelial compartments may develop extrathymically (30) or mature early in fetal thymic ontogeny, before expression of the alloantigen thereby circumventing or escaping intrathymic deletion. Second, some thymically derived TCR- $\gamma/\delta$  cells may be induced into a state of anergy and programmed for cell death but in some cases may be exported before intrathymic deletion. This is consistent with another TCR- $\gamma/\delta$  transgenic model where tolerance was maintained by intrathymic inactivation (29). In either case, it appears that potentially self-reactive TCR- $\gamma/\delta$  cells can be detected in peripheral tissue in a state of anergy. However, the results in Fig. 2 suggest that substantial elimination of anergic cells occurred in epithelial tissues over time. Therefore, it would appear that TCR- $\gamma/\delta$  cells in epithelial tissues may become anergic before apoptosis and clonal deletion, perhaps in much the same manner as thymic TCR- $\gamma/\delta$  cells. However, the observed phenotype (clonal deletion or clonal anergy) may depend on the window of observation. The kinetics of these events may be substantially different in these nonlymphoid tissues due to local factors such as the nature of the APC presenting the self-Ag in the periphery, the extrathymic development of iIELs, or soluble factors.

The reversal of previously tolerized self-reactive T cells may be instrumental in the initiation of autoimmune disease (31). For this reason, cells that are not deleted but rather persist in a state of anergy remain a potential risk to the host. Recently, it was shown that V $\beta$ 6-, V $\beta$ 8.1-, and V $\beta$ 11-expressing cells, normally deleted in the thymus of DBA/2 (Mls<sup>a</sup>, IE<sup>+</sup>) mice, were detected in the intestine although functional analyses were not assessed (32). Thus, if the observations in this Tg model reflect the normal mechanism for maintaining tolerance among T cells localizing to epithelial tissues, these cells may represent a reservoir of self-reactive T cells with the potential of mediating autoimmune disease.

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Address correspondence to Terrence Barrett, The Ben May Institute, The University of Chicago, 5841 South Maryland Avenue, Box 424, Chicago, IL 60637.

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