

# Tolerance of T Cell Receptor $\gamma/\delta$ Cells in the Intestine

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## Summary

The present study examined the mechanism(s) of tolerance induction for intestinal intraepithelial lymphocytes (iIELs) using an alloantigen (Ag)-specific  $\gamma/\delta$  T cell receptor (TCR  $\gamma/\delta$ ) transgenic (Tg) model. In Tg Ag-bearing H-2<sup>b/d</sup> mice (Tg<sup>b/d</sup>), Tg iIELs were Thy-1<sup>-</sup>, CD44<sup>+</sup>, CD45R (B220)<sup>+</sup>, and CD5<sup>+</sup>, whereas in syngeneic Tg<sup>d/d</sup> mice, iIELs were Thy-1<sup>+</sup>, CD44<sup>-</sup>, and CD45R<sup>-</sup> with a subset of CD5<sup>+</sup> cells. Previously, we had shown that tolerance for Tg<sup>b/d</sup> iIELs involved functional anergy and deletion (Barrett, T. A., M. L. Delvy, D. M. Kennedy, L. Lefrancois, L. A. Matis, A. L. Dent, S. M. Hedrick, and J. A. Bluestone. 1992. *J. Exp. Med.* 175:65). In this study we demonstrate that Tg<sup>b/d</sup> iIELs expressing dull levels of Thy-1 proliferated in the presence of exogenous rIL-2. A direct precursor-product relationship between the Thy-1<sup>+</sup>-responsive iIELs and the tolerant Thy-1<sup>dull/-</sup> iIELs was demonstrated by adoptive transfer into severe combined immunodeficient (SCID) mice. Tg Thy-1<sup>+</sup> iIELs reconstituting Ag<sup>+</sup> but not Ag<sup>-</sup> SCID mice downregulated Thy-1 after Ag exposure in vivo. Analysis of bone marrow (BM) chimeras demonstrated the persistence of Tg iELs in all Ag<sup>+</sup> chimeras although a modest degree of clonal deletion was apparent. The greatest percentage of Tg iELs were detected when Ag was restricted to radioresistant cells (e.g., epithelial cells) compared with BM-derived antigen-presenting cells (APC). This was especially apparent in thymectomized chimeric mice. Consistent with the notion that Ag-bearing epithelial cells may be poor APC, isolated intestinal epithelial cells from Ag-bearing mice failed to stimulate Tg iIELs compared with splenic APC. These studies suggest that the major population of TCR  $\gamma/\delta$  iIELs were probably extrathymically derived and encountered self-Ag on intestinal epithelial cells. The induction of tolerance likely involved an activation event resulting in downregulation of Thy-1. These mechanisms of tolerance for TCR  $\gamma/\delta$  iIELs led to the persistence of a reservoir of self-reactive T cells with the potential for mediating autoimmune disease.

The triggering of self-reactive T cells is thought to be the initiating event in many autoimmune diseases (1). The self-destructive process frequently targets nonlymphoid tissues such as synovium, pancreas, serosa, skin, and intestine. Several models have been proposed to explain these inappropriate antiseif immune reactions. First, self-proteins may be induced during viral infections or injuries that are recognized by immune T cells not previously rendered tolerant (2). Alternatively, antigenic mimicry by viral antigens may activate previously tolerant T cells that crossreact on self-tissue (3). In both cases, potentially self-reactive cells are present that have not undergone classical thymic deletion. Unlike the majority of autoreactive cells that interact with self-antigen in the thymus, the induction of tolerance for T cells in nonlymphoid tissue may involve nondeletional mechanisms (4). In these tissues, T cells may encounter self-antigen on "non-

professional" APC that lack the full capacity for antigen presentation (5). Therefore, the induction of tolerance in nonlymphoid tissue may lead to the persistence of T cells with the potential for mediating autoimmune disease.

T cells in the intestinal epithelial compartment are poised for potential autoimmune responses. Previous studies have shown that potentially self-reactive intestinal intraepithelial lymphocytes (iIELs) may be detected (6-9). Furthermore, it has been suggested that the self-reactive iIELs developed extrathymically and thus were not susceptible to normal clonal deletion mechanisms in the thymus (6-9). T cells that are derived in the extrathymic pathway may then interact with antigens presented on nonprofessional APC. Thus, clonal inactivation rather than activation may occur after antigen exposure.

Our approach to examining the potential for iIELs to mediate self-reactivity in the intestine has been the use of TCR transgenic (Tg) mice. The TCR transgenes used in these studies were derived from a  $\gamma/\delta$  T cell specific for an MHC

<sup>1</sup> Abbreviations used in this paper: Ag, alloantigen; BM, bone marrow; iIEL, intestinal intraepithelial lymphocyte; SI, stimulation index; Tg, transgenic.

class I Ag encoded in the TL region of H-2<sup>b</sup> and H-2<sup>k</sup> but not in syngeneic H-2<sup>d</sup> strains of mice (10). This Ag is expressed ubiquitously on all tissues, including those present in the intestine. Transgene-expressing TCR  $\gamma/\delta$  cells constituted a major component of the iIEL population in both syngeneic and Ag-bearing strains (6). This animal provided an excellent model for studying regulation of self-reactivity in this tissue, especially during extrathymic development.

Initial studies demonstrated that transgenic TCR  $\gamma/\delta$  cells were deleted in the thymus and spleen of Ag-bearing mice (10). However, Tg iIELs expressing normal levels of the transgenic TCR were present. Furthermore, Tg iIELs from Ag-bearing mice were unresponsive to activation and decreased in number over time (6). 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.

In this study, the phenotypic and functional consequences of self-Ag exposure on tolerance induction were examined. The results showed that Tg iIELs present in Ag-bearing mice expressed decreased levels of Thy-1 cell surface antigen as compared with syngeneic Tg mice. Bone marrow (BM) chimeras and adoptive transfer studies into SCID mice demonstrated that this phenotype resulted from downregulation of Thy-1 on iIELs. In addition, both clonal deletion and clonal anergy were evident in this model. Clonal deletion was most apparent in BM chimeras in which Ag was restricted to BM-derived cells, whereas clonal anergy dominated in chimeras expressing Ag only on radioresistant host cells. These results suggest that the mechanism for maintaining tolerance depends on the nature of the APC in the periphery, the presence of an extrathymic pathway for T cell differentiation, and the location of the potentially autoreactive T cells. Thus, this mechanism for maintaining tolerance in peripheral tissue such as the intestine may be important in autoimmune disease.

## Materials and Methods

**Animals.** Adult H-2<sup>d/d</sup> and H-2<sup>b/d</sup> Tg mice (Tg<sup>d/d</sup> and Tg<sup>b/d</sup>, respectively) were generated by breeding a Tg<sup>d/d</sup> (founder no. 75  $\times$  BALB/c)F<sub>1</sub> male (10) to either C57BL/10 (B10) or BALB/c females. BALB/c and CB6 F<sub>1</sub> (H-2<sup>b/d</sup>) mice were used as host animals for generation of BM chimeras. C3H SCID mice were bought from The Jackson Laboratory (Bar Harbor, ME) and C.B-17 SCID mice were a gift of M. Bosma (Fox Chase Cancer Center, Philadelphia, PA), bred at the University of Chicago (Chicago, IL). Mice were raised under specific pathogen-free conditions in the University of Chicago animal barrier facility.

**Cell Isolation and Immunofluorescence Analysis.** Intestines were removed from adult mice and iIELs isolated as previously described (11). Epithelial cells were isolated as iIELs with slight modifications. Epithelial cells were isolated by washing intestinal fragments after 15 min of digestion. In some cases digestion media containing Dispase (3 mg/ml) (Boehringer Mannheim Biochemicals, Indianapolis, IN) instead of EDTA was used for isolation. Cells were immediately washed in ice-cold DMEM. After washing, cells were suspended in 40% Percoll and layered over 66% Percoll, then centrifuged at 600 *g* for 15 min. Epithelial cells were collected at the interface and washed. Viable epithelial cells were then sorted on the basis of forward and 90° light scatter and staining with the

anti-epithelial mAb, G8.8a (12), followed by biotin (BIO)-coupled rabbit anti-rat (mouse-absorbed) (Vector Laboratories, Burlingame, CA) and Streptavidin (StAv)-PE (Southern Biotechnology Associates, Birmingham AL). The purified epithelial cells were >75% viable at 24 h and >60% viable at 48 h. The following mAbs coupled to BIO, FITC, or PE were used for flow cytometry studies: anti-V $\gamma$ 2 (UC3-10A6) (10), anti-CD44, anti-IL-2R $\alpha$  (13), anti-CD45R/B220, anti-CD5, and anti-Thy-1. Streptavidin-CyChrome was used to visualize in three colors (all mAbs and StAv-CyChrome were purchased from PharMingen, San Diego, CA). When two-color analysis was performed, dead cells were excluded from analysis on the basis of propidium iodide dye exclusion. Flow cytometric analysis was performed on a FACScan® (Becton Dickinson & Co., Mountain View, CA), and data were analyzed using Consort 30 software.

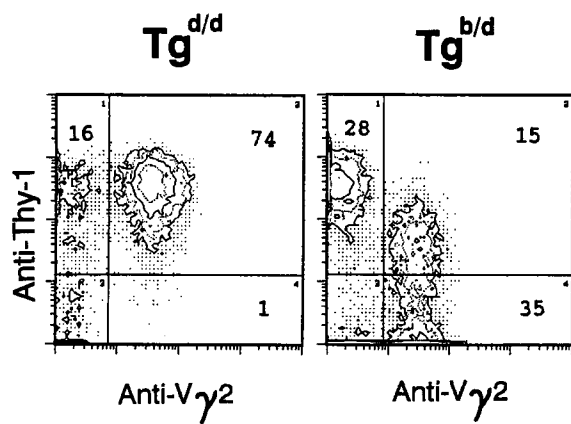
**Proliferation of iIELs.** Isolated iIELs were cultured with splenic or epithelial APC. The proliferative activity was assessed by [<sup>3</sup>H]thymidine uptake.  $2 \times 10^5$  irradiated, epithelial cells or anti-Thy-1 mAb (AT83A) (a gift from F. Fitch, University of Chicago, Chicago, IL) complement-treated splenic APC from H-2<sup>b</sup> or H-2<sup>d</sup> mice were cocultured with  $2 \times 10^5$  responder iIELs in 96-well flat-bottomed microtiter plates. Exogenous human rIL-2 (50 U/ml) (Cetus Corp., Emeryville, CA) was added when indicated on day 1 of culture. Responder iIELs were isolated as described (11) and separated from other intestinal cells (i.e., epithelial cells) on the basis of forward angle and 90° side scatter, and in some cases on the basis of staining with anti-V $\gamma$ 2-FITC and/or anti-Thy-1-PE. Sorting was performed on an Epics 753 flow cytometer (Coulter Immunology, Hiialeah, FL). At 48 h, cultures were pulsed for 18 h with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well). Cells were collected and analyzed in a scintillation counter.

**Chimeras.** BM chimeras were constructed by reconstituting gamma-irradiated (800 rad; Cesium source) BALB/c or CB6 F<sub>1</sub> mice with  $10^7$  BM cells intravenously. BM cells from Tg<sup>d/d</sup> and Tg<sup>b/d</sup> donors were T cell depleted using anti-Thy-1 mAb and complement. In some cases, host mice were thymectomized 1 wk before irradiation as described (14). Mice were analyzed 8 wk after reconstitution.

**SCID Mice.** C.B-17 or C3H SCID mice were injected intravenously with  $0.6-1 \times 10^7$  iIELs from Tg<sup>d/d</sup> donor mice. Donor iIELs were isolated and purified for Thy-1<sup>+</sup> cells using a magnetics-activated cell sorter (MACS) (Becton Dickinson & Co.) as described (15). Briefly, iIELs were stained with biotin-conjugated anti-Thy-1 mAb, counterstained with StAv-PE (Southern Biotechnology Associates), and biotin-magnetic beads were then separated on a MACS column. Intestinal iIELs were isolated from SCID mice 6 wk after reconstitution.

## Results

**Phenotype of Tg iIELs in Syngeneic and Ag-bearing Mice.** We have previously shown that functionally unresponsive Tg iIELs cells are present in Tg<sup>b/d</sup> mice despite evidence for deletion of Tg thymocytes (6). This suggested that Tg<sup>b/d</sup> iIELs may undergo tolerance induction in a developmentally distinct pathway from cells localizing to peripheral lymphoid tissue. Our initial approach to analyzing peripheral T cell tolerance in this tissue was to examine the cell surface phenotype of iIELs in Tg<sup>d/d</sup> and Tg<sup>b/d</sup> mice. It has been well documented that iIELs are a heterogeneous population that differ in Thy-1 expression (11). Interestingly, Thy-1 was expressed at uniformly high levels on iIELs isolated from Tg<sup>d/d</sup> mice. In con-

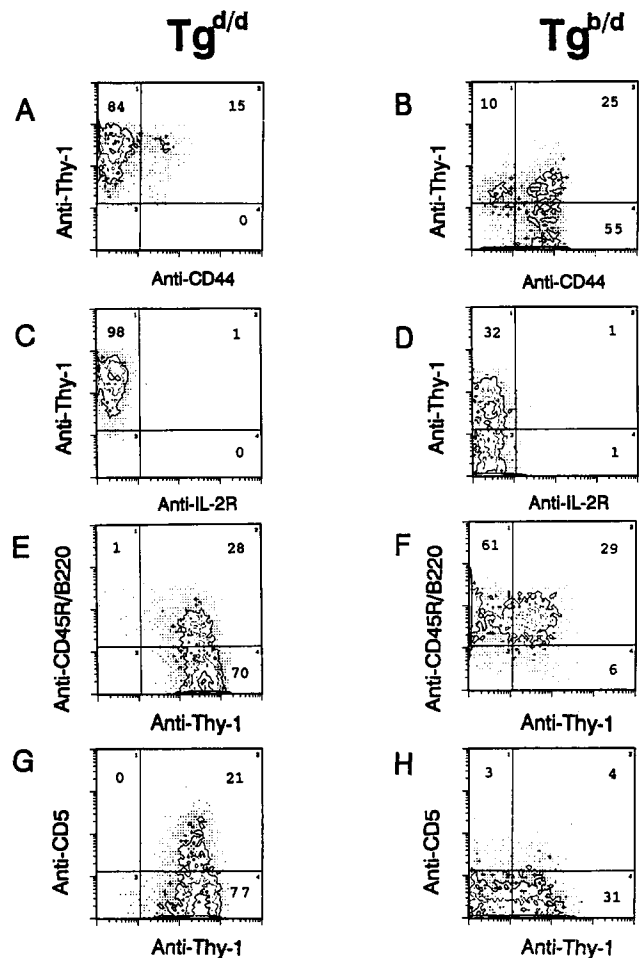


**Figure 1.** iIELs expressing lower levels of Thy-1 were detected in Ag-bearing ( $Tg^{b/d}$ ) but not syngeneic ( $Tg^{d/d}$ ) mice. Shown is the flow cytometric analysis of iIELs from  $Tg^{d/d}$  and  $Tg^{b/d}$  mice. Quadrants were determined on the basis of control staining with irrelevant hamster-FITC and rat-PE mAbs. The percentage of positive cells in each quadrant is shown.

trast, Thy-1 was expressed on only a small subset of  $Tg^{b/d}$  iIELs, and the intensity was 6% of that observed in  $Tg^{d/d}$  iIELs (Fig. 1; mean fluorescence index [MFI] = 25 compared with 398, respectively). Thus, Thy-1 was expressed at lower levels on functionally anergic  $Tg^{b/d}$  iIELs compared with functionally intact  $Tg^{d/d}$  iIELs.

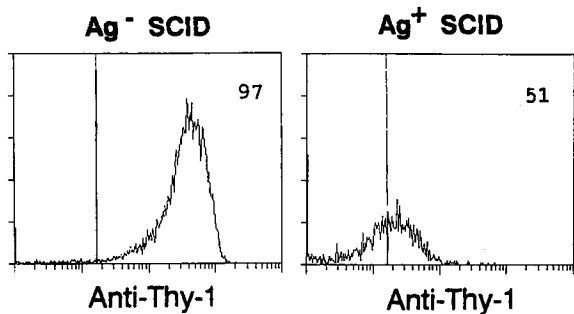
To find out whether  $Tg^{b/d}$  iIELs had encountered self-Ag, we examined them for expression of cell surface activation markers using three-color staining. A mAb directed at the  $V\gamma 2$  TCR chain expressed by Tg cells (10) was used to gate on Tg iIELs. The expression of each marker was then compared with that of Thy-1 (Fig. 2). Staining of  $Tg^{b/d}$  iIELs with anti-IL-2 receptor (IL-2R $\alpha$ ) mAb was not different from that seen in  $Tg^{d/d}$  mice. However, Tg iIELs from  $Tg^{b/d}$  mice expressed greater levels of the activation marker, CD44 (Pgp-1), as compared with  $Tg^{d/d}$  iIELs. The increased CD44 expression on  $Tg^{b/d}$  iIELs suggested that these cells had been previously activated, possibly by Ag (16). Alternatively, CD44 expression may indicate that  $Tg^{b/d}$  iIELs are immature, as observed in the thymus (17). We have previously shown that B220, a CD45 isoform, is differentially expressed by Thy-1<sup>+</sup> as compared with Thy-1<sup>-</sup> iIELs (11). In several systems, expression of B220 on T cells appears to correlate with the degree of T cell activation (18). These markers were then compared with the level of Thy-1 seen on Tg iIELs (Fig. 2). Nearly all  $Tg^{b/d}$  iIELs expressed B220 whereas the majority of  $Tg^{d/d}$  iIELs were B220<sup>-</sup>. The marker CD5 was also differentially expressed in Tg mice as 21% of  $Tg^{d/d}$  iIELs were CD5<sup>+</sup>, compared with 7% of  $Tg^{b/d}$  iIELs. Together, these results suggest that in  $Tg^{b/d}$  mice, TCR  $\gamma/\delta$  iIELs undergo a phenotypic change consistent with an activation of the cells in response to antigenic exposure.

*Tg Thy-1<sup>+</sup> iIELs Transferred Into Ag-bearing SCID Mice Home to the Intestine and Downregulate Thy-1.* The previous results suggested that the level of Thy-1 expression was correlated with the induction of tolerance. One mechanism that could explain this finding was that Thy-1 was downregulated when iIELs encountered self-Ag. Alternatively,



**Figure 2.** Tg iIELs from Ag-bearing mice express surface activation markers. Cells were gated on the basis of staining with anti- $V\gamma 2$ -biotin followed by StAv-CyChrome, and results for staining with FITC- and PE-conjugated mAbs are shown. The percent of gated cells staining positive is denoted based on control staining.

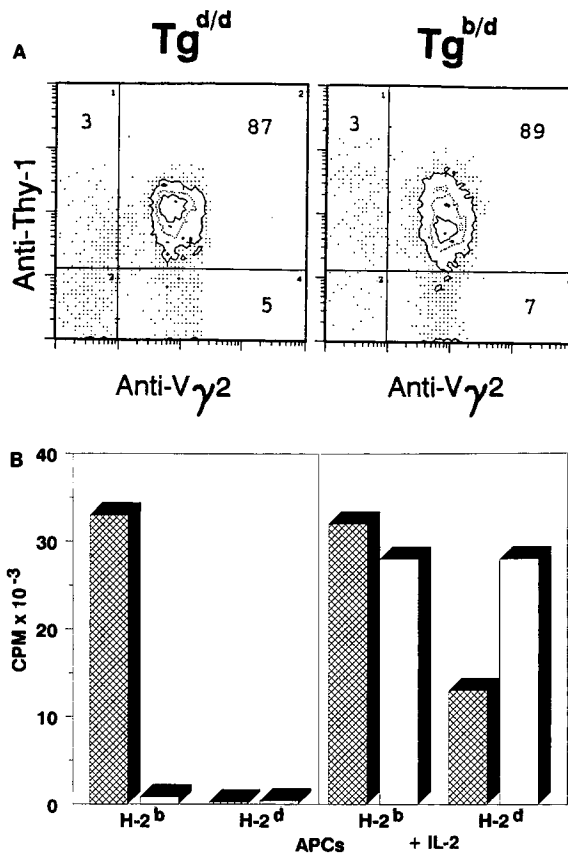
Thy-1<sup>+</sup> iIELs may be deleted, resulting in the enrichment of immature Thy-1<sup>-</sup> iIELs. To distinguish these two possibilities, a direct relationship between Ag exposure and Thy-1 downregulation was established. Freshly isolated iIELs from  $Tg^{d/d}$  mice were sorted on the basis of Thy-1 expression, and Thy-1<sup>+</sup> iIELs (contamination <1%) were transferred into either H-2<sup>d</sup> or H-2<sup>k</sup> (Ag-bearing) SCID mice. At 6 wk, iIELs were isolated and analyzed by flow cytometry. As shown in Fig. 3, Tg iIELs adoptively transferred into syngeneic C.B-17 SCID mice (H-2<sup>d</sup>) were able to home to the intestinal epithelial compartment where they expressed high levels of Thy-1. In contrast, Tg iIELs isolated from reconstituted C3H SCID mice were Thy-1<sup>del</sup>. No Tg cells could be detected in thymus or spleen of iIELs-reconstituted SCID mice, and control age-matched SCID mice did not have CD3<sup>+</sup> iIELs (data not shown). Therefore, a direct relationship between downregulation of Thy-1 and Ag exposure was demonstrated for Tg iIELs.



**Figure 3.** Tg iELs home to the intestine and downregulate Thy-1 in Ag-bearing SCID mice. Thy-1<sup>+</sup> iELs from Tg<sup>d/d</sup> mice were injected into syngeneic, H-2<sup>d</sup> and Ag-bearing, H-2<sup>k</sup> SCID mice. Flow cytometric analysis of iELs from SCID mice was performed 6 wk after reconstitution. Cells were gated on the basis of staining with FITC-conjugated anti-V $\gamma$ 2 mAb, and results for staining with anti-Thy 1-PE are shown. The percent of gated cells staining positive is denoted based on control staining.

*Purified Tg Thy-1<sup>dull</sup> iELs from Tg<sup>b/d</sup> Mice Proliferate to Ag Only in the Presence of Exogenous rIL-2.* We and others have shown that Thy-1<sup>-</sup> iELs are functionally inactive as compared with Thy-1<sup>+</sup> iELs with regard to cytolytic activity (19), lymphokine production, and proliferation (11). Interestingly, iELs from Tg<sup>b/d</sup> mice had a small population of Thy-1 dull (Thy-1<sup>dull</sup>) cells. These cells may represent cells undergoing clonal inactivation in the Tg<sup>b/d</sup> environment. To examine the proliferative response of Tg iELs that express lower levels of Thy-1, Thy-1<sup>dull</sup> iELs from both Tg<sup>d/d</sup> and Tg<sup>b/d</sup> mice were purified (Fig. 4 A) and cocultured with Ag-bearing splenic cells. As seen in Fig. 4 B, Thy-1<sup>dull</sup> Tg<sup>d/d</sup> iELs proliferated vigorously in response to H-2<sup>b</sup> but not H-2<sup>d</sup> APC (stimulation index [SI] = 120). There was no significant allogeneic mixed lymphocyte reaction detected in non-Tg iEL cultures at 48 h (data not shown). In contrast, purified Thy-1<sup>dull</sup> iELs from Tg<sup>b/d</sup> mice proliferated 50-fold less well to H-2<sup>b</sup> splenic APC (SI = 2.1) or anti-V $\gamma$ 2 mAb (data not shown), indicative of an unresponsive or anergic state (19). Thus, Tg iELs expressing equivalently low levels of Thy-1 were unresponsive in Tg<sup>b/d</sup> but not Tg<sup>d/d</sup> mice. Since the anti-V $\gamma$ 2 mAb activates independently of  $\delta$  usage, the non-responsiveness of the Tg<sup>b/d</sup> iELs is not due to their inability to recognize antigen.

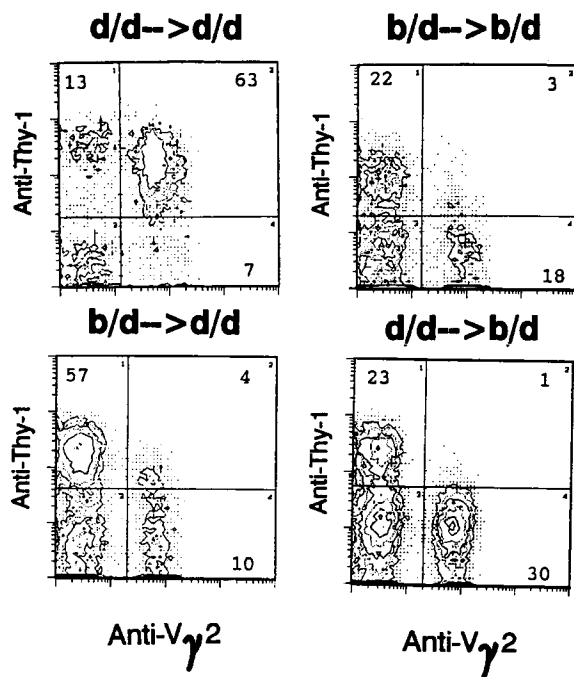
In several examples of clonal anergy in vitro, exogenous IL-2 will reconstitute the aborted proliferative response to antigen-stimulated T cells (20). Consistent with a state of clonal anergy (20), rIL-2 reconstituted the proliferative response of Thy-1<sup>dull</sup> Tg<sup>b/d</sup> iELs independent of the presence of allogeneic H-2<sup>b</sup> splenic cells. These results appear to conflict with the data presented in Fig. 2 that suggested that cells were IL-2R $\alpha$ <sup>-</sup>. The absence of staining of Tg<sup>b/d</sup> iELs with anti-IL-2R $\alpha$  may be the result of low sensitivity of the mAb, especially for the detection of the physiologically relevant, high affinity IL-2R $\alpha/\beta/\delta$  complex. Alternatively, we have previously shown that IL-2R $\alpha$  is upregulated on Tg<sup>b/d</sup> iELs after an 18-h culture with H-2<sup>d</sup> or H-2<sup>b</sup> APC (6). It is possible that similar upregulation occurred during the in



**Figure 4.** Proliferation of Thy-1<sup>dull</sup> iELs from Ag-bearing Tg mice with exogenous rIL-2. iELs were isolated and sorted for Thy-1<sup>dull</sup> expression. Flow cytometric analysis of the resulting iEL subsets is shown (A). Results are reported for Thy-1<sup>dull</sup> iELs from Tg<sup>d/d</sup> (crosshatched bars) and Tg<sup>b/d</sup> (open bars) mice pulsed on day 2 (B) after culture with splenic APC from H-2<sup>d</sup> and H-2<sup>b</sup> mice without and with rIL-2. The proliferative capacity was determined by [<sup>3</sup>H]thymidine uptake over 18 h. All measurements were performed in triplicate and the data expressed as the mean. The SE was <20%.

vitro culture accounting for the proliferation observed in Fig. 4.

*Development of Tg iELs in BM Chimeras.* In studies of thymic tolerance of TCR  $\alpha/\beta$  cells, BM chimeras have been used to demonstrate that BM-derived APC are responsible for clonal deletion, whereas tolerance to Ag expressed by non-BM-derived cells is usually mediated by nondeletional mechanisms (21). Radiation BM chimeras were used to examine the role of individual APC in mediating tolerance induction for iELs. As shown in Fig. 5, BM from Tg<sup>b/d</sup> or Tg<sup>d/d</sup> mice was injected into ( $\rightarrow$ ) irradiated (800 rad) Ag-bearing (H-2<sup>b/d</sup>) or nonbearing (H-2<sup>d</sup>) hosts. Mice were analyzed 8 wk after reconstitution to allow for the replacement of host BM-derived elements. The total yields of iEL preparations did not differ significantly between individual animals (data not shown). Tg iELs were present in d/d $\rightarrow$ d/d BM chimeras, demonstrating that Tg TCR  $\gamma/\delta$  iELs could develop in adult irradiated hosts. As shown in Fig. 5, the pattern of Thy-1 expression in d/d $\rightarrow$ d/d and b/d $\rightarrow$ b/d BM chimeras resem-



**Figure 5.** Analysis of tolerance in radiation chimeras. Flow cytometric analysis of iIELs isolated from radiation BM chimeras 8 wk after reconstitution is shown. BM from Tg donors was injected into irradiated host mice as follows: Tg<sup>d/d</sup> BM into BALB/c (H-2<sup>d</sup>) mice (d/d → d/d), Tg<sup>b/d</sup> BM into BALB/c mice (b/d → d/d), Tg<sup>d/d</sup> BM into CB6 F<sub>1</sub> (H-2<sup>b/d</sup>) mice (d/d → b/d), and Tg<sup>b/d</sup> BM into CB6 F<sub>1</sub> mice (b/d → b/d). Representative results from one of four experiments are shown. The percent of iIELs staining positive is denoted based on control staining.

bled that of the intact Tg<sup>d/d</sup> and Tg<sup>b/d</sup> mice, respectively. The majority of the Vγ2<sup>+</sup> iIELs were Thy-1<sup>+</sup> in d/d → d/d BM chimeras, whereas Tg iIELs in b/d → b/d chimeras were Thy-1<sup>-</sup>. In addition there were fewer Tg iIELs in b/d → b/d chimeras. These data are consistent with previous results in intact animals that showed a modest decline in iIELs in Ag-bearing mice over time (6).

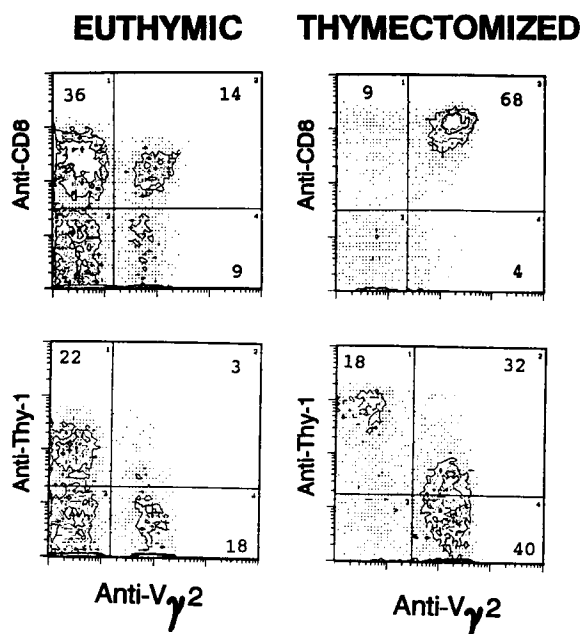
Next, we examined tolerance induction to Ag expressed by either BM-derived (b/d → d/d) or radioresistant (d/d → b/d) cells. In both instances, the majority of Tg iIELs were Thy-1<sup>-</sup>. However, clonal deletion was especially evident in BM chimeras where Ag was restricted to BM-derived APC (b/d → d/d). Together, these results suggest that clonal deletion dominated when Ag was expressed on BM-derived cells, whereas clonal anergy persisted in animals expressing antigen only on radioresistant host cells. In all BM chimeras where Tg iIELs developed in the presence of Ag, the patterns of expression of CD44, IL-2Rα, CD45R, and CD5 were similar to intact Tg<sup>b/d</sup> mice (data not shown). Furthermore, Tg cells were not detected in the peripheral lymphoid tissue in any of the Ag-bearing BM chimeras despite the presence of these cells in d/d → d/d chimeras (Y. Tatsumi and J. A. Bluestone, manuscript in preparation).

**Phenotype of iIELs in Thymectomized BM Chimeras.** The development of CD8α<sup>+</sup> iIELs can occur extrathymically. We and others have suggested that this feature of iIEL devel-

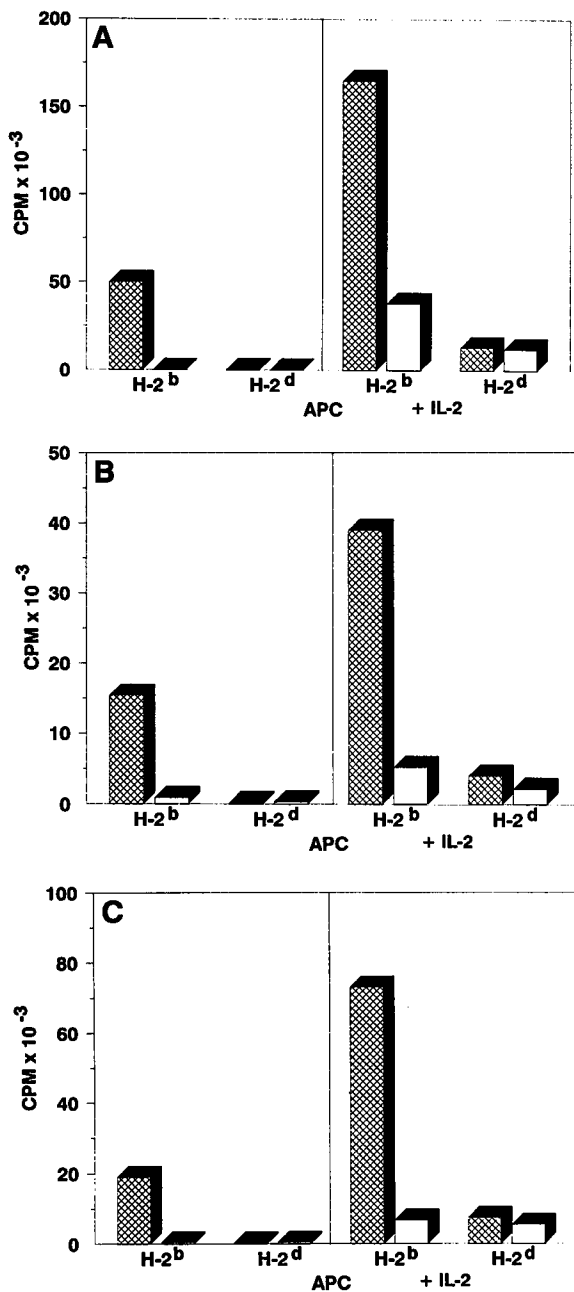
opment may explain why potentially self-reactive iIELs can persist while thymically derived T cells are deleted (6–9). To examine whether iIEL tolerance induction was thymic dependent, euthymic and thymectomized BM chimeras were constructed. In general, Tg iIELs isolated from thymectomized b/d → b/d BM chimeras resembled euthymic b/d → b/d BM chimeras (Fig. 6) and intact Tg<sup>b/d</sup> mice (6) (Fig. 1). First, Tg iIELs were present in Ag-bearing, thymectomized BM chimeras despite their absence in peripheral lymphoid tissue. Second, Tg iIELs in Ag-bearing mice were largely Thy-1<sup>-</sup>, whereas Thy-1<sup>+</sup> Tg iIELs predominated in syngeneic Tg<sup>d/d</sup> mice. Third, Tg iIELs in thymectomized and euthymic b/d → b/d BM chimeras were largely CD8<sup>+</sup>, whereas Tg iIELs in Tg<sup>d/d</sup> mice were predominantly CD8<sup>-</sup> (6). In either case, the persistence of Tg iIELs in Ag-bearing mice is likely the result of extrathymic development.

One difference was observed between the iIELs in thymectomized versus euthymic BM chimeras. Tg cells represented a significantly greater percentage of the iIELs isolated from thymectomized Ag-bearing BM chimeras compared with euthymic chimeras. This difference was not due to a change in the absolute number of Tg iIELs but rather a severe reduction in the thymus-dependent TCR α/β iIELs in these animals. Thus, similar numbers of Tg iIELs persisted in euthymic chimeric and thymectomized chimeric mice.

**Tg iIELs Developing in Ag-bearing Chimeras Are Functionally Unresponsive.** To determine whether functional tolerance was maintained in Ag-bearing chimeras, Tg iIELs were isolated and stimulated in vitro. Tg iIELs were enriched by



**Figure 6.** The induction of tolerance for TCR γ/δ iIELs independent of the thymus. Flow cytometric analysis of iIELs isolated from BM chimeras 8 wk after Tg<sup>b/d</sup> bone marrow was used to reconstitute irradiated euthymic or thymectomized H-2<sup>b/d</sup> hosts (b/d → b/d). Representative results from one of three experiments are shown. The percent of iIELs staining positive is denoted based on control staining.



**Figure 7.** Unresponsiveness of iIELs from Ag-bearing BM chimeras as measured by proliferation. Purified Tg iIELs from d/d→d/d BM chimeras (crosshatched bars) and b/d→b/d (A), b/d→d/d (B), and d/d→b/d (C) BM chimeras (open bars) were cultured with syngeneic H-2<sup>d</sup> or allogeneic H-2<sup>b</sup> APC without and with rIL-2. The proliferative capacity was determined by [<sup>3</sup>H]thymidine uptake over 18 h on day 2. All measurements were made in triplicate and the data expressed as a mean. The SE was <20%.

cell sorting (see Materials and Methods) and cocultured with Ag-bearing splenic APC. As shown in Fig. 7, purified Tg iIELs from d/d→d/d BM chimeras proliferated vigorously when cultured with Ag-bearing spleen. In contrast, Tg iIELs developing in Ag-bearing chimeras, regardless of the Ag-bearing APC population, were unresponsive. Overall, these

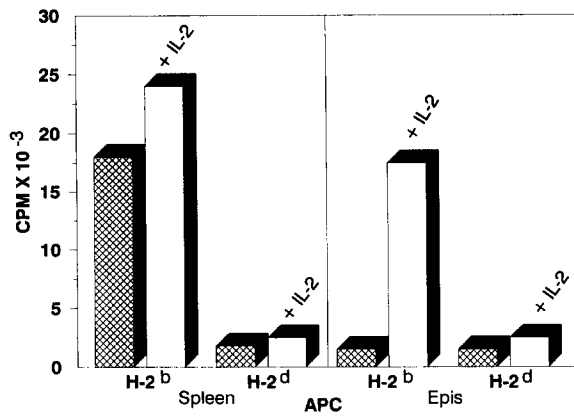
results suggested that the induction of tolerance for Tg<sup>+</sup> iIELs involved functional inactivation regardless of the APC population presenting the self-Ag.

*Tg iIELs Respond Poorly to Epithelial Cells in the Absence of Exogenous rIL-2.* Taken together the previous results suggested that exposure to Ag extrathymically results in downregulation of Thy-1 and functional inactivation of iIELs. As iIELs may develop in the epithelial compartment, the induction of tolerance may also occur locally on Ag-bearing epithelial cells. Therefore, the ability of epithelial and splenic APC from Ag-bearing mice to activate Tg<sup>d/d</sup> iIELs was examined (Fig. 8). Although Tg<sup>d/d</sup> iIELs proliferated vigorously in response to splenic APC (SI = 10), Tg<sup>d/d</sup> iIELs failed to respond to epithelial-derived APC sorted on the basis of staining with an epithelial cell-specific mAb (G8.8a, a gift from A. Farr; University of Washington, Seattle, WA [12]) (contamination <1%). Similar results were obtained using epithelial cells isolated with dispase (data not shown). The epithelial cells expressed the Tg-specific TL-encoded alloantigen based on the ability of Tg<sup>+</sup> iIELs to respond to allogeneic but not syngeneic epithelial cells in the presence of exogenous rIL-2. These results suggested that the failure of iIELs to proliferate to the allogeneic epithelial cells was due to lack of IL-2 secretion by the iIELs. In summary, these results suggest that epithelial cells may not be as effective as BM-derived APC in presenting the TL-encoded Ag. Thus, the failure to delete extrathymically derived Tg<sup>b/d</sup> iIELs may be related to the nature of the APC (in this case epithelial cells) first encountered by the iIELs.

## Discussion

Normal iIELs can be divided into subpopulations based on a variety of phenotypic markers. It has been previously suggested that the Thy-1<sup>-</sup> TCR  $\gamma/\delta$  iIELs represent a functionally immature, extrathymically derived subset that mature within the intestine after exposure to antigen (22). However, the data presented here suggest that the Thy-1<sup>-</sup> population, especially the Thy-1<sup>du1</sup> iIELs, may be derived from a potentially self-reactive pool of cells. These conclusions are supported by several pieces of data. First, Thy-1<sup>+</sup> Tg<sup>d/d</sup> iIELs downregulated Thy-1 after transfer into Ag-bearing SCID mice (Fig. 3). Second, the Thy-1<sup>-</sup> iIELs from Tg<sup>b/d</sup> mice express cell surface markers consistent with an activation event (Fig. 2). Namely, these cells express CD44 and CD45R (14, 16). Finally, a subset of Thy-1<sup>du1</sup> iIELs from Ag-bearing mice responded to exogenous rIL-2 even when cocultured with syngeneic spleen (Fig. 4 B). Proliferation in response to IL-2 alone is also observed in several in vitro (20) and some TCR  $\alpha/\beta$  transgenic models (23) of anergy. The response to exogenous rIL-2 suggests that Thy-1<sup>du1</sup> Tg<sup>b/d</sup> iIELs had previously encountered Ag, which led to upregulation of high affinity IL-2R.

As we have previously reported, the maintenance of tolerance of the TCR  $\gamma/\delta$  iIELs in these Tg mice involved clonal anergy as well as clonal deletion. In fact, the preferential decrease of Thy-1<sup>+</sup> Tg<sup>+</sup> iIELs in Ag-bearing mice suggests that



**Figure 8.** Tg iIELs respond poorly to Ag-bearing intestinal epithelial cells. Tg iIELs from Tg<sup>b/d</sup> mice were cultured with splenic APC and intestinal epithelial cells (*Epis*) from allogeneic H-2<sup>b</sup> or syngeneic H-2<sup>d</sup> mice without or with rIL-22. The proliferative capacity was determined by [<sup>3</sup>H]thymidine uptake over 18 h on day 2. All measurements were made in triplicate and the data expressed as a mean. The SE was <20%.

the functionally active Thy-1<sup>+</sup> subset of TCR  $\gamma/\delta$  IELs is either deleted or the Thy-1 Ag is downregulated. Previous studies have suggested a role for Thy-1 molecules in anti-TCR mAb-induced apoptosis (24). Therefore, expression of Thy-1 by Tg iIELs in Ag-bearing mice as well as in BM chimeras may contribute to the deletion of Tg<sup>+</sup> iIELs observed in these mice. It is also possible that Thy-1 downregulation may contribute to functional inactivation of Tg<sup>b/d</sup> iIELs. Crosslinking of surface Thy-1 with mAb has been shown to synergize with TCR-mediated activation and can activate T cells in the absence of specific TCR-mediated stimulation (25). Therefore, the downregulation of Thy-1 may be a mechanism for decreasing the functional capacity of the cell. In normal mice, Thy-1<sup>-</sup> iIELs fail to secrete lymphokine upon TCR-mediated stimulation despite significant levels of several lymphokines released from Thy-1<sup>+</sup> iIELs (11). Thus, during the induction of tolerance, iIELs may undergo a differentiation event leading to the loss of Thy-1 expression and function.

The fate of Ag-reactive iIELs appears to depend on the

nature of the APC as well as the pathway of development. In Tg<sup>b/d</sup> mice, Tg cells were eliminated in the spleen and lymph of Ag-bearing mice by a mechanism of clonal deletion in the thymus. However, the maintenance of tolerance in the intestine resulted in a functionally unresponsive population of Tg iIELs that expressed low levels of Thy-1. The major population of tolerant iIELs were probably extrathymically derived and encountered Ag in the epithelial compartment. The results from BM chimeras demonstrate that when Ag expression was restricted to radioresistant cells the greatest numbers of anergic Tg<sup>+</sup> iIELs were present. The induction of anergy by epithelial cells may have been related to the inability of epithelial cells to effectively present the TL-encoded alloantigen (Fig. 8). Previous reports have shown that engagement of the TCR in the absence of appropriate second signals can lead to clonal inactivation (20). Furthermore, the induction of anergy has been observed in vitro when T cells encounter Ag on nonconventional APC, such as islet cells (5) or keratinocytes (26). In fact, preliminary results of staining with the CTLA4-Ig fusion protein suggest that epithelial cells do not express the costimulatory molecule B7/BB1 (27) (data not shown). Thus, the induction of anergy in Tg<sup>b/d</sup> iIELs may have been due to epithelial cells that lack the full capacity to present Ag. Whether self-reactive TCR  $\gamma/\delta$  IELs may be activated in states of intestinal inflammation is unclear. In these states, appropriate second signals may be upregulated on epithelial cells leading to stimulation rather than inactivation or deletion of self-reactive iIELs. Thus, autoimmunity in the intestine may involve the breakdown of the mechanisms of tolerance identified in this study.

In this study we examined tolerance to self-Ag for TCR  $\gamma/\delta$  iIELs. These results suggest that TCR  $\gamma/\delta$  iIELs were tolerized to self-Ag presented by radioresistant elements leading to the persistence of an anergic clonal population of T cells. The maintenance of tolerance has also been examined for TCR  $\alpha/\beta$  iIELs. In athymic radiation chimeras, significant deletion was observed of self-reactive CD4<sup>+</sup> and CD8  $\alpha/\beta$ <sup>+</sup> TCR  $\alpha/\beta$  IELs. Thus, it is possible that tolerance may be maintained by distinct mechanisms for TCR  $\gamma/\delta$  and TCR  $\alpha/\beta$  lineages of iIELs. However, it is equally likely that these differences may be related to the different antigens studied in these models.

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