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Immune Deviation of 2C Transgenic Intraepithelial Lymphocytes in Antigen-bearing Hosts

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Summary

The present study examined self-tolerance for T cell receptor (TCR)αβ intestinal intraepithelial lymphocytes (iIELs) using the 2C transgenic (Tg) mouse model specific for a peptide antigen (Ag) presented by the class I major histocompatibility complex H-2L^d. Although Tg$^+$ T cells were largely deleted from the periphery of Ag$^+$ mice, equivalent numbers of Tg iIELs were present in Ag$^+$ compared to Ag$^-$ mice. Tg iIELs in Ag$^-$ mice contained CD8αβ, CD8αα, and CD4$^-$CD8$^-$ subsets, whereas only CD8αα and CD4$^-$CD8$^-$ Tg iIEL subsets were detected in Ag$^+$ mice. Analysis of surface markers revealed that Tg iIELs in Ag$^+$ mice expressed decreased levels of Thy-1 and increased CD45R/B220 as compared to Ag$^-$ Tg iIELs. In response to activation with exogenous peptide or immobilized anti-TCR mAb, iIELs from Ag$^-$ mice proliferated at high levels and produced interleukin (IL)-2 and interferon (IFN)-γ, while Tg$^+$ iIELs from Ag$^+$ mice proliferated at low levels and failed to produce detectable IL-2 or IFN-γ. Activation of sorted iIEL subsets from Ag$^-$ mice revealed that CD8αα and CD4$^-$CD8$^-$ subsets produced low levels of IL-2 and IFN-γ in response to activation with antigen-presenting cells and added peptide or immobilized anti-TCR mAb, while CD8αβ$^+$ iIELs responded to endogenous levels of peptide. In response to APC and exogenous peptide, sorted iIEL subsets from Ag$^+$ mice produced IL-2 and IFN-γ, and proliferated at greatly reduced levels compared to corresponding subsets from Ag$^-$ mice. Analysis of cytokine mRNA levels revealed that activation in vitro induced IL-2 mRNA only in Ag$^-$, but not Ag$^+$ iIELs, whereas a high level of IL-4 mRNA induction was detected in Tg$^+$ iIELs from Ag$^+$ mice, and to a lesser degree, from Ag$^-$ mice. These data suggest that tolerance for Tg$^+$ iIELs resulted in the deletion of CD8αβ$^+$ subsets and the persistence of Tg$^+$ iIEL subsets with decreased sensitivity to endogenous levels of self-peptide. A comparison of the cytokine profiles expressed by Tg$^+$ iIEL subsets in Ag$^-$ and Ag$^+$ mice suggested that tolerance induction had involved the functional deviation of cells from TC1 (T helper-1–like) to a less inflammatory TC2 (T helper-2–like) phenotype capable of mediating humoral immune responses in the mucosa.

The mucosal immune system provides the first line of defense for the elimination of enteric pathogens. It is, therefore, essential that a vigorous immune response be delivered at the mucosal surface to combat infectious agents. To maintain protective immunity at the mucosal surface, T lymphocytes and antibodies are potent and prearmed. For example, intestinal intraepithelial lymphocytes (iIELs)[1] expressing αβ or γδ TCRs exhibit spontaneous cytolytic activity against infected target cells (1–3). TCRαβ iIELs also display a diverse TCR repertoire capable of recognizing a broad array of foreign peptides in an MHC-restricted fashion (4–6). In addition, TCRγδ iIELs recognize, in part, a unique set of antigens as whole proteins in an MHC-independent manner (7–10). These findings suggest that the mucosal immune system exists in a dynamic state of activation prepared for the constant assault of pathogens. However, exposure of the mucosal immune system to antigenic stimuli may induce dysfunctional levels of inflammation, as seen in inflammatory bowel disease. Therefore, the control of lymphocyte reactivity is essential.

Studies by our laboratory (11) and others (12–14) have suggested that tolerance of iIELs to self-Ag in the mucosal system may be regulated differently from T cells in periph-

[1] *Abbreviations used in this paper:* HPRT, hypoxanthine-guanine phophoribosyltransferase; iIEL, intestinal intraepithelial lymphocyte; PLN, peripheral lymph node; Tg, transgenic.

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eral lymphoid tissue. Previously, we used a TCRγδ transgenic mouse (G8) specific for a nonclassical class I molecule, H-2T10^b, to show that transgenic (Tg) iIELs in Ag$^+$ mice were present but functionally tolerant, as evidenced by a decrease in proliferation and IL-2 production (15). Transgenic TCRγδ iIELs resident in Ag$^+$ mice expressed distinct surface phenotypes compared to Tg iIELs from syngeneic mice (11). The Tg iIELs in Ag$^+$ mice expressed decreased levels of Thy-1 and increased levels of CD45R/B220, as compared to iIELs in Ag$^-$ mice. Results from other models where tolerance of TCRαβ iIELs was examined confirmed that, although thymic deletion predominated in peripheral lymphoid tissue of Ag-bearing hosts, nondeletional mechanisms operated to allow persistence of potentially self-reactive TCR iIEL populations. In mice expressing the Mls-1^a and MMTV Ags, Vβ6-, Vβ8.1-, and Vβ11-expressing lymph node and splenic T cells were deleted intrathymically. Potentially reactive Vβ-bearing iIELs persisted in these same mice (13, 14). Likewise, iIELs expressing self-reactive TCR were detected in male H-Y TCR Tg mice (12). In Ag$^+$ male H-Y mice, CD8αα and CD4$^+$CD8$^+$ self-reactive iIELs persisted, while reactive CD4$^+$ and CD8αβ iIELs were deleted. In addition, results suggested that potentially self-reactive iIELs that persisted in these models were functionally unresponsive to activating stimuli. These studies raised the issue of whether iIEL subsets in Ag$^+$ mice were unresponsive because of antigen-induced tolerance or developmental immaturity (16–18).

In the present study, we used the 2C TCR Tg mouse strain to investigate the tolerizing effects of a ubiquitously expressed class I MHC antigen on peripheral and intraepithelial T cells. The 2C Tg mouse strain was derived from a CD8$^+$ T cell clone positively selected by the class I MHC protein, H-2K^b, and specific for a H-2L^d–restricted self-peptide derived from α-ketoglutarate dehydrogenase protein (19, 20). The use of the 2C TCR Tg system provided several advantages: (*a*) a TCR-specific mAb, 1B2, was available for monitoring Tg$^+$ T cells (21); (*b*) the T cell clonotype was derived from a CD8$^+$ clone, a phenotype expressed by a large percentage of iIELs; and (*c*), the peptide sequence was defined, and unlike the H-Y Ag (the target of TCR Tg mice used in previous studies), the α-ketoglutarate dehydrogenase protein is expressed ubiquitously in the gut (20, 22).

The present studies demonstrate that 2C Ag$^-$ (H-2^b) mice develop large numbers of Tg$^+$ CD4$^-$CD8$^-$, CD8αβ, and CD8αα iIEL. In contrast, the Tg$^+$ CD8αβ iIEL were deleted in Ag$^+$ (H-2$^{b/d}$) mice. In addition, the presence of Ag affected functional responses of CD4$^-$CD8$^-$ and CD8αα H-2$^{b/d}$ iIELs, as reflected by proliferation, cytokine production, and expression of surface activation markers, suggesting that Tg iIELs had undergone immune deviation in Ag$^+$ hosts rather than persisting as functionally immature subsets. In fact, Tg iIELs residing in Ag$^+$ mice expressed an activated, IL-4–producing, TC2-like phenotype as, described by Mosmann and colleagues (23). Thus, tolerance of iIELs in the 2C Tg model involved deletion of CD8αβ$^+$ Tg T cells in the periphery and intestine. For CD4$^-$CD8$^-$ and CD8αα-expressing iIEL subsets, tolerance involved functional differentiation to less inflammatory cell types capable of participating in local humoral immune responses.

Materials and Methods

Mice. Adult H-2^b and H-2$^{b/d}$ Tg mice (Ag$^-$ and Ag$^+$, respectively) were generated by breeding a 2C Tg$^+$ H-2^b male (a gift from Dr. Dennis Loh, Nippon Research Center, Kanagawa, Japan) to either C57BL/10 or BALB/c females obtained from the National Cancer Institute (Frederick, MD) animal stock. Animals were raised under specific pathogen-free conditions in the Veterans Administration Lakeside Medical Center, Medical Science Building.

Culture Medium. Culture medium consisted of DME, 10 mM Hepes, 5% FCS, 2-ME, glutamine, antibiotics, and nonessential amino acids, as previously described (24).

Cell Isolation. Inguinal, axillary, and mesenteric LN cells were mechanically disassociated and fat was eliminated by passage of the cell suspensions through a nylon mesh. Cells suspensions were washed, pelleted, and resuspended in 5% DMEM and stored on ice. Intestines were removed from 6–8-wk old mice, and iIELs were isolated as described previously (24), with minor modifications. Briefly, small intestines were removed and flushed with cold PBS. Intestines were opened longitudinally and cut into 1-cm pieces. After multiple rinses with cold PBS and brief vortexing, the pieces were resuspended in 50 ml digestion buffer containing 10% newborn calf serum (GIBCO BRL, Gaithersburg, MD), 0.3 mg/ml dithioerythritol (GIBCO BRL), with 5 mM EDTA in PBS. Pieces, suspended in this buffer, were gently agitated at 40–50 rpm in a closed 75-ml digestion flask (Fisher Scientific, Itasca, IL) with a stir bar at 37°C for 40 min. Pieces were washed with cold PBS, and the supernatant was collected and pelleted. Pellets were resuspended in 5% DMEM and kept at 4°C overnight. The cells were resuspended in 50% Percoll (Pharmacia, Piscataway, NJ) and 0.3 mg/ml DTT, layered onto a discontinuous Percoll gradient (75% density), and centrifuged for 20 min at 20°C at 400 × *g*. The cells concentrated at the interface of the 50 and 75% layers, and were then pipetted off and washed in 4 vol of PBS. The purity of Tg iIELs within preps was assessed by flow cytometry on the basis of forward angle and 90° light scatter, as well as using the fluorochrome-coupled Tg clonotypic mAb, 1B2.

Antibodies, Three-color Immunofluorescence, and Immunofluorescence Analysis. The following mAbs coupled to FITC, PE, or biotin were used: anti–Thy-1, anti-CD8α, anti-CD8β, anti-CD45R/B220, and anti-CD44 (Pharmingen, San Diego, CA) and 1B2 (a gift from Dr. Dennis Loh) (21). Biotin-labeled Abs were followed by streptavidin-CyChrome or streptavidin-PE (Pharmingen). Dead cells were excluded from analysis on the basis of forward and side angle scatter, and in some cases, by propidium iodide (Sigma Chemical Co., St. Louis, MO). Approximately 5 × 10^5 cells were stained per sample for 20 min with a concentration of mAb titered to maximize specific staining and limit background. A total of 10,000 gated events were collected for analysis. Acquisition of FCM data was performed on a FACScan® (Becton Dickinson & Co., Mountain View, CA), and cell sorting was performed on a FACStarPlus® (Becton Dickinson). Data were analyzed using the CellQuest program (Becton Dickinson). To purify CD8αβ, CD8αα, and CD4$^-$CD8$^-$ IEL subsets, cells were simultaneously stained with 1B2-FITC, anti–CD8α-PE, and

anti–CD8β-biotin, and counterstained by streptavidin-CyChrome. This sorting resulted in >98% pure subsets (data not shown).

Proliferation Assays. Isolated iIELs or LN cells were cocultured in triplicate with splenic APC. For each condition, 3×10^5 irradiated, anti–Thy-1 mAb (AT83A, a gift from Dr. F. Fitch, University of Chicago, Chicago, IL) plus C′-treated splenic APC from H-2^b or H-2^d mice were cocultured with 10^5 responder Tg iIELs or LN T cells in 96-well round-bottomed microtiter plates in triplicate. In some experiments, T cells were stimulated with immobilized 1B2 mAb coated overnight on the microtiter wells at 4°C with the mAb. Coated wells were washed three times with PBS before use. Exogenous p2Ca peptide was added to some experiments using H-2^d APC. The p2Ca peptide sequence used was LSPFPFDL (19, 20) (Bio-Synthesis, Lewisville, TX). Exogenous human rIL-2 (50 U/ml; Genzyme, Cambridge, MA) was added when indicated on day 1 of culture. At 48 h, cultures were pulsed for 18 h with [^{3}H]thymidine (1 μCi/well). Cells were harvested and analyzed with a liquid scintillation counter (Packard Instrument Co., Meriden, CT).

Lymphokine Assays. Isolated iIELs and LN cells were isolated and cultured in 96-well plates, as described above. After 48 h, supernatants were harvested and analyzed for IL-2, IL-4, and IFN-γ,

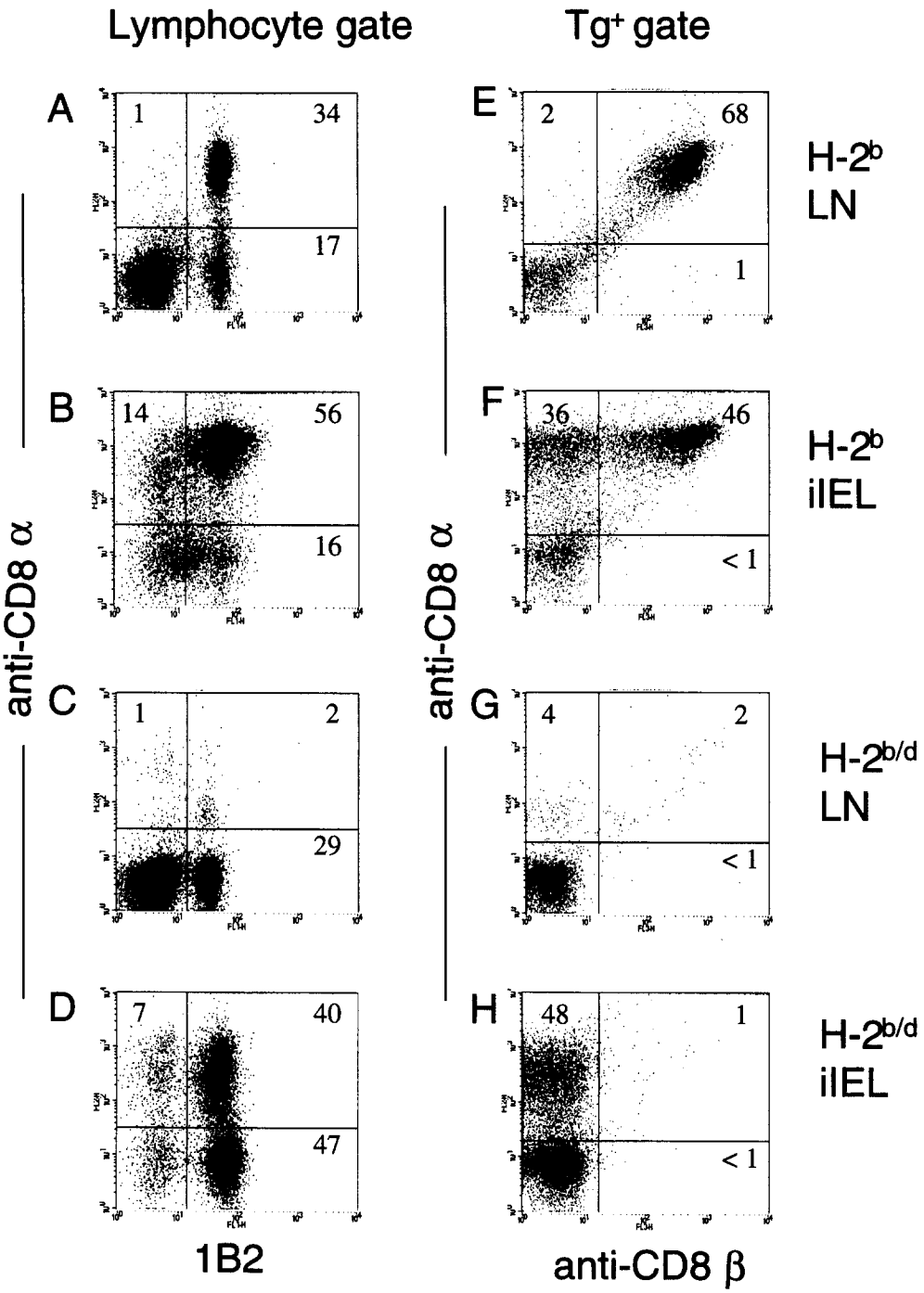

Figure 1. Transgenic T cell subsets in 2C mice. Peripheral and iIEL populations were analyzed by FCM for Tg TCR (1B2) expression vs. CD8α (*A–D*), gating on all lymphocytes by standard forward- and side-scatter values. To assess CD8α vs. CD8β expression by Tg cells (*E–H*), cells stained with anti–CD8α-PE (*y*-axis), 1B2-FITC and anti–CD8β-biotin followed by streptavidin-CyChrome were gated for 1B2-FITC–positive cells. Quadrants were determined on the basis of control staining and percentages of positively stained cells in each quadrant are shown.

using murine cytokine ELISA MiniKits (Endogen, Cambridge, MA). The sensitivity of these ELISAs were as follows: >10 pg/ml for IL-2, >10 pg/ml for IL-4, and >100 pg/ml for IFN-γ.

Competitive Reverse Transcription (RT-PCR). Total RNA from 10–20 × 10^4 iIEL/sample was extracted in TRIzol according to the manufacturer's directions (GIBCO BRL). Reverse transcription was performed using murine Moloney leukemia virus reverse transcriptase (GIBCO BRL) and oligo dT primers (GIBCO BRL) as described (25). Qualitative PCR (Q-PCR) was performed using a multiple cytokine-containing competitor construct (PQRS) as described previously (25). Briefly, aliquots of cDNA were assayed for levels of a constitutively expressed mRNA, hypoxanthine-guanine phosphoribosyltransferase (HPRT), by using a range of concentrations of the PQRS mimic and a constant dilution of 1/100 of the cDNA samples. After gauging the relative concentration of the experimental cDNA for HPRT by comparing with the competitor band intensity range, experimental samples of cDNA dilutions were adjusted to yield the equivalent of 50 fg/reaction. For assessment of cytokine mRNA, parallel samples were diluted based on relative levels of HPRT. The competitor PQRS cDNA was kept constant at 20 fg/reaction. Amplification products were separated on a 2.0% ethidium bromide–stained agarose gel. Imaging of the gels was performed using an Eagle Eye II imager (Stratagene, La Jolla, CA) and Adobe Photoshop software.

Results

Phenotype of Tg iIELs in Syngeneic and Ag-bearing Mice. The distribution and surface phenotype of T cells in Ag^- and Ag^+ mice was examined to determine the effect of self-Ag on the development and activation state of Tg^+ T cells. Consistent with previous reports (26–28), the results in Fig. 1 indicate that Tg^+ T cells populated the peripheral LN (PLN) in high numbers and expressed the CD8αβ heterodimer on a majority of the Tg T cells (Fig. 1, *A* and *E*). Total $CD4^+$ PLN and iIEL T cells were <2% of Tg^+ cells in H-2^b and H-$2^{b/d}$ mice (data not shown). Similar populations were observed for Tg^+ iIELs in H-2^b (Ag^-) mice. The largest subset of Tg^+ iIELs in Ag^- mice were CD8αβ.

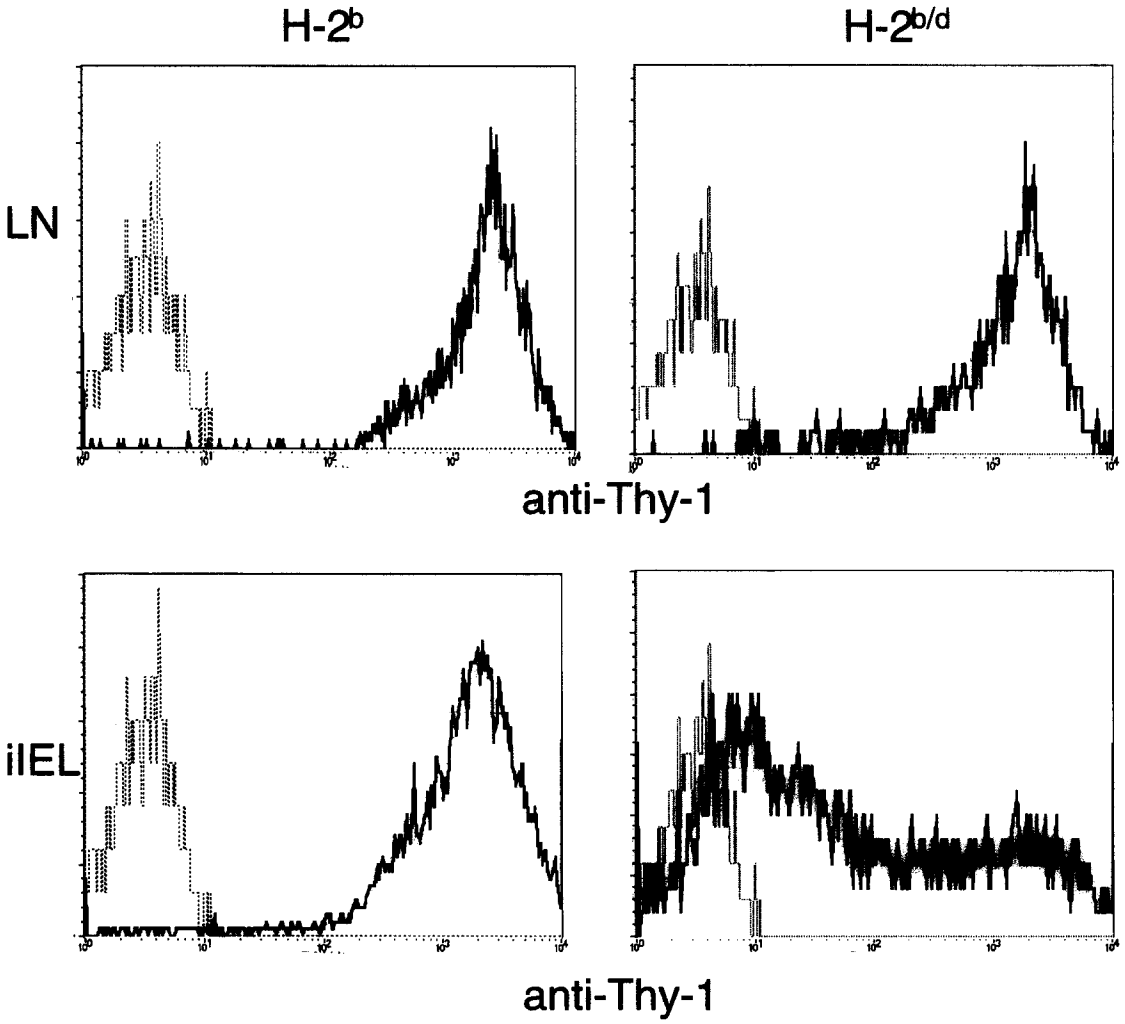

Figure 2. Differential expression of Thy-1 by LN and iIEL in Ag^- and Ag^+ Tg mice. LN and iIEL cells were gated on the basis of Tg TCR expression using 1B2-FITC, and the results for staining with anti–Thy-1–PE are shown. Control staining with an irrelevant rat-PE mAb is indicated by the dotted line.

The remaining iIELs were either CD8αα (36%) or $CD4^-$ $CD8^-$ (16%) (Fig. 1 *F*). By comparison, $CD8\alpha\beta^+$ Tg T cells in the periphery and intestines of Ag^+ mice were deleted. In PLNs, deletion of $CD8\alpha\beta^+$ Tg T cells correlated with an overall decrease in Tg^+ T cell yields. The few Tg^+ T cells remaining in PLNs were either CD8αα or $CD4^-$ $CD8^-$ (Fig. 1, *C* and *G*). In contrast to PLNs, there was no decrease in Tg^+ T cell yield from the intestinal epithelial compartment of Ag^+ compared to Ag^- mice (data not shown). Although the CD8αβ Tg iIELs were absent in Ag^+ mice, a compensatory increase in the percentages and absolute numbers of $CD4^-CD8^-$ and CD8αα iIELs were observed (Fig. 1, *D* and *H*).

Previous results from the G8 TCRγδ Tg model suggested that Thy-1 downregulation and CD45R/B220 upregulation correlated with tolerance for Tg^+ iIELs (2, 11). Similarly, Tg^+ iIELs isolated from 2C Ag^+ Tg^+ mice expressed decreased levels of Thy-1 (Fig. 2). The pattern of Thy-1 expression did not differ between CD8αα and $CD4^-CD8^-$ subsets in Ag^+ mice (data not shown). In contrast, Thy-1 was expressed at uniformly high levels on Tg^+ PLN and iIEL from Ag^- mice (Fig. 2), as well as the few remaining Tg^+ PLN T cells in Ag-bearing mice (Fig. 2). Thus, the presence of Ag in 2C mice correlated with reduced levels of Thy-1 expression on Tg^+ iIELs, but normal Thy-1 expression on residual PLN Tg^+ T cells.

It has been suggested by others (14, 29, 30) that iIELs expressing low levels of Thy-1 represent an immature population. Thus, one interpretation of the Thy-1 staining of Tg^+ iIELs in Ag^+ mice was that these cells were an immature population of iIELs incapable of responding to Ag. An alternative explanation was that the cells had been exposed to Ag and had downregulated Thy-1. In addition to Thy-1 modulation upon exposure to Ag, several systems have

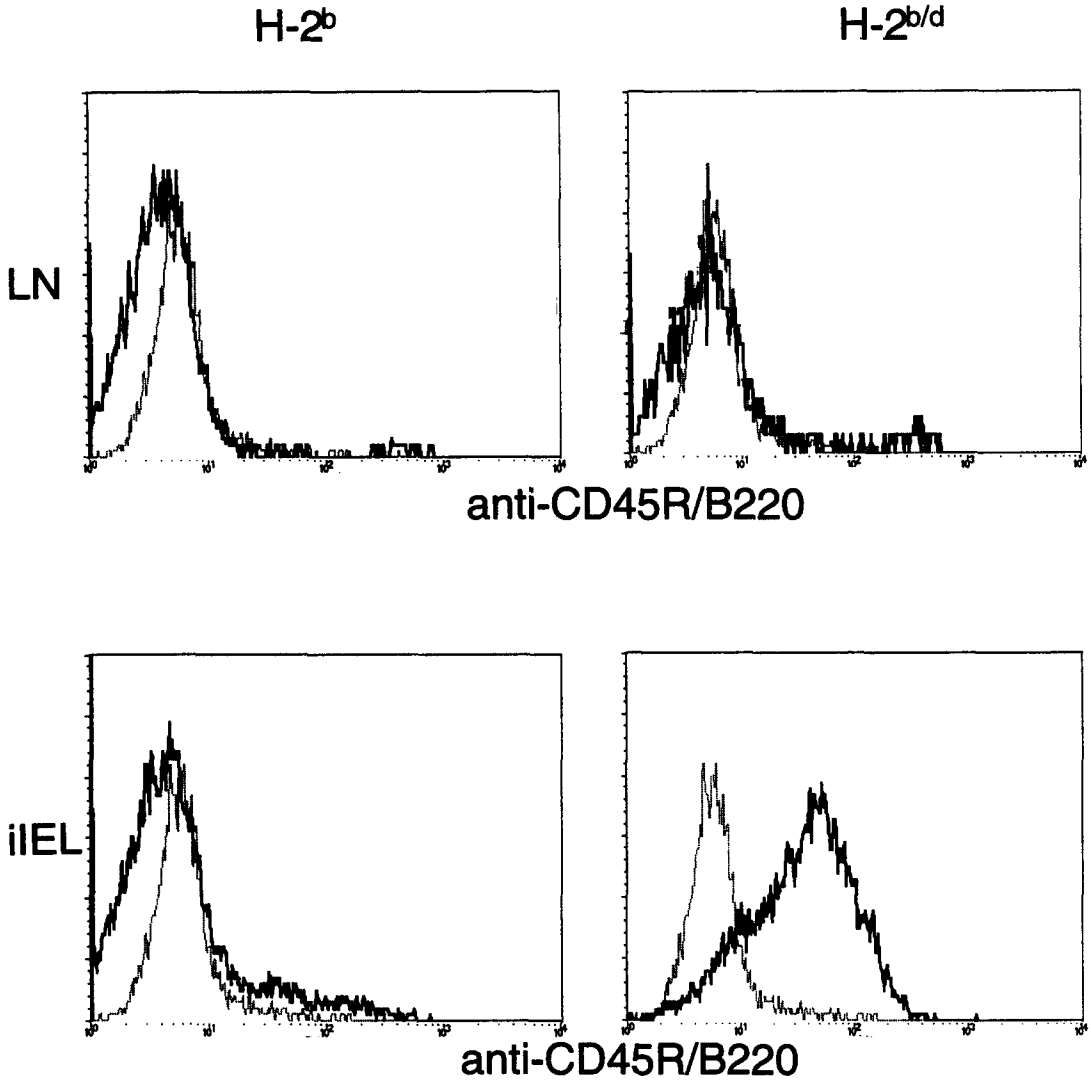

Figure 3. Differential expression of CD45R/B220 by LN and iIEL in Ag^- and Ag^+ Tg mice. LN and iIEL cells were gated on the basis of Tg TCR expression using 1B2-FITC, and the results for staining with CD45R/B220-PE are shown. Control staining with an irrelevant rat-PE mAb is indicated by the dotted line.

shown that the expression of CD45R/B220 on T cells correlates with the degree of T cell activation (11, 31, 32). As shown in Fig. 3, LN T cells and iIELs isolated from Ag^- mice were $B220^-$, a typical phenotype for resting naive peripheral T cells. In contrast, Tg^+ iIELs from Ag^+ mice expressed CD45R/B220 at higher levels overall compared to Ag^- mice (Fig. 3; mean fluorescence index (MFI) = 90 compared to 6, respectively). The pattern of CD45R/B220 expression did not differ between CD8αα and $CD4^-CD8^-$ subsets in Ag^+ mice (data not shown). Together, these results suggested that Tg iIELs in Ag^+ mice had responded to self-Ag in vivo.

Proliferative Responses of Tg Cells from LN and IEL of Ag^- and Ag^+ Mice. Since Tg LN and iIELs from Ag^+ mice expressed surface phenotypes consistent with previous exposure to Ag, we assessed the relative proliferative responses of these subsets to stimulation with Ag or immobilized anti-TCR mAb. Proliferative responses were assessed for Tg^+ T cells cultured with Ag-bearing APCs and increasing levels of exogenous peptide Ag. As seen in Fig. 4, addition of exogenous peptide to Ag^+ APC augmented proliferation for iIELs and LN from Ag^- mice (Fig. 4 *A*). However, proliferative responses of Tg LN and iIELs from Ag^+ mice were reduced by 58 and 95%, respectively. Response to a control peptide for all four groups showed no proliferation (data not shown), and all cultures were normalized for Tg^+ T cells.

To assess the relative proliferative responses of distinct iIEL subsets, CD8αβ, CD8αα, and $CD4^-CD8^-$, Tg iIEL populations were purified by cell sorting and stimulated with Ag^+ APC with and without exogenous peptide (1 μg/ml). Of the three subsets detected in Ag^- mice, only the $CD8\alpha\beta^+$ Tg iIELs responded to Ag^+ APC without exogenous peptide. None of the Tg^+ T cells isolated from Ag^+ mice proliferated to Ag^+ APC, however, addition of exogenous peptide induced proliferation on all T cell subsets from Ag^- mice. Interestingly, addition of peptide increased proliferative responses for CD8αα and $CD4^-CD8^-$ iIELs from Ag^- mice, but not CD8αβ iIELs. This was not caused by the increased IFN-γ produced by this CD8αβ subset, since proliferative responses were not enhanced with the addition of blocking mAb to IFN-γ. $CD4^-CD8^-$ Tg iIELs from Ag^+ mice proliferated in response to APC and peptide (Fig. 5 *B*), whereas CD8αα iIELs from Ag^+ mice remained unresponsive to Ag^+ APC despite the addition of high doses of peptide. These results indicated that unresponsiveness was quantitative with increased proliferative responses evident for Tg iIELs from Ag^+ mice activated with allogenic MHC containing high levels of peptide.

To confirm that the inability of iIELs from Ag^+ 2C Tg^+ mice to respond was not caused by lack of CD8 expression, the proliferative responses of the iIELs to immobilized anti-TCR mAb were assessed. Tg iIELs from Ag^+ mice responded 25-fold less well to 1B2 mAb compared to iIELs from Ag^- mice (Fig. 4 *B*). Interestingly, the few residual Tg^+ PLN cells in Ag^+ mice (*closed squares*) responded similarly to 1B2-induced signals, suggesting that the residual

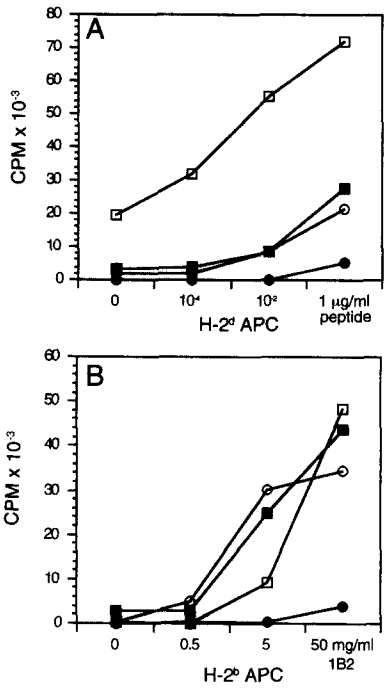

Figure 4. Differential proliferative responses by Tg^+ LN and iIEL from Ag^- and Ag^+ mice. Proliferative responses were measured for Tg^+ iIELs and LN T cells in response to Ag^+ APC and increasing levels of exogenous peptide (*A*) or to increasing concentrations of immobilized 1B2 mAb (*B*). All measurements were performed in triplicate, and the data are expressed as the mean with an SE <15%. The data shown are representative of three experiments. —□—, $H\text{-}2^b$ LN; —○—, $H\text{-}2^b$ iIEL; —■—, $H\text{-}2^{b/d}$ LN; —●—, $H\text{-}2^{b/d}$ iIEL.

cells (although $CD8^-$) could respond to antigenic stimuli (Fig. 4 *B*). Finally, the addition of rIL-2 (50 U/ml) did not reconstitute the proliferative responses of iIEL isolated from Ag^+ mice (data not shown). Thus, whole populations of Tg^+ iIELs isolated from Ag^+ mice appeared significantly less responsive as compared to iIELs isolated from Ag^- mice. Taken together with the results of Fig. 5, these findings suggest that although some subsets may not have downregulated proliferative responses during tolerance induction (e.g., $CD4^-CD8^-$), proliferative responses were decreased overall in Ag^+ mice.

Cytokine Production by Ag^- and Ag^+ LN and IEL in Response to Peptide Ag. Although proliferative responses of iIELs from Ag^+ mice were diminished, it was possible that they were functionally competent, as measured by criteria such as cytokine production. Cytokine production was assessed after stimulation with increasing concentrations of exogenous peptide to ensure maximum responses. The results in Fig. 6 *A* indicate that Tg^+ LN and iIEL from Ag^- but not Ag^+ mice produced IL-2 in response to addition of peptide to Ag^+ APC. Increasing concentrations of peptide resulted in high IL-2 production for the Ag^- PLN Tg^+ T cells, while iIELs produced modest levels of IL-2 only at the highest concentrations. For IFN-γ production, Tg PLN cells in Ag^+ mice produced equivalent levels com-

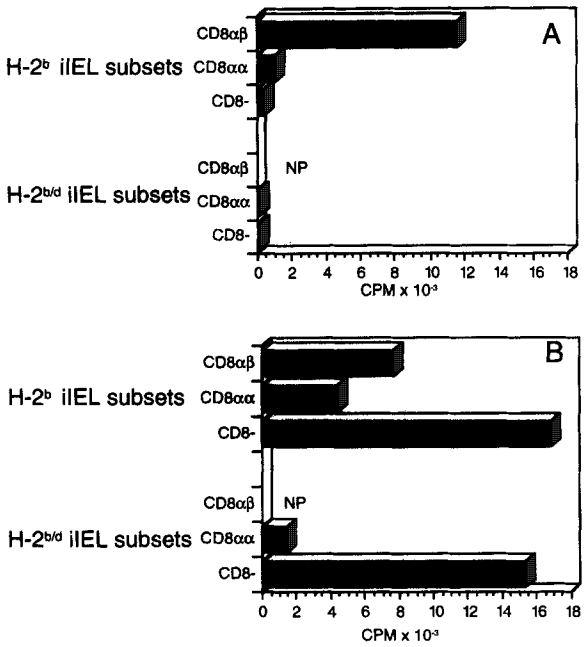

Figure 5. Ag^+ iIEL subsets require high levels of exogenous peptide for proliferative responses. Intestinal iIEL subsets were sorted on the basis of staining with 1B2, CD8α, and CD8β, attaining a purity of >98% for the subsets of Tg^+ iIEL indicated. Proliferative responses to APC without exogenous Ag (*A*) and with 1 μg/ml added exogenous Ag (*B*) were measured. All measurements were performed in triplicate, and the data are expressed as the mean with an SE <15%. *NP*, not present. The data shown are representative of three experiments.

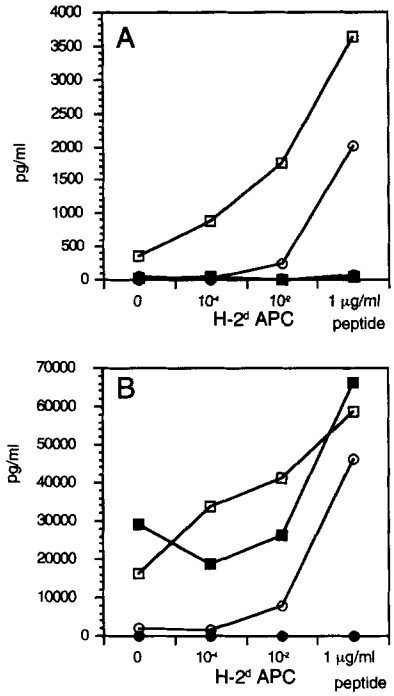

Figure 6. Effect of self-Ag on IL-2 and IFN-γ production in 2C mice. Equivalent Tg^+ numbers of T cells from LN and iIEL of Ag^- and Ag^+ mice were stimulated with endogenous antigen presented by irradiated Ag^+ APC ($H\text{-}2^d$) or increasing doses of added peptide. Culture supernatants were collected at 48 h, and levels of IL-2 (*A*) and IFN-γ (*B*) were measured by ELISA. The data shown are representative of three experiments. ⊟, $H\text{-}2^b$ LN; ⊖, $H\text{-}2^b$ iIEL; ■, $H\text{-}2^{b/d}$ LN; ●, $H\text{-}2^{b/d}$ iIEL.

pared to those from Ag^- mice (Fig. 6 *B*). In contrast, iIEL populations of Tg^+ cells from Ag^+ mice failed to produce either IL-2 or IFN-γ, even at high levels of peptide plus APC. Taken together, these data suggested that tolerance led to decreased IL-2 production for both PLN and iIEL populations, but decreased IFN-γ production for only iIELs.

To maximize the ability to detect small amounts of cytokines produced by the individual iIELs subsets, iIEL populations were sorted to >98% purity before stimulation in vivo, and cytokine production was assessed for each subpopulation. In comparison to results with whole iIELs, low levels of IFN-γ and IL-2 were detected from Tg iIEL subsets from Ag^+ mice (Fig. 7). Addition of peptide increased IL-2 and IFN-γ production for iIELs from Ag^+ mice, but failed to induce levels comparable to those observed for iIEL subsets from Ag^- mice. Thus, tolerance led to decreased production of both IL-2 and IFN-γ for CD8αα and $CD4^-CD8^-$ Tg^+ iIEL subsets detected in Ag^+ mice.

Assessment of Cytokine mRNA by RT-PCR. One interpretation for the decreased proliferation and cytokine production by the Ag^+ iIEL subsets was that they had been rendered anergic by the presence of Ag. Alternatively, Tg^+ iIELs in Ag^+ mice may have altered their cytokine profile subsequent to encounter with Ag. Downregulation of IL-2 and IFN-γ production may have been accompanied by upregulation of IL-4 production. Our assessment of IL-4 levels using ELISA assays were variable from experiment to experiment, so we assessed IL-4 cytokine responses by measuring mRNA by competitive RT-PCR. As seen in Fig. 8, Tg^+ iIELs from Ag^- mice responded to in vitro stimulation with induction of IL-2, but limited IL-4 mRNA. In contrast, stimulation of Tg^+ iIELs from Ag^+ mice failed to induce IL-2 mRNA. These results were consistent with in vitro results (Figs. 6 and 7), and suggested that tolerance to self-Ag had effectively downregulated activation-induced IL-2 gene expression. An examination of IL-4 mRNA levels revealed that activation induced high levels of IL-4 for Tg^+ iIELs from Ag^+ mice, with a lesser induction observed for Tg^+ iIELs from Ag^- mice. These results suggested that Tg^+ iIELs in Ag^+ mice had differentiated to express TC2 (Th-2–like) cytokines.

Discussion

The purpose of this study was to determine the mechanisms responsible for the maintenance of self-tolerance of TCRαβ iIEL subsets. To address this question, we used the 2C TCR Tg model, which allowed us to examine the responses of T cells specific for a known antigen. The data suggested that tolerance for Tg^+ T cells involved deletional or nondeletional mechanisms, depending on expression of

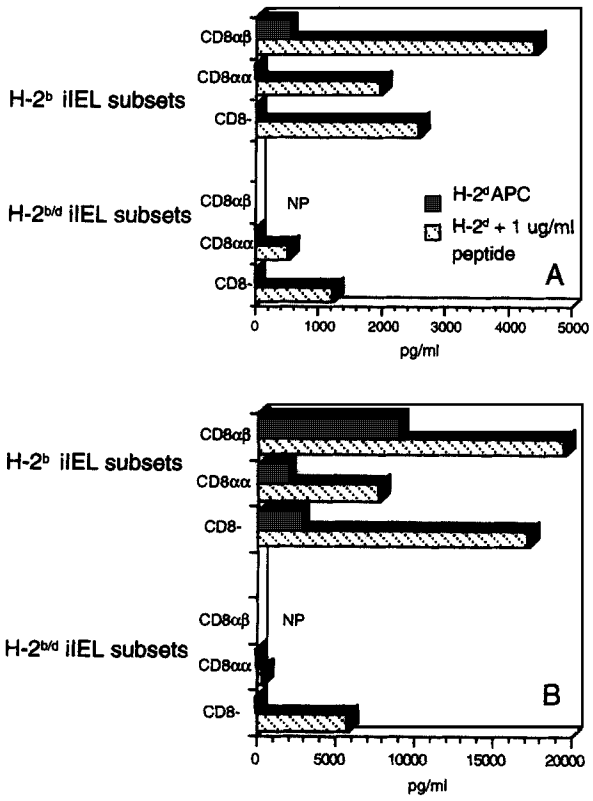

Figure 7. Cytokine production by Tg^+ iIEL subsets. Sorted iIEL subsets from Ag^- and Ag^+ mice were stimulated with endogenous antigen presented by irradiated Ag^+ APC without or with 1 μg/ml added peptide. Culture supernatant were collected at 48 h, and levels of IL-2 (*A*) and IFN-γ (*B*) were measured by ELISA. The data shown are representative of three experiments. *NP*, not present.

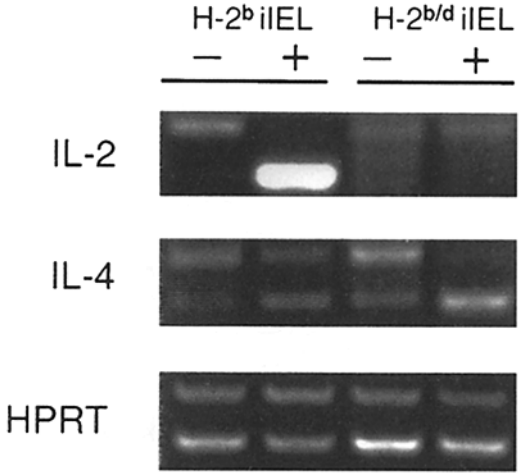

Figure 8. IL-4 and IL-2 cytokine mRNA expressed by activated Tg^+ iIEL in Ag^+ mice. Purified Tg^+ iIELs from Ag^+ and Ag^- mice were cultured for 3 h at 37°C in wells coated with clonotypic anti-TCR mAb, 1B2 (10 μg/ml) (+), or PBS only (−). The RNA was extracted, reverse transcribed, and analyzed for cytokines using competitive PCR with the PQRS cytokine mimic. Samples were normalized based on levels of mRNA for HPRT. The data shown are representative of three experiments.

CD8αβ molecules. In Ag^+ mice, $CD8\alpha\beta^+$ Tg^+ T cells were absent, suggesting that this population had been deleted. This resulted in decreased numbers of Tg^+ T cells in the periphery of 2C mice. However, Tg^+ iIEL numbers were equivalent between Ag^- and Ag^+ mice. The maintenance of Tg^+ numbers in Ag^+ mice despite the absence of CD8αβ iIELs may have resulted from downregulation of CD8β expression; however, no intermediate CD8β dull cells were observed. Another possibility was that the remaining CD8αα and $CD4^-CD8^-$ subsets had expanded. Nondeletional mechanisms of tolerance were observed for iIEL subsets which did not express CD8αβ. Effects of self-Ag on CD8αα and $CD4^-CD8^-$ iIEL subsets were indicated by modulation of surface activation markers and deviation of cytokine profiles. Transgenic iIELs in Ag^+ mice expressed decreased levels of Thy-1 and increased CD45R/B220 compared to iIELs from Ag^- mice. Functionally, iIELs in Ag^+ mice produced lower levels of IL-2 and IFN-γ compared to Ag^- mice. The decreased IL-2 and IFN-γ production correlated with the induction of IL-4 mRNA for Tg iIELs in Ag^+ mice, suggesting that tolerance involved the transition from IL-2–producing to an IL-4–producing phenotype. Interestingly, IL-4 production by iIELs from normal mice has been reported by Kiyono and colleagues (33). Taken together, these data suggest that tolerance involved distinct mechanisms for Tg^+ T cell populations in peripheral lymphoid and intestinal epithelial tissues. Whereas $CD8\alpha\beta^+$ Tg^+ T cells were absent from both populations, tolerance for CD8αα and $CD4^-CD8^-$ iIELs involved nondeletional mechanisms leading to the survival of functionally distinct subsets that expressed Th-2–like cytokines.

The distinct effects of tolerance observed for populations of Tg^+ iIELs may have been directly related to the differential sensitivities of iIEL subsets to self-Ag. Intestinal IEL subsets expressing surface CD8αβ, but not $CD4^-CD8^-$ and CD8αα, responded to Ag-bearing splenocytes (Fig. 5). CD8αβ-expressing iIELs also produced greater IL-2 and IFN-γ to added peptide compared to $CD4^-CD8^-$ and CD8αα iIELs in Ag^- mice. Previous reports have shown that CD8αβ molecules support adhesion and signaling for T cell responses to class I MHC–restricted antigens (34–36). During thymic development, increased levels of CD8 led to negative selection (37, 38). Thus, expression of CD8αβ molecules by Tg^+ iIELs may have influenced the effect of tolerance induction by increasing the sensitivity of this subset to endogenous levels of self-Ag.

In models of self-tolerance for iIELs, it has been difficult to distinguish functionally immature from mature phenotypes. This issue is particularly relevant when addressing effects of tolerance for CD8αα iIEL. Poussier and Julius have found that iIELs from normal and Tg mice expressing the CD8αα phenotype were unresponsive to stimulation by anti-TCR mAb (H597), suggesting that this subset was immature or required factors for activation not provided in vitro (13). These results raised the issue of whether self-reactive CD8αα iIELs in mice expressing $Mls\text{-}1^a$ or the male H-Y antigen had been tolerized to self-Ag or had persisted because of a failure in TCR-mediated signaling. We

addressed this issue by examining the responses of CD8αα and $CD8^-$ Tg iIEL subsets in Ag^- and Ag^+ mice. The results showed that, in fact, CD8αα iIELs mice were responsive to Ag, as assessed by proliferation and cytokine production, suggesting that Tg^+ CD8αα iIELs were not immature. In contrast, the corresponding CD8αα iIEL subsets in Ag^+ mice were unresponsive, even when cultured with exogenous peptide Ag. A comparison of responses by iIELs in Ag^- and Ag^+ mice suggested that the CD8αα subset in Ag^+ mice was a functionally mature population that had been tolerized to self-Ag.

Local environmental factors in the intestine may have been involved in tolerance induction for iIELs. Epithelial cells providing low levels of stimulation (39, 40) for Tg^+ iIELs may have contributed to the poor proliferative responses and the decreased IL-2 and IFN-γ production that was observed. Previous studies using Th-1 T cell clones have suggested that self-Ag presented by APCs with poor costimulatory function induced decreased IL-2 production in vitro (41–43). Thus, chronic, low levels of stimulation provided by intestinal epithelial cells may have helped to decrease some of the functional responses (IL-2, IFN-γ) observed for Tg^+ iIELs in Ag^+ mice. However, our results with iIEL from Ag^+ mice indicated that these cells were not completely unresponsive, but rather produced IL-4 in response to stimulation. These data suggested that exposure to self-Ag had induced the differentiation of Tg^+ iIELs to Th-2-like cells. $CD8^+$ T cells producing a cytokine profile typical of $CD4^+$ Th-2 cells have been described by others (44–46) and referred to by Mosmann and colleagues as TC2 cells (23). In the present model of self-tolerance in 2C mice, differentiation of iIELs in Ag^+ mice may have been directed down a TC2 pathway of differentiation in response to local cytokines as well. We have found (Barrett, T.A., unpublished observations), as well as others (33, 47–49), that $CD4^+$ lamina propria T cells and $CD8^+$ iIELs produce IL-4 and IL-5 cytokines. In addition, mucosal mast cell populations releasing IL-4 in response to stimulation may contribute to local levels of the cytokine (50). Intestinal epithelial cells also have the ability to make IL-10, which may be an important regulator of iIEL functional development (51). In recent reports by Forsthuber et al. and Ridge et. al. (52, 53), it has been suggested that conversion of tolerant populations of peripheral T cells down Th-2/TC2 pathways of differentiation may be an important mechanism for neonatal tolerization in vivo. These reports suggested that immune deviation was determined by the conditions under which Ag was presented. Thus, immune deviation for iIELs in Ag^+ mice may have occurred because of developmental pressures that eliminated TC1 precursors ($CD8\alpha\beta^+$ iIELs) and local factors that promoted TC2 differentiation.

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