

# T Cells with $\gamma/\delta$ T Cell Receptors (TCR) of Intestinal Type Are Preferentially Expanded in TCR- $\alpha$ -deficient *lpr* Mice

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## Summary

Fas-mediated apoptosis is essential for activation-induced cell death of  $\alpha/\beta$  T cells, but it is not clear what role, if any, it plays in regulating other components of the immune system. To study the role of Fas in  $\gamma/\delta$  T cell development, Fas-deficient *lpr* mice were bred with T cell receptor  $\alpha$  gene-ablated (TCR- $\alpha$   $-/-$ ) mice to generate mice deficient in one or both genes. The TCR- $\alpha$   $-/-$ , *lpr/lpr* mice had a nearly 10-fold increase in total lymph node cell (LNC) number compared with Fas-intact TCR- $\alpha$   $-/-$  mice, because of expansion of TCR- $\gamma/\delta$ <sup>+</sup> and TCR- $\beta$ <sup>+</sup> cells. In Fas-intact TCR- $\alpha$   $-/-$  mice, approximately one third of the LNCs expressed TCR- $\gamma/\delta$ . These were evenly divided between the CD4<sup>-</sup>, CD8- $\alpha$ <sup>+</sup> and the CD4<sup>-</sup>, CD8<sup>-</sup> subsets, and rarely expressed the B220 epitope of CD45. In contrast, in TCR- $\alpha$   $-/-$ , *lpr/lpr* mice, TCR- $\gamma/\delta$ <sup>+</sup> cells comprised half of the LNCs and were primarily CD4<sup>-</sup>, CD8<sup>-</sup>, and B220<sup>+</sup>. Moreover, Fas deficiency in TCR- $\alpha$   $-/-$  mice caused a preferential expansion of  $\gamma/\delta$  T cells expressing variable region genes characteristic of intestinal intraepithelial lymphocytes. These results demonstrate a role for Fas in regulating the  $\gamma/\delta$  T cell contribution to peripheral lymph nodes. This mechanism may be most important in limiting the access of activated intestinal intraepithelial lymphocytes to the peripheral lymphoid system.

Fas is a cell surface receptor, signaling through which induces apoptosis in the Fas-bearing cell (1–3). In mice the gene is expressed at its highest level in the thymus, with lower levels detected in liver, heart, and ovary (4). It has also been reported on mature T cells with an activated or memory phenotype (5, 6) and has been shown to be essential in activation-induced cell death (AICD)<sup>1</sup> of  $\alpha/\beta$  T cells (7).

Much of what is known about Fas has come from studies of a naturally occurring Fas-deficient mutant, the *lpr* mouse. Watanabe-Fukunaga et al. (8), and subsequently others (9), have shown that *fas* is disrupted in *lpr* mice by the insertion of an early transposable element (Etn) in the second intron of the gene. These mutant mice express little or no Fas on their thymocytes or mature T cells (10, 11) and exhibit a number of abnormalities of T cell development. The most obvious effects of this mutation are T cell autoimmunity (12),

and the massive accumulation of polyclonal  $\alpha/\beta$  T cells, which express a reduced level of TCR (13) and express neither CD4 nor CD8 ( $\alpha/\beta$  double-negative [DN] T cells) (14). These cells express the B220 epitope of CD45 (14), which is normally found only on B cells.

While the molecular basis of the *lpr* defect is now known and the phenotype of the accumulating  $\alpha/\beta$  DN T cells in these mice has been well described, the mechanism by which Fas deficiency leads to this accumulation remains unclear. Since Fas is expressed at high levels in the thymus (11), it has been suggested that the lack of Fas-mediated apoptosis during thymic repertoire selection gives rise to the abnormalities found in *lpr* mice (8, 15–17). However, recent experiments crossing the *lpr* mutation with a CD8-restricted transgenic TCR (Tg TCR) specific for the male antigen H-Y presented by H-2D<sup>b</sup> have shown by examining the LN T cells that positive and negative selection in the thymus was mostly intact (18–20). Even expression of a Tg TCR- $\beta$  chain alone, where endogenous TCR- $\alpha$  can pair with the transgene to form receptors of many specificities, did not develop DN T cell lymphadenopathy (21). More recently, Singer and Abbas have used an *lpr* mouse with a CD4-restricted Tg TCR specific for a pep-

<sup>1</sup> Abbreviations used in this paper: AICD, activation-induced cell death; DN, double negative; Etn, early transposable element; IHL, intrahepatic lymphocytes; iIEL, intestinal intraepithelial lymphocytes; LNC, LN cells; Neo<sup>R</sup>, neoresistance gene; SP, single positive; TCR- $\alpha$   $-/-$ , TCR- $\alpha$  gene ablated; Tg TCR, transgenic TCR.

tide from pigeon cytochrome C to show that deletion induced by exogenous peptide antigen was intact in the thymus of these mice (7). Thus, if the  $\alpha/\beta$  DN T cells in *lpr* mice arise in the thymus, they must come from an unselected pool of double-positive thymocytes that have failed to make a complete TCR complex.

An alternative possibility is that the phenotypic similarity between *lpr*  $\alpha/\beta$  DN T cells and immature thymocytes is misleading, and that these cells develop instead from activated peripheral single-positive (SP) T cells. When a T cell is exposed to its cognate antigen, it becomes activated and clonally expands, leading to an effector response. After this response, most of the responder cells are removed from the system by AICD (22). Fas-mediated apoptosis is probably essential for AICD of T cells. Several groups have shown that in *lpr* mice it is more difficult to induce peripheral tolerance to a superantigen (23–25) and the *lpr* mice do not maintain neonatal tolerance (23). More recently, Singer and Abbas used pigeon cytochrome C Tg TCR *lpr* mice to show that AICD was defective in Fas-deficient mice (7). These mice failed to delete their peripheral T cells in response to a large dose of antigenic peptide in vivo, whereas Fas-intact Tg TCR mice deleted these cells. Thus, in the absence of Fas, both peripheral tolerance and AICD were severely impaired.

Expression of the B220 epitope of CD45 by T cells may be a hallmark of AICD, and we have shown recently that the liver is an important site for its expression on T cells before deletion (26). Using mice with a Tg TCR specific for a peptide of SV40 large T antigen in the context of H-2<sup>k</sup>, we have shown that exposure of T cells to their cognate antigen in vivo induced expression of a variety of activation markers on the responding cells, including B220. These activated cells trafficked to the liver, down-regulated their expression of CD8, and underwent apoptosis. These findings suggest that the liver is a major site for elimination of T cells in AICD.

In examination of intrahepatic lymphocytes (IHLs) of normal, unmanipulated mice, we found a population of T cells that resembled the  $\alpha/\beta$  DN T cells of *lpr* mice (27). These cells expressed B220 but neither coreceptor and had a reduced level of TCR- $\alpha/\beta$  on their surface. The B220<sup>+</sup> but not the B220<sup>-</sup> population of IHLs contained cells that were proliferating and cells undergoing apoptosis. In *lpr* mice these cells were detectable in the liver before they could be found in the peripheral LNs (28). Taken together, these data suggest the hypothesis that B220 expression in T cells is a marker of incipient Fas-mediated cell death and that a failure of Fas-mediated apoptosis in IHLs in *lpr* mice leads to their accumulation and eventual export into the LNs as B220<sup>+</sup>  $\alpha/\beta$  DN T cells.

While the role of Fas and AICD in controlling peripheral  $\alpha/\beta$  T cell number is thus becoming clearer, little is known about the regulation of  $\gamma/\delta$  T cell number. In fact, very little is known about the antigen(s) that normally activate  $\gamma/\delta$  T cells, the normal role of  $\gamma/\delta$  T cell effector responses, or the capacity of  $\gamma/\delta$  T cells to be regulated by activation and apoptosis. Because of their prevalence in the skin and mucosal

surfaces, it has been proposed that they may act as a first line of defense against invading pathogens, though, again, how they might be regulated is uncertain.

The  $\alpha/\beta$  T cells may play a role in regulating  $\gamma/\delta$  T cells, at least in the LN. Ablation of TCR- $\alpha$  gene expression (TCR- $\alpha$  -/-) eliminated the normal  $\alpha/\beta$  T cell pool and all of its effector responses and allowed for an increase in the number of  $\gamma/\delta$  T cells developing in peripheral LNs compared with mice with wild-type TCR- $\alpha$  genes (TCR- $\alpha$  intact) (29, 30). This expansion of  $\gamma/\delta$  T cells in TCR- $\alpha$  -/- mice has greatly facilitated the study of this normally rare population of lymphocytes. To study the role of Fas in regulating  $\gamma/\delta$  T cells, we bred TCR- $\alpha$  -/- mice with *lpr* mice to generate animals deficient in expression of both genes. We examined the LN cells (LNCs) of these mice and compared them to those of their littermates with wild-type expression of one or both genes. With this system we were able to examine the role of Fas in regulating peripheral  $\gamma/\delta$  T cells and at the same time to test the hypothesis that unselected DP thymocytes give rise to  $\alpha/\beta$  DN T cells in *lpr* mice.

## Materials and Methods

**Mice.** TCR- $\alpha$  -/- mice of the 129 strain have been described (29). MRL-*lpr/lpr* mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred with the TCR- $\alpha$  -/- mice in a specific pathogen-free environment in the Immunobiology Mouse Unit (Yale University School of Medicine, New Haven, CT). Progeny of this mating were interbred to generate F2 mice, which were used at 16 wk of age.

**PCR Typing of TCR- $\alpha$  and *fas/lpr* Gene Loci.** Tail DNA from F2 mice were resuspended in 100  $\mu$ l of Tris-EDTA, and this suspension was diluted 1:20 in H<sub>2</sub>O to make tail template DNA. The following primers were used for PCR: TCRCAF1, TCCAGAACC-CAGAACCCTGCTGTG; TCRCAR1, CCTGAACTGGGGTAG-GTGGGG; NEOMS1, CCTTGCGCAGCTGTGCTCGACGTTG; FAS12FX, ACAGCATAGATTCCATTTGCTGCT; FAS12REV, TGAGTAATGGGCTCAGTCAGCA; and FASZ8XTR, CAA-ATTTTATTGTTGCGACACCA (modified from Adachi et al. [31]). PCR reactions contained 5  $\mu$ l tail DNA template, 20 mM Tris-HCL (pH 8.4), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, Inc., Piscataway, NJ), 100 ng of each of four primers, and 1 U Taq Polymerase (Gibco BRL, Gaithersburg, MD) in a final volume of 50  $\mu$ l, and were run for 30 cycles. Each cycle consisted of 45 s at 94°C, 1 min at 61.5°C, and 1 min at 72°C. 25  $\mu$ l of each PCR reaction was electrophoresed through a 1.2% agarose gel and visualized by ethidium bromide staining under UV light. Wild-type, *lpr*, and heterozygous mice were typed in two separate reactions. The forward primer (FAS12FX), complementary to the second intron of *fas*, 5' of the Etn insertion on *lpr* mice, paired with FAS12REV, which also primed from within the second intron, 3' to the Etn insertion, to give a product of ~200 bp in the wild-type reaction. No product was seen from *lpr* template. The *lpr*-specific primer FASZ8XTR reacted with FAS12FX to give a product of ~250 bp. Mice were typed for TCR- $\alpha$  genotype by PCR, again using two reactions. The forward primer TCRCAF1 paired with either the knock-out-specific (neoresistance gene [*Neo*<sup>r</sup>]-specific) reverse primer NEOMS1 (mutant reaction, ~750 bp) or with TCRCAR1 (wild-type reaction, ~300 bp). The wild-type reaction performed

using TCR- $\alpha$   $-/-$  template often gave a band of  $\sim 1,800$  bp because of priming through the Neo<sup>R</sup> gene. The two wild-type reactions and the two mutant reactions were used together to type F2 mice for both genes.

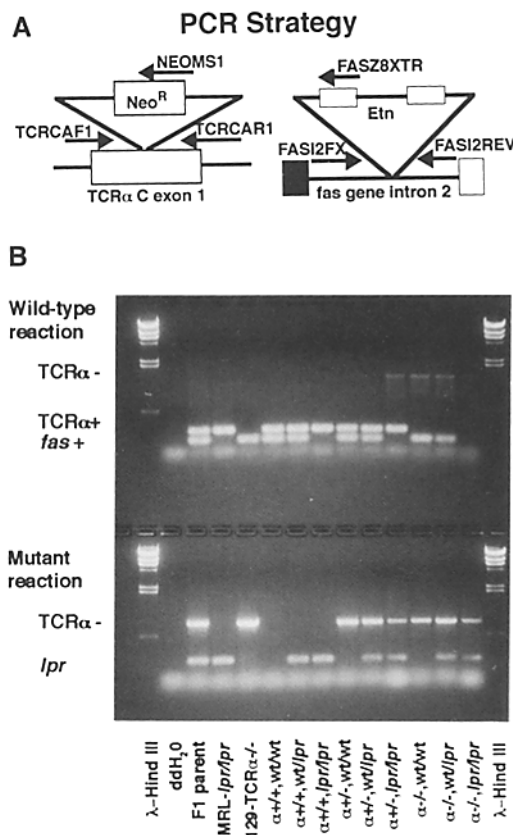
**Cells, Antibodies, and FACS<sup>®</sup> Analysis.** F2 mice were killed at 16 wk of age and 10 peripheral (nonmesenteric) LNs were obtained from each mouse. These LN were ground in a tissue homogenizer and the LNCs were washed in Bruff's medium with 2% FCS before use. Viable cells were counted by nigrosin dye exclusion to determine total cell yield from each mouse.

The following antibodies were used: H57-597-PE (anti-TCR C $\beta$ ; Pharmingen, San Diego, CA) (32), RA3-3A1-FITC (TIB-146, anti-B220, kind gift of Kim Bottomly) (33), 3.168-FITC (anti-CD8- $\alpha$ , conjugated and tested in our laboratory) (34), H129-19-Red 613 (anti-CD4; Gibco BRL) (35), 53-6.7-Red 613 (anti-CD8- $\alpha$ , Gibco BRL) (36), GL-3-PE (anti-pan-TCR- $\gamma/\delta$ ; Pharmingen) (37), UC3-10A6-PE (anti-TCRV $\gamma$ 4; Pharmingen) (38), 536-PE (anti-V $\gamma$ 5; Pharmingen) (39), GL-1-biotin (anti-V $\gamma$ 6; kind gift of Leo LeFrancis) (37, 40) followed by streptavidin-PE (Biomed, Foster City, CA) and GL-2-PE (anti-V $\delta$ 4; Pharmingen) (37, 40). To study the subsets of TCR- $\beta$ <sup>+</sup> cells,  $1 \times 10^6$  LNCs were suspended in 100 ml Bruff's/FCS and stained with H57-597-PE with either: (a) RA3-3A1-FITC plus 53-6.7-Red 613, to study CD8<sup>+</sup> cell phenotype; (b) RA3-3A1-FITC plus H129-19-Red 613, to study CD4<sup>+</sup> cell phenotype; (c) RA3-3A1-FITC plus 53-6.7 Red 613 plus H129-19-Red 613, to study DN cell phenotype; or (d) 3.168-FITC plus H129-19-Red 613, for CD8 vs CD4 expression. The same staining pattern was used with GL-3-PE to study subsets of  $\gamma/\delta$  T cells. Data were acquired on a FACScan<sup>®</sup> flow cytometer (Becton Dickinson and Co., Cockeysville, MD) using FACScan<sup>®</sup> Research Software and analyzed using Lysis 1.7 software (Becton Dickinson and Co.). The percentage of cells with each phenotype was determined using the TCR- $\beta$ <sup>+</sup> or TCR- $\gamma/\delta$ <sup>+</sup> cell gate and quadrant regions shown. The percentage of cells with each phenotype was multiplied by the total cell yield to determine the absolute yield for each subset.

## Results

**Expansion of T Cells in Fas-deficient TCR- $\alpha$   $-/-$  Mice.** To generate Fas-deficient mice lacking TCR- $\alpha$  expression, we bred TCR- $\alpha$   $-/-$  mice of strain 129 with MRL-*lpr/lpr* mice and examined mice of the F2 generation. The TCR- $\alpha$  and *fas* genotypes of these mice were determined by PCR analysis of tail DNA (Fig. 1). A single forward primer complementary to the 5' end of the first exon of the constant region of the TCR- $\alpha$  gene was paired with either a primer from the 3' end of that exon (TCR- $\alpha$  wild-type reaction) or with a Neo<sup>R</sup>-specific primer (TCR- $\alpha$  mutant reaction), identifying the genotype of the mice in two reactions. A similar strategy was used for the *fas* gene, where a single forward primer in the second intron was paired with either a reverse primer, which is 3' of the Etn insertion in *lpr* mice (*fas* wild-type reaction), or with an Etn-specific reverse primer (*lpr* mutant reaction). The two sets of reactions were used together, and the specificity of the typing was checked by FACS<sup>®</sup> analysis of Fas, CD4, CD8, and TCR expression in the thymus (data not shown).

The F2 mice were killed at 16 wk, and the lymphocytes from 10 peripheral (nonmesenteric) LNs were pooled. These



**Figure 1.** PCR typing of F2 (TCR- $\alpha$   $-/-$   $\times$  MRL-*lpr/lpr*) mice. (A) PCR typing strategy. PCR between the primers TCRCAF1 and TCR-CAR1 identifies intact TCR- $\alpha$  genes, whereas TCRCAF1 to NEOMS1 identifies the insert in the TCR C $\alpha$  knock-outs. Similarly, PCR between the primers FAS12FX and FAS12REV identifies the second intron of the normal *fas* gene, whereas PCR from FAS12FX to FAS28XTR identifies the mutant *fas* genes containing the Etn transposon. (B) Typing reaction. The four PCR products unambiguously type all nine genotypes of F2 (TCR- $\alpha$   $-/-$   $\times$  MRL-*lpr/lpr*) mice in two PCR reactions. The TCR- $\alpha$ -specific product runs above the *fas/lpr*-specific product in both reactions. All four products were seen using F1 parental DNA, and only the expected products were seen when MRL-*lpr/lpr* or strain 129-TCR- $\alpha$   $-/-$  templates were used alone.

lymphocytes were stained for TCR- $\alpha/\beta$  or TCR- $\gamma/\delta$  and analyzed by FACS<sup>®</sup>. The percentage of LNCs expressing either marker was then multiplied by the cell yield for each mouse to determine the yield for each cell type. Pooled data from 16 litters are shown in Table 1. Heterozygotes for either gene were identical in phenotype of homozygous wild-type mice for that gene; the data presented here are from homozygotes. By 16 wk of age, the *lpr* mice with wild-type TCR- $\alpha$  had developed massive lymphadenopathy due to  $\alpha/\beta$  DN T cell accumulation;  $\sim 10\%$  of these mice were moribund. As previously described (29, 41), ablation of the TCR- $\alpha$  gene led under specific pathogen-free conditions to an increase in the absolute number of peripheral TCR- $\gamma/\delta$ <sup>+</sup> T cells but a decrease in the total number of peripheral lymphocytes because of the elimination of  $\alpha/\beta$  T cells. Massive

**Table 1.** T Cell Yield from LNs

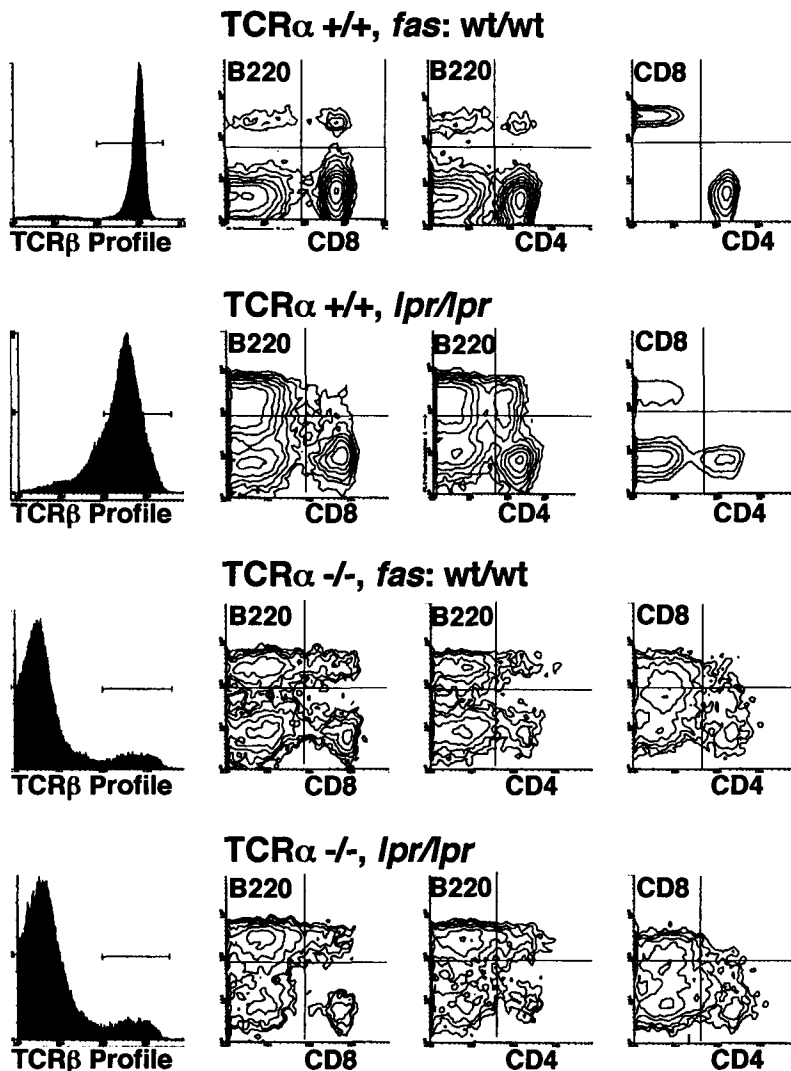
Genotype	n	LNC	TCR- $\beta^+$ LNC	TCR- $\gamma/\delta^+$ LNC
$\alpha +/+ , wt/wt$	14	57.72 $\pm$ 17.9	51.66 $\pm$ 13.0	0.64 $\pm$ 0.36
$\alpha +/+ , lpr/lpr$	8	2,680.00 $\pm$ 1570	2,220.00 $\pm$ 1280	32.00 $\pm$ 13.6
$\alpha -/- , wt/wt$	12	9.43 $\pm$ 2.77	1.30 $\pm$ 0.65	3.14 $\pm$ 0.89
$\alpha -/- , lpr/lpr$	9	85.67 $\pm$ 38.8	7.91 $\pm$ 2.97	44.60 $\pm$ 22.5

The yield of live lymphocytes from 10 peripheral (nonmesenteric) LNs are shown in millions. Values are mean  $\pm$  SD. Genotype of mice was determined by PCR as shown in Fig. 1. The yield of TCR- $\beta^+$  cells was determined by multiplying the total yield of LNCs by the percentage of LNCs stained with anti-TCR- $\beta$  mAb. The yield of TCR- $\gamma/\delta^+$  cells was determined by multiplying the total yield of LNCs by the percentage of LNCs stained with anti-TCR- $\gamma/\delta$  mAb. *wt*, wild type.

lymphadenopathy was also eliminated in TCR- $\alpha -/- , lpr/lpr$  mice. However, absence of Fas in these mice caused a 15-fold expansion of TCR- $\gamma/\delta^+$  T cells, as well as a 6-fold increase in TCR- $\beta^+$  cells when compared with Fas-intact TCR- $\alpha -/-$  mice. The TCR- $\beta^+$  cells in TCR- $\alpha -/-$  mice have recently been described (41) and are the subject of ongoing

study. The increased number of TCR- $\gamma/\delta^+$  cells was detectable in TCR- $\alpha +/+ , lpr/lpr$  as well as TCR- $\alpha -/- , lpr/lpr$  mice, and is examined in detail here.

**Fas Deficiency Causes Expansion of TCR- $\beta^+$  Cells with Abnormal Phenotypes.** To determine which subsets of TCR- $\beta^+$  cells were expanding in TCR- $\alpha -/- , lpr/lpr$  mice, we stained



**Figure 2.** Fas deficiency increases the proportion of B220<sup>+</sup> DN cells in TCR- $\beta^+$  cells of TCR- $\alpha -/-$  mice. Single cell suspensions of LNCs were stained in anti-TCR- $\beta$ -PE and either (a) anti-B220-FITC plus anti-CD8-Red 613; (b) anti-B220-FITC plus anti-CD4-Red 613; or (c) anti-CD8-FITC and anti-CD4-Red 613, and analyzed by multicolor FACS<sup>®</sup>. TCR- $\beta^+$  cells were identified using the histogram gate shown to generate these plots. The quadrant regions shown were used to calculate the percentage of lymphocytes in each subset.

the LNCs of 16-wk-old mice with antibodies TCR- $\beta$ , CD4, CD8, and B220 and analyzed these cells by FACS<sup>®</sup>. Representative FACS<sup>®</sup> plots are shown in Fig. 2. Using the TCR- $\beta$ <sup>+</sup> gate and quadrant regions shown, we determined the percentage in each subset of TCR- $\beta$ <sup>+</sup> cells. As has been described previously by many groups, Fas deficiency led to the abnormal development of B220<sup>+</sup> TCR- $\beta$ <sup>+</sup> DN cells in TCR- $\alpha$ -intact *lpr* mice (for review see reference 42). Although ablation of TCR- $\alpha$  gene expression eliminated the normal  $\alpha/\beta$  T cell pool in Fas-intact TCR- $\alpha$ <sup>-/-</sup> mice, a small population of TCR- $\beta$ <sup>+</sup> cells persisted in the peripheral LNs. These cells were CD8<sup>+</sup>, DN, or CD4<sup>+</sup>. Approximately one quarter of the TCR- $\beta$ <sup>+</sup> cells in a Fas-intact TCR- $\alpha$ <sup>-/-</sup> mouse expressed B220. These cells were mostly DN, with a small proportion of CD8<sup>lo</sup> cells. In TCR- $\alpha$ <sup>-/-</sup>, *lpr/lpr* mice, the lack of Fas increased the proportion of B220<sup>+</sup> DN cells in TCR- $\beta$ <sup>+</sup> cells, skewing the TCR- $\beta$ <sup>+</sup> population toward a DN, B220<sup>+</sup> phenotype.

The skewing of TCR- $\beta$ <sup>+</sup> cells toward a DN, B220<sup>+</sup> phenotype in Fas-deficient TCR- $\alpha$ <sup>-/-</sup> mice was due to a preferential expansion of the B220<sup>+</sup> subsets in this population (Table 2). The percentage of lymphocytes for each subset was determined using the quadrants shown in Fig. 2, and this number was multiplied by the total cell yield for that mouse to determine the absolute yield for each subset. Most of the TCR- $\beta$ <sup>+</sup> cells in a Fas-intact TCR- $\alpha$ <sup>-/-</sup> mouse did not express B220 and fell within the DN cell quadrant shown in Fig. 2, though many in fact expressed CD8 at a low level. Absence of Fas in TCR- $\alpha$ <sup>-/-</sup>, *lpr/lpr* mice led to an expansion of most TCR- $\beta$ <sup>+</sup> cell subsets, but the B220<sup>+</sup> subsets, especially the DN B220<sup>+</sup> pool, increased far more than did the B220<sup>-</sup> subsets. Fas deficiency led to a 4-fold expansion of the DN B220<sup>-</sup> subset, a 10-fold increase in the CD4<sup>+</sup> B220<sup>+</sup> and the DN B220<sup>+</sup> subsets, and a 5-fold expansion of the CD8<sup>+</sup> B220<sup>+</sup> subset. This accumulation of B220<sup>+</sup> TCR- $\beta$ <sup>+</sup> cells is similar to the preferential accumulation of  $\alpha/\beta$  DN T cells seen in TCR- $\alpha$ -intact *lpr* mice.

**TCR- $\gamma/\delta$ <sup>+</sup> Cells in Fas-deficient Mice Accumulate and Acquire an Abnormal Phenotype.** Since the majority of the expanding cells in the TCR- $\alpha$ <sup>-/-</sup>, *lpr/lpr* mice expressed TCR- $\gamma/\delta$ , it was important to learn which subsets of  $\gamma/\delta$  T cells were expanding in these mice. We stained LNCs from

16-wk-old mice for TCR- $\gamma/\delta$ , CD4, CD8, and B220 and analyzed these cells by three-color FACS<sup>®</sup>. Representative FACS<sup>®</sup> plots are shown in Fig. 3. Using the TCR- $\gamma/\delta$ <sup>+</sup> gate and quadrant regions shown, we determined the percentage of LNCs in each subset of  $\gamma/\delta$  T cells. Ablation of TCR- $\alpha$  gene expression eliminated the TCR- $\alpha/\beta$ <sup>+</sup> cell population and caused an increase in the proportion and absolute number of TCR- $\gamma/\delta$ <sup>+</sup> cells in TCR- $\alpha$ <sup>-/-</sup> mice, as has been reported (29). The phenotype of the TCR- $\gamma/\delta$ <sup>+</sup> cells in these mice was identical to that seen in TCR- $\alpha$ -intact mice. These cells were primarily divided between CD8<sup>+</sup> cells and DN cells, with a small population expressing CD4 and not CD8. Most  $\gamma/\delta$  T cells from Fas-intact mice did not express B220. In *lpr* mice, however, the majority of  $\gamma/\delta$  T cells did express B220 but were CD8<sup>-</sup>. Thus, Fas deficiency skewed the  $\gamma/\delta$  T cell pool toward a B220<sup>+</sup>, DN phenotype.

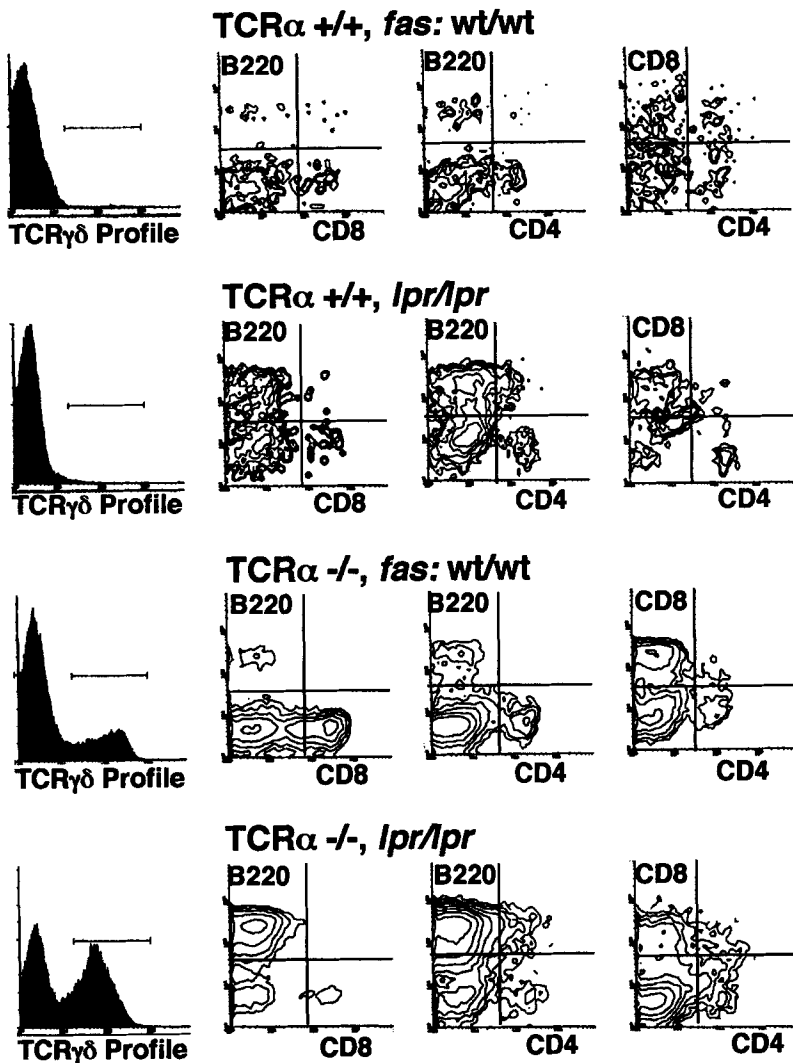
This skewing of coreceptor and B220 expression of the  $\gamma/\delta$  T cell pool was due to the preferential expansion of the DN B220<sup>+</sup> subset in TCR- $\alpha$ <sup>-/-</sup>, *lpr/lpr* mice. We multiplied the percentage of lymphocytes in each subset by the total cell yield from each mouse to determine the absolute number of cells in each subset (Table 3). While all subsets of  $\gamma/\delta$  T cells were expanded by Fas deficiency, the expansion within the B220<sup>+</sup> DN subset was far more dramatic than that of any other subpopulation, leading to the skewed phenotype. Similar numbers of abnormal TCR- $\gamma/\delta$ <sup>+</sup> cells were observed in TCR- $\alpha$ -intact *lpr* mice. The CD8<sup>+</sup> B220<sup>-</sup>  $\gamma/\delta$  T cell subset and the CD4<sup>+</sup> B220<sup>-</sup>  $\gamma/\delta$  T cell subset increased 1.5-fold with Fas deficiency, but the B220<sup>-</sup> DN  $\gamma/\delta$  T cells increased 20-fold. The CD4<sup>+</sup> B220<sup>+</sup> subset also increased 20-fold, while the CD8<sup>+</sup> B220<sup>+</sup> subset expanded 6-fold. The 300-fold expansion of the DN B220<sup>+</sup>  $\gamma/\delta$  T cell subset, however, obscured the expansion of the other subsets. This effect on  $\gamma/\delta$  T cells is similar to that seen in  $\alpha/\beta$  T cells in TCR- $\alpha$ -intact *lpr* mice.

**Fas Deficiency Skews the  $\gamma/\delta$  T Cell Repertoire Toward Expression of Variable Genes Characteristic of Gut-derived  $\gamma/\delta$  T Cells.** In TCR- $\alpha$ -intact mice, almost all peripheral LN T cells express TCR- $\alpha/\beta$ , and  $\gamma/\delta$  T cells are a very small population, making these cells difficult to study. This problem is further compounded by the massive accumulation of  $\alpha/\beta$  DN T cells in TCR- $\alpha$ -intact *lpr* mice. The absence of TCR-

**Table 2.** Yield of TCR- $\beta$ <sup>+</sup> Subsets

Genotype	CD4 <sup>+</sup> , B220 <sup>-</sup>	CD4 <sup>+</sup> , B220 <sup>+</sup>	CD8 <sup>+</sup> , B220 <sup>-</sup>	CD8 <sup>+</sup> , B220 <sup>+</sup>	DN, B220 <sup>-</sup>	DN, B220 <sup>+</sup>
$\alpha$ + / +, wt/wt	28.0 ± 8.10	0.161 ± 0.024	22.4 ± 5.86	0.405 ± 0.042	1.26 ± 0.486	0.349 ± 0.097
$\alpha$ + / +, <i>lpr/lpr</i>	160.0 ± 110.8	102.0 ± 36.2	102.0 ± 36.2	4.93 ± 1.12	164.0 ± 138.0	1,968 ± 833
$\alpha$ - / -, wt/wt	0.129 ± 0.075	0.048 ± 0.007	0.301 ± 0.185	0.062 ± 0.042	0.600 ± 0.280	0.399 ± 0.187
$\alpha$ - / -, <i>lpr/lpr</i>	0.189 ± 0.111	0.290 ± 0.160	0.290 ± 0.087	2.64 ± 1.28	2.64 ± 1.28	4.65 ± 2.13

Expansion of subsets of TCR- $\beta$ <sup>+</sup> cells in Fas-deficient mice. The yields of cells of each phenotype are shown in millions per mouse. Values are mean ± SD. Yields were calculated by multiplying the percentage of cells with each phenotype by the total yield of LNCs. Percentages for CD4 SP and CD8 SP cells were determined using the TCR- $\beta$ <sup>+</sup> cell gate and quadrant regions shown in Fig. 2. Fas deficiency expands the B220<sup>+</sup> subsets of TCR- $\beta$ <sup>+</sup> cells in TCR- $\alpha$ <sup>-/-</sup> as well as TCR- $\alpha$ -intact mice. Data were obtained from the same mice described in Table 1. *wt*, wild type.



**Figure 3.** Fas deficiency skews the expanded  $\gamma/\delta$  T cells in TCR- $\alpha^{-/-}$  mice towards a B220<sup>+</sup>, DN phenotype. TCR- $\gamma/\delta$  cells were identified using the histogram gate shown to generate the contour plots. The quadrant regions shown were used to calculate the percentage of lymphocytes in each subset.

$\alpha/\beta^+$  cells, especially the vastly expanded  $\alpha/\beta$  DN T cells of *lpr* mice, in TCR- $\alpha^{-/-}$  mice allowed us to make a more detailed examination of the effect of Fas deficiency on the development of  $\gamma/\delta$  T cells. To determine the potential source of the abnormally expanded  $\gamma/\delta$  T cells in Fas-deficient mice, we examined the expression of specific V $\gamma$  and V $\delta$  genes by FACS<sup>®</sup> in LNCs of 16-wk-old Fas-intact and *lpr* TCR- $\alpha^{-/-}$  mice. Representative FACS<sup>®</sup> plots are shown in Fig. 4. TCR V $\gamma$ 4 (also known as V $\gamma$ 2) is normally expressed in peripheral LN  $\gamma/\delta$  T cells (38). In Fas-intact TCR- $\alpha^{-/-}$  mice  $\sim$ 20% of LN  $\gamma/\delta$  T cells expressed this V region. In contrast,  $\sim$ 6% of the  $\gamma/\delta$  T cell pool in TCR- $\alpha^{-/-}$ , *lpr/lpr* mice was stained with anti-V $\gamma$ 4 antibodies. Dendritic epidermal T cells characteristically express TCR V $\gamma$ 5 (also known as V $\gamma$ 3) in the mouse and are not normally found in the LNs of adult mice (39). Less than 0.5% of LN  $\gamma/\delta$  T cells in either Fas-intact or *lpr* TCR- $\alpha^{-/-}$  mice were stained with antibodies to V $\gamma$ 5, indicating that dendritic epidermal T cells did not contribute significantly to the peripheral LN  $\gamma/\delta$  T cell pool in TCR- $\alpha^{-/-}$  mice.

Intestinal intraepithelial lymphocytes (iIELs) are a heterogeneous population of mononuclear cells, primarily T cells, which reside in the epithelium of the gut. Approximately half of iIELs are TCR- $\gamma/\delta^+$ , and these cells characteristically express TCR V $\gamma$ 7 (also known as V $\gamma$ 5) and TCR V $\delta$ 4 (37, 40). To determine if cells of this lineage made a significant contribution to the expansion of  $\gamma/\delta$  T cells in TCR- $\alpha^{-/-}$ , *lpr/lpr* mice, we stained peripheral LNCs from 16-wk-old mice with antibodies to TCR V $\gamma$ 7 and TCR V $\delta$ 4 (Fig. 4). In Fas-intact TCR- $\alpha^{-/-}$  mice,  $\sim$ 8% of the  $\gamma/\delta$  T cells were stained with anti-V $\gamma$ 7. TCR V $\delta$ 4 expression was slightly more abundant in these mice:  $\sim$ 18% of  $\gamma/\delta$  T cells. Expression of both these variable regions was much higher in *lpr* mice. Approximately 15% of  $\gamma/\delta$  T cells expressed V $\gamma$ 7, and  $>$ 25% of  $\gamma/\delta$  T cells expressed TCR V $\delta$ 4. These percentages were multiplied by the total LNC count to determine the cell yield for each subset of  $\gamma/\delta$  T cells (Fig. 5). This calculation revealed a dramatic expansion of  $\gamma/\delta$  T cells expressing variable genes characteristic of iIELs. Although Fas deficiency caused only a threefold increase in V $\gamma$ 4-expressing cells, from

**Table 3.** Yield of TCR- $\gamma/\delta^+$  Subsets

Genotype	CD4 <sup>+</sup> , B220 <sup>-</sup>	CD4 <sup>+</sup> , B220 <sup>+</sup>	CD8 <sup>+</sup> , B220 <sup>-</sup>	CD8 <sup>+</sup> , B220 <sup>+</sup>	DN, B220 <sup>-</sup>	DN, B220 <sup>+</sup>
$\alpha +/+$ , wt/wt	0.044 $\pm$ 0.016	0.012 $\pm$ 0.002	0.051 $\pm$ 0.014	0.010 $\pm$ 0.004	0.482 $\pm$ 0.115	0.025 $\pm$ 0.011
$\alpha +/+$ , <i>lpr/lpr</i>	0.248 $\pm$ 0.235	0.392 $\pm$ 0.224	0.213 $\pm$ 0.101	0.061 $\pm$ 0.010	7.921 $\pm$ 5.755	18.38 $\pm$ 9.44
$\alpha -/-$ , wt/wt	0.211 $\pm$ 0.070	0.017 $\pm$ 0.001	1.000 $\pm$ 0.383	0.033 $\pm$ 0.016	1.803 $\pm$ 0.923	0.124 $\pm$ 0.070
$\alpha -/-$ , <i>lpr/lpr</i>	0.316 $\pm$ 0.048	0.303 $\pm$ 0.149	1.454 $\pm$ 0.757	0.198 $\pm$ 0.105	6.724 $\pm$ 1.924	35.50 $\pm$ 26.90

Expansion of subsets of TCR- $\gamma/\delta^+$  cells in Fas-deficient mice. The yield of cells with each phenotype is shown in millions per mouse. Values are mean  $\pm$  SD. The yield of cells of each phenotype was calculated by multiplying the percentage of cells with that phenotype by the total cell yield from the mouse. Percentages for CD4 SP and CD8 SP cells were determined using the TCR- $\gamma/\delta^+$  cell gate and quadrant regions shown in Fig. 3. Data shown were obtained from the same mice described in Table 1. wt, wild type.

an average of 0.6 to 1.7 million per mouse, there was a 12-fold increase in V $\gamma$ 7 and V $\delta$ 4-expressing cells from 0.28 to 3.4 million and from 0.76 to 9.6 million, respectively. Both the V $\gamma$ 4<sup>+</sup> and V $\gamma$ 7/V $\delta$ 4<sup>+</sup> subsets contributed to the abnormal B220<sup>+</sup>  $\gamma/\delta$  T cells observed in TCR- $\alpha -/-$ , *lpr/lpr* mice (data not shown). Thus, Fas deficiency causes an abnormal phenotype in all subsets of  $\gamma/\delta$  T cells normally observed in the LN, but leads to a preferential expansion of those  $\gamma/\delta$  T cells using variable genes normally associated with iIELs. It is not clear if these abnormal LNCs arise from cells that were resident in the gut or if they simply arise from the same precursor pool.

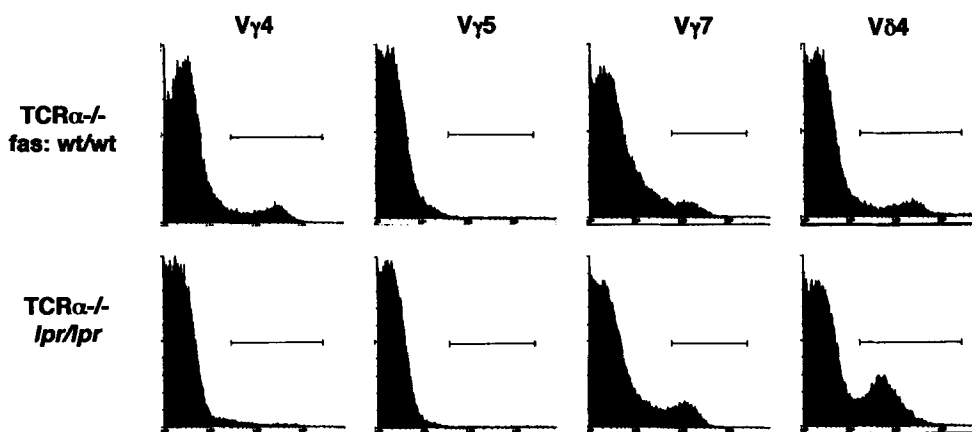
### Discussion

The absence of massive lymphadenopathy in TCR- $\alpha -/-$ , *lpr/lpr* mice demonstrates that double-positive thymocytes with incomplete TCR complexes do not give rise to the  $\alpha/\beta$  DN T cells. If unselectable T cells gave rise to  $\alpha/\beta$  DN T cells in *lpr*, they should have accumulated faster in TCR- $\alpha -/-$ , *lpr/lpr* mice. This potential precursor pool was abundant in the thymus of these mice (data not shown), but TCR- $\beta^+$  DN cells were reduced >100-fold by the elimination of TCR- $\alpha$  chain expression in *lpr* mice. Thus, unselected thymocytes with incomplete TCR complexes can be removed by a Fas-independent mechanism. These findings support the

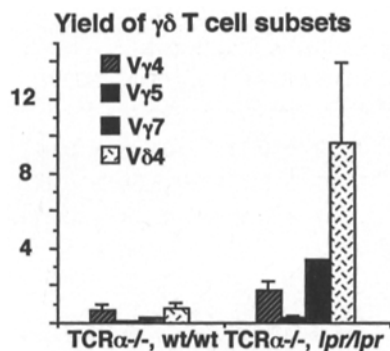
mounting evidence that points to an essential role for Fas in AICD in the periphery but a redundant role, if any at all, for Fas in the thymus (7, 22).

The data presented here clarify the origin of  $\alpha/\beta$  DN T cells in *lpr* and identify an important role for Fas in regulating peripheral  $\gamma/\delta$  T cells. The discovery of B220<sup>+</sup>  $\gamma/\delta$  T cells was quite surprising. This marker has not been reported on  $\gamma/\delta$  cells before, and these cells are not known to be regulated by AICD. Indeed, little is known about the antigen(s) recognized by  $\gamma/\delta$  T cells, nor how their development and responses are regulated. The data presented here make it clear, however, that Fas-mediated apoptosis plays an important role in  $\gamma/\delta$  T cell regulation. The identification of this same unusual population in TCR- $\alpha$ -intact *lpr* mice shows that this phenotype was not an artifact of TCR- $\alpha$  gene ablation. If the parallel between  $\alpha/\beta$  T cells and  $\gamma/\delta$  T cells holds true, these data strongly suggest that  $\gamma/\delta$  T cells are also subject to AICD in normal mice. However, the findings reported here describe only unmanipulated mice differing in the expression of two genes, so these data do not directly demonstrate AICD in  $\gamma/\delta$  T cells.

There is support for the idea of Fas-mediated AICD regulation of  $\gamma/\delta$  T cells in the preferential expansion of cells expressing iIEL-associated V $\gamma$  and V $\delta$  genes. Although little is known about the antigen(s) recognized by  $\gamma/\delta$  TCRs, it is not difficult to imagine that  $\gamma/\delta$  T cells in the gut are



**Figure 4.** Fas deficiency skews the  $\gamma/\delta$  T cell repertoire towards the use of variable genes characteristic of gut-derived  $\gamma/\delta$  T cells. Representative histograms are shown. The gates shown were used to calculate the percentage of LNCs expressing each variable gene region.



**Figure 5.** Selective expansion of gut-derived  $\gamma/\delta$  T cells in Fas-deficient TCR- $\alpha^{-/-}$  mice. Data are the numbers of cells expressing each variable region gene, in millions per mouse. Values shown are mean yields from three mice; error bars show SD.

more often exposed to their cognate antigen(s) than are  $\gamma/\delta$  T cells residing in the LNs or the skin. Digestive enzymes are constantly reducing dietary and flora-associated proteins to potentially antigenic peptides, and the high concentration of these may allow for their presentation by APCs. Further, the exposure of the gut to bacteria is the highest exposure of any tissue in the body, and many bacteria are known to produce superantigens and other agents capable of inducing T cell activation. This continual barrage of antigenic stimulation could lead to the frequent activation of  $\gamma/\delta$  T cells in the gut, and these multiplying cells may need to be removed somehow, either to maintain homeostasis or to prevent harmful effects when they traffic beyond the gut. If Fas-mediated AICD fulfills this need, one would expect iIEL-derived  $\gamma/\delta$  T cells to accumulate in *lpr* mice. This is exactly

what was observed, with more than four times as many V $\gamma$ 7/V $\delta$ 4-expressing cells as V $\gamma$ 4-expressing cells accumulating in *lpr* mice.

If this unusual phenotype in  $\gamma/\delta$  T cells is a result of AICD, it is probably a general feature of  $\gamma/\delta$  T cells and is not unique to iIELs. As stated above, Fas deficiency led to an expansion of both V $\gamma$ 4 and V $\gamma$ 7/V $\delta$ 4-expressing cells, and both these subsets contributed to the B220<sup>+</sup> cell pool. We suggest that this B220 expression is a hallmark of incipient AICD (7, 22), and its presence in both subsets of  $\gamma/\delta$  T cells in *lpr* mice shows the importance of this process in regulating all  $\gamma/\delta$  T cells in the periphery. If this is true, the differential expansion of those cells expressing iIEL-associated V $\gamma$  and V $\delta$  variable regions would be due to a difference in exposure to antigen(s) and in activation, and not to a difference in the process of AICD in the two subsets of cells.

Further work must be done to demonstrate directly that  $\gamma/\delta$  T cells are regulated by AICD and that B220 expression is its hallmark. If these hypotheses are true, then the B220<sup>+</sup>  $\gamma/\delta$  T cells of Fas-intact mice should contain a higher percentage of cells in cycle than the B220<sup>-</sup> subset, as well as a significant number of cells with less than a diploid amount of DNA, indicating active apoptosis. Direct activation of  $\gamma/\delta$  T cells should be able to induce this B220<sup>+</sup> phenotype. It would be interesting to see if a disruption of this process were observable in any of the diseases of tissues with high numbers of  $\gamma/\delta$  T cells, such as the inflammatory bowel diseases. Finally, the source of the major population of B220<sup>+</sup>  $\alpha/\beta$  DN T cells in TCR- $\alpha$ -intact *lpr* mice has not been resolved. An intriguing possibility, raised by these findings, is that many of these cells may come from the gut.

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