T Cells with γ/δ T Cell Receptors (TCR) of Intestinal Type Are Preferentially Expanded in TCR-α-deficient lpr Mice

By Dennis P. M. Hughes, Adrian Hayday,* Joseph E. Craft,† Michael J. Owen,§ and I. Nicholas Crispe

From the Section of Immunobiology, Yale University School of Medicine, the *Department of Biology, Yale University; the †Section of Rheumatology, Yale University School of Medicine, New Haven, Connecticut 06520-8011, and the §Imperial Cancer Research Fund, London, United Kingdom

Summary

Fas-mediated apoptosis is essential for activation-induced cell death of α/β 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 γ/δ T cell development, Fas-deficient lpr mice were bred with T cell receptor α gene-ablated (TCR-α-/-) mice to generate mice deficient in one or both genes. The TCK-α-/-, lpr/lpr mice had a nearly 10-fold increase in total lymph node cell (LNC) number compared with Fas-intact TCK-α-/- mice, because of expansion of TCR-γ/δ+ and TCR-β+ cells. In Fas-intact TCK-α-/- mice, approximately one third of the LNCs expressed TCR-γ/δ+. These were evenly divided between the CD4-, CD8+, + and the CD4-, CD8- subsets, and rarely expressed the B220 epitope of CD45. In contrast, in TCK-α-/-, lpr/lpr mice, TCR-γ/δ+ cells comprised half of the LNCs and were primarily CD4-, CD8-, and B220+. Moreover, Fas deficiency in TCK-α-/- mice caused a preferential expansion of γ/δ T cells expressing variable region genes characteristic of intestinal intraepithelial lymphocytes. These results demonstrate a role for Fas in regulating the γ/δ 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)† of α/β 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 α/β T cells, which express a reduced level of TCR (13) and express neither CD4 nor CD8 (α/β 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 α/β 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-2Db 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-β chain alone, where endogenous TCR-α 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-
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 α/β 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 α/β 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-2k, 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 remarkable population of T cells that resembled the α/β DN T cells of lpr mice (27). These cells expressed B220 but neither coreceptor and had a reduced level of TCR-α/β on their surface. The B220- but not the B220+ 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+ α/β DN T cells.

While the role of Fas and AICD in controlling peripheral α/β T cell number is thus becoming clearer, little is known about the regulation of γ/δ T cell number. In fact, very little is known about the antigen(s) that normally activate γ/δ T cells, the normal role of γ/δ T cell effector responses, or the capacity of γ/δ 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 α/β T cells may play a role in regulating γ/δ T cells, at least in the LN. Ablation of TCR-α gene expression (TCR-α−/−) eliminated the normal α/β T cell pool and all of its effector responses and allowed for an increase in the number of γ/δ T cells developing in peripheral LNs compared with mice with wild-type TCR-α genes (TCR-α intact) (29, 30). This expansion of γ/δ T cells in TCR-α−/− mice has greatly facilitated the study of this normally rare population of lymphocytes. To study the role of Fas in regulating γ/δ T cells, we bred TCR-α−/− 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 γ/δ T cells and at the same time to test the hypothesis that unselected DP thymocytes give rise to α/β DN T cells in lpr mice.

Materials and Methods

Mice. TCR-α−/− 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-α−/− 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-α and fas/lpr Gene Loci. Tail DNA from F2 mice were resuspended in 100 μl of Tris-EDTA, and this suspension was diluted 1:20 in H2O to make tail template DNA. The following primers were used for PCR: TCRCAF1, TTCAGAACC-CAGAACCCTGCTGTTG; TCRRA1, CCTGAACTGGGTAGGTG-GG; NEOMS1, CTTTCCGAGCTGTCGACGTGTTG; FAS2REV, AACAGCATAGATTCCATTTGCTGCT; and FASZ8XTR,CAA-ACCATGATTCCATTTGCTGCT; FAS2REV, TGATGATGCGTCCATGAGCA; and FASZ8XTR,CAA-ACCATGATTCCCATGAGCA. PCR reactions contained 5 μl tail DNA template, 20 mM Tris-HCl (pH 8.4), 50 mM KC1, 2.5 mM MgCl2, 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 μl, 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 μl 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 (FAS2FX), complementary to the second intron of fas, 5′ of the Etn insertion on lpr mice, was paired with FAS2REV, 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 FAS2XTR reacted with FAS2FX to give a product of ~250 bp. Mice were typed for TCR-α genotype by PCR, again using two reactions. The forward primer TCRCAF1 paired with either the knock-out-specific (neoresistance gene [Neo]−specific) reverse primer NEOMS1 (mutant reaction, ~750 bp) or with TCRRA1 (wild-type reaction, ~300 bp). The wild-type reaction performed...
using TCR-α/-/- template often gave a band of ~1,800 bp because of priming through the Neo 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® Analysis.** F2 mice were killed at 16 wk of age and 10 peripheral (nonmesenteric) LNIs were obtained from each mouse. These LNIs were ground in a tissue homogenizer and the LNCs were washed in Brulf/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 Cδ; Pharmingen, San Diego, CA) (32), RA3-3A1-FITC (TIB-146, anti-CD20, kind gift of Kim Bottomly) (33), 3.168-FITC (anti-CD8-α, conjugated and tested in our laboratory) (34), H129-19-Red 613 (anti-CD4; Gibco BRL) (35), 53-6.7-Red 613 (anti-CD8-α, Gibco BRL) (36), GL3-PE (anti-pan-TCR-γ/δ; Pharmingen) (37), UC3-10A6-PE (anti-TCR V4; Pharmingen) (38), 536-PE (anti-V5; Pharmingen) (39), GL1-biotin (anti-V6; kind gift of Leo LeFrancois) (37, 40) followed by streptavidin-PE (Biomeda, Foster City, CA) and GL2-PE (anti-Vδ; Pharmingen) (37, 40). To study the subsets of TCR-β- cells, 1 × 10⁶ LNCs were suspended in 100 ml Brulf/FCS and stained with H57-597-PE with either: (a) RA3-3A1-FITC plus 53-6.7-Red 613, to study CD8- cell phenotype; (b) RA3-3A1-FITC plus H129-19-Red 613, to study CD4- 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 GL3-PE to study subsets of γ/δ T cells. Data were acquired on a FACSscan® flow cytometer (Becton Dickinson and Co., Cockeysville, MD) using FACSscan® Research Software and analyzed using Lysys 1.7 software (Becton Dickinson and Co.). The percentage of cells with each phenotype was determined using FACScan® Research Software

**Results**

**Expansion of T Cells in Fas-deficient TCR-α -/- Mice.** To generate Fas-deficient mice lacking TCR-α expression, we bred TCR-α -/- mice of strain 129 with MRL-lpr/lpr mice and examined mice of the F2 generation. The TCR-α 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-α gene was paired with either a primer from the 3' end of that exon (TCR-α wild-type reaction) or with a Neo-specific primer (TCR-α 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® 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) LNIs were pooled. These lymphocytes were stained for TCR-α/β or TCR-γ/δ and analyzed by FACScan®. 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 identified in phenotype of homozygous wild-type mice for that gene; the data presented here are from homozygous mice. By 16 wk of age, the lpr mice with wild-type TCR-α had developed massive lymphadenopathy due to α/β DN T cell accumulation; ~10% of these mice were moribund. As previously described (29, 41), ablation of the TCR-α gene led under specific pathogen-free conditions to an increase in the absolute number of peripheral TCR-γ/δ- T cells but a decrease in the total number of peripheral lymphocytes because of the elimination of α/β T cells. Massive
Table 1. T Cell Yield from LNs

| Genotype          | n | LNC      | TCR-β⁺ LNC | TCR-γ/δ⁺ LNC |
|-------------------|---|----------|------------|--------------|
| α+/+, wt/wt       | 14| 57.72 ± 17.9 | 51.66 ± 13.0 | 0.64 ± 0.36 |
| α+/+, lpr/lpr     | 8 | 2,680.00 ± 1570 | 2,220.00 ± 1280 | 32.00 ± 13.6 |
| α−/−, wt/wt       | 12| 9.43 ± 2.77  | 1.30 ± 0.65 | 3.14 ± 0.89 |
| α−/−, lpr/lpr     | 9 | 85.67 ± 38.8 | 7.91 ± 2.97 | 44.60 ± 22.5 |

The yield of live lymphocytes from 10 peripheral (nonmesenteric) LNs are shown in millions. Values are mean ± SD. Genotype of mice was determined by PCR as shown in Fig. 1. The yield of TCR-β⁺ cells was determined by multiplying the total yield of LNCs by the percentage of LNCs stained with anti-TCR-β mAb. The yield of TCR-γ/δ⁺ cells was determined by multiplying the total yield of LNCs by the percentage of LNCs stained with anti-TCR-γ/δ mAb. wt, wild type.

lymphadenopathy was also eliminated in TCR-α−/−, lpr/lpr mice. However, absence of Fas in these mice caused a 15-fold expansion of TCR-γ/δ⁺ T cells, as well as a 6-fold increase in TCR-β⁺ cells when compared with Fas-intact TCR-α−/− mice. The TCR-β⁺ cells in TCR-α−/− mice have recently been described (41) and are the subject of ongoing study. The increased number of TCR-γ/δ⁺ cells was detectable in TCR-α−/−, lpr/lpr as well as TCR-α−/−, lpr/lpr mice, and is examined in detail here.

Fas Deficiency Causes Expansion of TCR-β⁺ Cells with Abnormal Phenotypes. To determine which subsets of TCR-β⁺ cells were expanding in TCR-α−/−, lpr/lpr mice, we stained single cell suspensions of LNCs stained in anti-TCR-β-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®. TCR-β⁺ 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.

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

The skewing of TCR-β+ cells toward a DN, B220− phenotype in Fas-deficient TCR-α−/− mice was due to a preferential expansion of the B220− 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-β+ cells in a Fas-intact TCR-α−/− 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-α−/−, lpr/lpr mice led to an expansion of most TCR-β+ cell subsets, but the B220− subsets, especially the DN B220− pool, increased far more than did the B220+ subsets. Fas deficiency led to a 4-fold expansion of the DN B220− subset, a 10-fold increase in the CD4− B220+ and the DN B220− subsets, and a 5-fold expansion of the CD8+ B220− subset. This accumulation of B220− TCR-β+ cells is similar to the preferential accumulation of α/β DN T cells seen in TCR-α-intact lpr mice.

TCR-γ/δ Cells in Fas-deficient Mice Accumulate and Acquire an Abnormal Phenotype. Since the majority of the expanding cells in the TCR-α−/−, lpr/lpr mice expressed TCR-γ/δ, it was important to learn which subsets of γ/δ T cells were expanding in these mice. We stained LNCs from 16-wk-old mice for TCR-γ/δ, CD4, CD8, and B220 and analyzed these cells by three-color FACS®. Representative FACS® plots are shown in Fig. 3. Using the TCR-γ/δ+ gate and quadrant regions shown, we determined the percentage of LNCs in each subset of γ/δ T cells. Ablation of TCR-α gene expression eliminated the TCR-α/β+ cell population and caused an increase in the proportion and absolute number of TCR-γ/δ+ cells in TCR-α−/− mice, as has been reported (29). The phenotype of the TCR-γ/δ+ cells in these mice was identical to that seen in TCR-α-intact mice. These cells were primarily divided between CD8+ cells and DN cells, with a small population expressing CD4 and not CD8. Most γ/δ T cells from Fas-intact mice did not express B220. In lpr mice, however, the majority of γ/δ T cells did express B220 but were CD8−. Thus, Fas deficiency skewed the γ/δ T cell pool toward a B220−, DN phenotype.

This skewing of co-receptor and B220 expression of the γ/δ T cell pool was due to the preferential expansion of the DN B220− subset in TCR-α−/−, 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 γ/δ T cells were expanded by Fas deficiency, the expansion within the B220− DN subset was far more dramatic than that of any other subpopulation, leading to the skewed phenotype. Similar numbers of abnormal TCR-γ/δ+ cells were observed in TCR-α-intact lpr mice. The CD8+ B220− γ/δ T cell subset and the CD4− B220− γ/δ T cell subset increased 1.5-fold with Fas deficiency, but the B220− DN γ/δ T cells increased 20-fold. The CD4+ B220− subset also increased 20-fold, while the CD8+ B220− subset expanded 6-fold. The 300-fold expansion of the DN B220+ γ/δ T cell subset, however, obscured the expansion of the other subsets. This effect on γ/δ T cells is similar to that seen in α/β T cells in TCR-α-intact lpr mice.

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

| Genotype | CD4+, B220− | CD4+, B220+ | CD8+, B220− | CD8+, B220+ | DN, B220− | DN, B220+ |
|----------|-------------|-------------|-------------|-------------|------------|------------|
| α+/+, 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 |
| α+/+, lpr/lpr | 160.0 ± 110.8 | 102.0 ± 36.2 | 102.0 ± 36.2 | 4.93 ± 1.12 | 164.0 ± 138.0 | 1.968 ± 0.833 |
| α−/−, 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 |
| α−/−, lpr/lpr | 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-β+ 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-β+ cell gate and quadrant regions shown in Fig. 2. Fas deficiency expands the B220− subsets of TCR-β+ cells in TCR-α−/− as well as TCR-α-intact mice. Data were obtained from the same mice described in Table 1. wt, wild type.
TCRα+/-, fas: wt/wt

TCRα+/-, lpr/lpr

TCRα-/-, fas: wt/wt

TCRα-/-, lpr/lpr

Figure 3. Fas deficiency skews the expanded γ/δ T cells in TCR-α-/- mice towards a B220+, DN phenotype. TCR-γ/δ 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.

α/β+ cells, especially the vastly expanded α/β DN T cells of lpr mice, in TCR-α-/- mice allowed us to make a more detailed examination of the effect of Fas deficiency on the development of γ/δ T cells. To determine the potential source of the abnormally expanded γ/δ T cells in Fas-deficient mice, we examined the expression of specific Vγ and Vδ genes by FACS in LNCs of 16-wk-old Fas-intact and lpr TCR-α-/- mice. Representative FACS plots are shown in Fig. 4. TCR Vγ4 (also known as Vγ2) is normally expressed in peripheral LN γ/δ T cells (38). In Fas-intact TCR-α-/- mice, ~20% of LN γ/δ T cells expressed this V region. In contrast, ~6% of the γ/δ T cell pool in TCR-α-/-, lpr/lpr mice was stained with anti-Vγ4 antibodies. Dendritic epidermal T cells characteristically express TCR Vγ5 (also known as Vγ3) in the mouse and are not normally found in the LN5 of adult mice (39). Less than 0.5% of LN γ/δ T cells in either Fas-intact or lpr TCR-α-/- mice were stained with antibodies to Vγ5, indicating that dendritic epidermal T cells did not contribute significantly to the peripheral LN γ/δ T cell pool in TCR-α-/- 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-γ/δ+, and these cells characteristically express TCR Vγ7 (also known as Vγ5) and TCR Vδ4 (37, 40). To determine if cells of this lineage made a significant contribution to the expansion of γ/δ T cells in TCR-α-/-, lpr/lpr mice, we stained peripheral LNCs from 16-wk-old mice with antibodies to TCR Vγ7 and TCR Vδ4 (Fig. 4). In Fas-intact TCR-α-/- mice, ~8% of the γ/δ T cells were stained with anti-Vγ7. TCR Vδ4 expression was slightly more abundant in these mice: ~18% of γ/δ T cells. Expression of both these variable regions was much higher in lpr mice. Approximately 15% of γ/δ T cells expressed Vγ7, and >25% of γ/δ T cells expressed TCR Vδ4. These percentages were multiplied by the total LNC count to determine the cell yield for each subset of γ/δ T cells (Fig. 5). This calculation revealed a dramatic expansion of γ/δ T cells expressing variable genes characteristic of iIELs. Although Fas deficiency caused only a threefold increase in Vγ4-expressing cells, from
Table 3. Yield of TCR-γ/δ+ Subsets

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

Expansion of subsets of TCR-γ/δ+ cells in Fas-deficient mice. The yield of cells with each phenotype is shown in millions per mouse. Values are mean ± 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-γ/δ+ 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 Vγ7 and Vδ4-expressing cells from 0.28 to 3.4 million and from 0.76 to 9.6 million, respectively. Both the Vγ4+ and Vγ7/Vδ4+ subsets contributed to the abnormal B220+ γ/δ T cells observed in TCR-α−/−, lpr/lpr mice (data not shown). Thus, Fas deficiency causes an abnormal phenotype in all subsets of γ/δ T cells normally observed in the LN, but leads to a preferential expansion of those γ/δ 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-α−/−, lpr/lpr mice demonstrates that double-positive thymocytes with incomplete TCR complexes do not give rise to the α/β DN T cells. If unselectable T cells gave rise to α/β DN T cells in lpr, they should have accumulated faster in TCR-α−/−, lpr/lpr mice. This potential precursor pool was abundant in the thymus of these mice (data not shown), but TCR-β+ DN cells were reduced >100-fold by the elimination of TCR-α 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 α/β DN T cells in lpr and identify an important role for Fas in regulating peripheral γ/δ T cells. The discovery of B220+ γ/δ T cells was quite surprising. This marker has not been reported on γ/δ cells before, and these cells are not known to be regulated by AICD. Indeed, little is known about the antigen(s) recognized by γ/δ 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 γ/δ T cell regulation. The identification of this same unusual population in TCR-α-intact lpr mice shows that this phenotype was not an artifact of TCγ-α gene ablation. If the parallel between α/β T cells and γ/δ T cells holds true, these data strongly suggest that γ/δ 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 γ/δ T cells.

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

![Figure 4. Fas deficiency skews the γ/δ T cell repertoire towards the use of variable genes characteristic of gut-derived γ/δ T cells. Representative histograms are shown. The gates shown were used to calculate the percentage of LNCs expressing each variable gene region.](image-url)
more often exposed to their cognate antigen(s) than are γδ T cells residing in the LN 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 γδ 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 γδ T cells to accumulate in lpr mice. This is exactly what was observed, with more than four times as many Vγ7/Vδ4-expressing cells as Vγ4-expressing cells accumulating in lpr mice.

If this unusual phenotype in γδ T cells is a result of AICD, it is probably a general feature of γδ T cells and is not unique to iIELs. As stated above, Fas deficiency led to an expansion of both Vγ4 and Vγ7/Vδ4-expressing cells, and both these subsets contributed to the B220+ cell pool. We suggest that this B220+ expression is a hallmark of incipient AICD (7, 22), and its presence in both subsets of γδ T cells in lpr mice shows the importance of this process in regulating all γδ T cells in the periphery. If this is true, the differential expansion of those cells expressing iIEL-associated Vγ and Vδ 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 γδ T cells are regulated by AICD and that B220 expression is its hallmark. If these hypotheses are true, then the B220+ γδ T cells of Fas-intact mice should contain a higher percentage of cells in cycle than the B220− subset, as well as a significant number of cells with less than a diploid amount of DNA, indicating active apoptosis. Direct activation of γδ T cells should be able to induce this B220+ 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 γδ T cells, such as the inflammatory bowel diseases. Finally, the source of the major population of B220+ α/β DN T cells in TCR-α− 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.

We thank Leo LeFrancois, Kim Bottomly, and Mark Shlomchik for generously providing reagents.

This work was supported by a BSRG Fluid Research Funds award to I. N. Crispe from Yale Medical School. D. P. M. Hughes is a student of the MSTP program at the Yale University School of Medicine. J. E. Craft is supported in part by grants from the National Institutes of Health and from the Arthritis and the Lupus Foundations. I. N. Crispe is an Investigator of the Cancer Research Institute.

Address correspondence to Dr. Dennis P. M. Hughes, Section of Immunobiology, Yale University School of Medicine, 330 Cedar Street, PO Box 208011, New Haven, CT 06520-8011.

Received for publication 21 December 1994.

References

1. Itoh, N., S. Yonehara, S. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for the human cell surface antigen Fas can mediate apoptosis. Cell. 66:233–243.

2. Yonehara, S., and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp Med. 169:1747–1756.

3. Trauth, B. C., C. Klas, A. M. J. Peters, S. Matzku, P. Moller, W. Falk, K.-M. Debatin, and P. H. Krammer. 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science (Wash. DC). 245:301–305.

4. Watanabe-Fukunaga, R., C.I. Brannan, M. Itoh, S. Yonehara, N.G. Copeland, N. Jenkins, and S. Nagata. 1992. The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J. Immunol. 148:1274–1279.

5. Miyawaki, T., T. Uehara, R. Nibu, T. Buji, A. Yachie, S. Yonehara, and N. Taniguchi. 1992. Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. J. Immunol. 149:3753–3758.

6. Klas, C., K.-M. Debatin, R.R. Jonker, and P.H. Krammer. 1993. Activation interferes with the APO-1 pathway in mature human T cells. Int. Immunol. 5:625–630.

7. Singer, G.G., and A.K. Abbas. 1994. The Fas antigen is in...
volved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunology.* 1:365–371.

8. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (Lond.)* 356:314–356.

9. Wu, J., T. Zhou, J. He, and J.D. Mountz. 1993. Autoimmune disease in mice due to integration of an endogenous retrovirus in an apoptosis gene. *J. Exp. Med.* 178:461–468.

10. Ogasawara, J., K. Watanabe-Fukunaga, M. Adachi, A. Matsumawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effect of the anti-Fas antibody in mice. *Nature (Lond.)* 364:806–809.

11. Drappa, J., N. Brot, and K.B. Eklon. 1993. The Fas protein is expressed at high levels on CD4+CD8+ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL lpr/lpr. *Proc. Natl. Acad. Sci. USA.* 90:10340–10344.

12. Giese, T., and W.F. Davidson. 1992. Evidence for early onset, polyclonal activation of T cell subsets in mice homozygous for lpr. *J. Immunol.* 149:3097–3106.

13. Nemazee, D.A., S. Studier, M. Steinmetz, Z. Dembic, and M. Kiefer. 1985. The lymphoproliferating cells of MRL/lpr/lpr mice are a polyclonal population that bear the T lymphocyte receptor for antigen. *Eur. J. Immunol.* 15:760–764.

14. Morse, H.C., W.F. Davidson, R.A. Yetter, E.D. Murphy, J.B. Roth, and R.L. Coffman. 1988. Abnormalities induced by the mutant gene lpr. Expansion of a unique lymphocyte subset. *J. Immunol.* 129:2612–2615.

15. Miescher, G.C., R.C. Budd, R.K. Lees, and R.H. MacDonald. 1987. Abnormal expression of T cell receptor genes in Lyt-2-L3T4-lymphocytes of lpr mice: comparison with normal immature thyroglobins. *J. Immunol.* 138:1959–1967.

16. Budd, R.C., M. Schreyer, G.C. Miescher, and H.R. MacDonald. 1987. T cell lineages in the thymus of lpr/lpr mice. Evidence for parallel pathways of normal and abnormal T cell development. *J. Immunol.* 139:2200–2210.

17. Seth, A., R.H. Pyle, M. Nagarkatti, and P.S. Nagarkatti. 1988. Expression of the J1d marker on peripheral T lymphocytes of MRL/lpr/lpr mice. *J. Immunol.* 141:1120–1125.

18. Mountz, J.D., T. Zhou, J. Eldridge, K. Berry, and H. Bluthmann. 1990. Transgenic rearranged T cell receptor gene inhibits lymphadenopathy and accumulation of CD4+CD8+ T cells in lpr/lpr mice. *J. Exp. Med.* 172:1805–1817.

19. Zhou, T., H. Bluthmann, J. Eldridge, M. Brockhaus, K. Berry, and J.D. Mountz. 1991. Abnormal thyrocyte development and production of autoantibody T cells in T cell receptor transgenic autoimmune mice. *J. Immunol.* 147:466–474.

20. Sidman, C.L., J.D. Marshall, and H. von Boehmer. 1992. Transgenic T cell receptor interactions in the lymphoproliferative and autoimmune syndromes of lpr and gld mutant mice. *Eur. J. Immunol.* 22:499–504.

21. Zhou, T., H. Bluthmann, J. Eldridge, K. Berry, and J.D. Mountz. 1993. Origin of CD4+CD8+ B220+ T cells in MRL-lpr/lpr mice: clues from a T cell receptor transgenic mouse. *J. Immunol.* 150:3651–3657.

22. Crispe, I.N. 1994. Fatal interactions: Fas-induced apoptosis of mature T cells. *Immunology.* 1:347–349.

23. Zhou, T., H. Bluthmann, J. Zhang, C.K. Edwards, and J.D. Mountz. 1992. Defective maintenance of T cell tolerance to a superantigen in MRL/lpr/lpr mice. *J. Exp. Med.* 176:1063–1072.

24. Scott, D.E., W.J. Kisch, and A.D. Steinberg. 1993. Studies of T cell deletion and T cell anergy following in vivo administration of SEB to normal and lupus-prone mice. *J. Immunol.* 150:664–672.

25. Russell, J.H., B. Rush, C. Weaver, and R. Wang. 1993. Mature T cells of autoimmune lpr/lpr mice have a defect in antigen-stimulated suicide. *Proc. Natl. Acad. Sci. USA.* 90:4409–4413.

26. Huang, L., G. Soldevilla, M. Leeket, R.A. Flavell, and I.N. Crispe. 1994. The liver eliminates T cells undergoing antigen-triggered apoptosis in vivo. *Immunology.* 1:741–740.

27. Huang, L., K. Sye, and I.N. Crispe. 1994. Proliferation and apoptosis of B220+CD4+CD8- TCRα/β intermediate T cells in the liver of normal mice: implications for lpr pathogenesis. *Int. Immunol.* 6:533–540.

28. Ohteki, T., S. Seki, T. Abo, and K. Kumagai. 1990. Liver is a possible site for the proliferation of abnormal CD3+4-8+ double-negative lymphocytes in autoimmune MRL/lpr/lpr mice. *J. Exp. Med.* 172:7–12.

29. Philpott, K.L., J.L. Viney, G. Kay, S. Rastan, E.M. Gardiner, S. Chae, A.C. Hayday, and M.J. Owen. 1992. Lymphoid development in mice congenitally lacking T-cell receptor alpha/beta expressing cells. *Science (Wash. DC).* 256:1448–1452.

30. Mallick, C.A., E.C. Dudley, J.L. Viney, M.J. Owen, and A.C. Hayday. 1993. Rearrangement and diversity of T cell receptor β chain genes in thyroglobins: a critical role for the β chain in development. *Cell.* 73:513–519.

31. Adachi, M., R. Watanabe-Fukunaga, and S. Nagata. 1993. Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc. Natl. Acad. Sci. USA.* 90:1756–1760.

32. Kubo, R.T., W. Born, J. Kappeler, P. Marrack, and M. Pigeon. 1989. Characterisation of an antibody which detects all murine alpha-beta T cell receptors. *J. Immunol.* 142:2736–2744.

33. Coffman, R.L., and I.V. Weissman. 1981. B220: A cell specific marker of T220 glycoprotein family. *Nature (Lond.).* 289:681–683.

34. Sarmiento, M., A.L. Glasebrook, and F.W. Fitch. 1980. IgG or IgM monoclonal antibodies reactive with different determinants in the molecular complex bearing Lyt-2 antigen block T-cell-mediated cytolysis in the absence of complement. *J. Immunol.* 125:2665–2672.

35. Cobbold, S.P., A. Jayasuriya, A. Nash, T.D. Prospero, and H. Waldman. 1984. Therapy with monoclonal antibodies by elimination of T cell subsets in vivo. *Nature (Lond.)* 312:548.

36. Ledbetter, J.A., and L.A. Herzenberg. 1979. Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.

37. Goodman, T., and L. Lefrancois. 1989. Intrathymic lymphocytes: anatomical site, not T cell receptor form, dictates phenotype and function. *J. Exp. Med.* 170:1569–1581.

38. Dent, A.L., L.A. Matis, and F. Hooshmand. 1990. Self-reactive γδ T cells are eliminated in the thymus. *Nature (Lond.)* 343:714–718.

39. Havran, W.L., and J.P. Allison. 1988. Developmentally ordered appearance of thyroglobins expressing different T-cell antigen receptors. *Nature (Lond.)* 335:433–445.

40. Lefrancois, L., R. LeCorre, J. Mayo, J.A. Bluestone, and T. Goodman. 1990. Extrathymic selection of TCR γ/δ T cells by class II major histocompatibility complex molecules. *Cell.* 63:333–340.

41. Viney, J.L., L. Dianda, S.J. Roberts, L. Wen, C.A. Mallick, A.C. Hayday, and M.J. Owen. 1994. Lymphocyte proliferation in mice congenitally deficient in T-cell receptor αβ cells. *Proc. Natl. Acad. Sci. USA.* 91:11948–11952.

42. van Houten, N., and R.C. Budd. 1994. Lessons from the lpr mouse: T lymphocyte development. *Semin. Immunol.* 6:1–2.