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The Death Receptor Fas (CD95/APO-1) Mediates the Deletion of T Lymphocytes Undergoing Homeostatic Proliferation

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Murine T cells adoptively transferred into syngeneic lymphopenic recipients undergo proliferation. Despite continued cell division, this lymphopenia-induced or homeostatic proliferation of a limited number of transferred T cells does not fill the T cell compartment. The continued expansion of the transferred T cells, even after stable T cell numbers have been reached, suggests that active cell death prevents further increase in T cell number. In this study, we show that wild-type T cells undergoing homeostatic proliferation are sensitive to Fas-mediated cell death. In the absence of Fas, T cells accumulate to significantly higher levels after transfer into lymphopenic recipients. As Fas-deficient lpr mice manifest no significant abnormalities in thymic negative selection or in foreign Ag-induced peripheral T cell deletion, their lymphadenopathy may result from unrestrained homeostatic proliferation.

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Basal homeostasis of the peripheral T lymphocyte compartment is a dynamic, tightly regulated process that maintains total T cell number at a remarkably stable level. The peripheral T cell compartment continually receives new T cells that have been selected in the thymus. In addition, peripheral T cells proliferate at a low but significant rate to self-peptide/self-MHC molecules (1). This daily efflux of thymic emigrants and peripheral expansion would result in a continual increase in T cell numbers if it were not balanced by the loss of existing T cells.

T cells undergo extensive homeostatic expansion in the periphery of neonates, where T cell numbers have not yet reached a steady state level, and also in lymphopenic adults (2–4). Experimentally, homeostatic proliferation is studied by transferring small numbers of T cells into irradiated or genetically lymphopenic mice. In addition to a T cell-deficient environment, this model of homeostatic proliferation requires self-peptide/MHC interactions and IL-7 (5–11). In these systems, the proliferating naive T cells up-regulate CD44 but, in contrast with stimulation by foreign Ag, do not express the activation markers CD69 or CD25 (9, 12). As a consequence, naive T cells expanding by homeostatic proliferation acquire only some of the appearance and function of effector memory T cells and as such manifest a unique phenotype.

Experimental studies of homeostatic proliferation in lymphopenic recipients revealed that the transferred lymphocytes from a single donor inoculum do not completely restore the T cell compartment (13–17). Although the number of T cells recovered increases, a plateau is reached at ~4 wk. The factors that limit further expansion in lymphopenic recipients have not been elucidated. It has been suggested that homeostatic expansion may be restricted by contact between neighboring T cells or by competition for limited resources, including IL-7 and access to APCs bearing appropriate self-peptide/MHC (7, 18–20). However, even at later time points when stable numbers of T cells have been achieved, a significant portion of the donor-derived T cells continue to proliferate as measured by BrdU incorporation (15). This suggests that it is not entirely limitations of proliferation but also active cell death that prevents further increase in T cell number. T cell elimination in vivo after foreign Ag stimulation is mediated by proapoptotic Bcl-2 family members such as Bim, Bax, and Bak (21, 22). In contrast, the molecules regulating active cell death during basal homeostasis have not been identified.

Although the phenotype of homeostatically expanding T cells does not precisely match that of T cells activated by foreign Ag, it does closely resemble that of Fas-deficient lpr T cells. lpr mice exhibit a profound age-dependent lymphopenopathy that includes CD4+/CD4- and CD8+/CD8- T cells, as well as an unusual population of CD4+ CD8- (CD4+ 8-) TCRβ T cells that express the B cell isofrom of CD45, CD45R (B220) (23). Despite over a decade of study of Fas, the source and explanation for the accumulating T cells in lpr mice remain a mystery. Little if any significant defect in thymic negative selection has been identified in lpr mice based on deletion by endogenous or exogenous superantigens (24–27). Although occasional reports have noted some retention of Ag or superantigen-reactive T cells within the thymus, this appears not to be sustained in the peripheral lymphoid compartment (28–30). Whereas Fas regulates Ag-induced lymphocyte death in vitro, most studies support the view that there is little if any delay in deletion of lpr T cells in response to Ags administered as exogenous proteins (31, 32). When foreign Ags were expressed endogenously, the role of Fas in deletion has been less clear. Deletion of hen egg lysozyme-specific CD4+ T cells lacking Fas was delayed after adoptive transfer to mice expressing soluble hen egg lysozyme under the control of the mouse metallothionein I promoter (33). In contrast, deletion of OVA-specific Fas-deficient CD8+ OT-I T cells was not affected after transfer to C57BL/6 mice expressing OVA under the control of the rat insulin promoter (34), but it was impaired if the recipient mice were of a mixed genetic background (35). These discrepancies may reflect differences in TCR/MHC/peptide affinities, CD4+ vs CD8+ T cells, location of...
the expressed Ag, or genetic background. Nonetheless, these observations raised the possibility that lpr T cells might accumulate in response to certain self-peptides, as occurs during homeostatic proliferation. Given the further phenotypic parallels between T cells undergoing homeostatic proliferation and the T cells that accumulate in lpr mice, we considered that the lymphopenopathy in lpr mice results from unregulated proliferation that occurs during normal basal T cell homeostasis. Consistent with this view, lpr mice raised in a germ-free environment, where their exposure to foreign Ags is minimal, still develop adenopathy (36).

To examine whether Fas regulates T cell homeostasis, we transferred Fas+ wild-type or Fas-deficient lpr donor T cells into RAG-1-deficient recipients. T cells undergoing homeostatic proliferation were sensitive to Fas-mediated cell death. Transferred lpr T cells accumulated to substantially increased numbers in lymphopenic recipients compared with Fas+ T cells. This accumulation was not the result of differences in rates of cell cycling between the Fas+ and lpr donor T cells. Furthermore, Fas ligand (FasL)-mutant gld mice did not accumulate to the levels of lpr T cells, suggesting that FasL expression by nonlymphoid cells is sufficient to regulate T cell homeostatic expansion. This data support the hypothesis that the accumulation of T cells in lpr mice is driven largely by self-Ags during homeostatic proliferation rather than by foreign Ags. Thus, Fas is likely an important regulator of basal T cell homeostasis.

Materials and Methods

*Mice*

Mice were bred and housed at the animal facilities of University of Vermont College of Medicine. Original breeding pairs of C57BL/6, C57BL/6 lpr, C57BL/6 gld, C57BL/6 RAG-1-deficient, and B6-PL-Thy1.2/Cy (CD90.1) mice were obtained from The Jackson Laboratory. Breeding of CD90.1 mice with C57BL/6 lpr (CD90.2) mice generated CD90.1 lpr mice. Offspring were screened for the lpr mutation by PCR as described previously (37) and for expression of CD90.1 and CD90.2 on peripheral blood lymphocytes by flow cytometry. All animal studies were conducted in accordance with the University of Vermont’s Animal Care and Use Committee.

Adoptive transfer

A total of 5 × 10⁶ lymph node cells from 5- to 6-wk-old C57BL/6 mice or the equivalent number of total CD4⁺ plus CD8⁺ T cells from age- and sex-matched C57BL/6 lpr or C57BL/6 gld mice were transferred i.v. via the tail vein into RAG-1-deficient mice. At this age, B6 lpr mice have very few CD4⁺/CD8⁺ T cells. In most experiments, CD90.1 congenic C57BL/6 and C57BL/6 lpr mice were used as the source of transferred cells.

To assess entry into cell cycle after adoptive transfer, donor cells were labeled with CFSE (Molecular Probes) before transfer. Lymph node cells were washed with PBS containing 0.1% BSA (PBS/0.1% BSA), resuspended at 10⁷ cells/ml, and incubated with 5 μM CFSE for 10 min at 37°C. Labeling was stopped by addition of ice-cold PBS/0.1% BSA. The cells were washed three times with PBS/0.1% BSA and resuspended in PBS for adoptive transfer.

To measure in vivo proliferation, mice received four i.p. injections of 1 mg of BrdU (100 μl of 10 mg/ml BrdU in sterile PBS, Sigma-Aldrich) during the 24 h before tissue harvest. Three injections were given on the day before tissue harvest and one injection was given on the day of sacrifice 1 h before tissue harvest.

Reagents and Abs

The following mAbs to murine cell surface molecules were purchased from Caltag Laboratories: PE-conjugated CD62L, TRI-COLOR-conjugated CD4, PE-conjugated CD8α, TRI-COLOR-conjugated CD8α, PE-Cy5.5-conjugated CD8α, TRI-COLOR-conjugated streptavidin, and PE-Texas Red-conjugated CD4. The following Abs were purchased from BD Biosciences: FITC-conjugated TCR-β, PE-conjugated TCR-β, allophycocyanin-conjugated TCR-β, PE-conjugated CD122, FITC-conjugated CD90.1, and PE-conjugated CD90.1.

Lyophilized rat IgG and hamster IgG (ICN/Cappel) were resuspended in PBS and stored at −80°C. Tissue culture supernatants used for T and B cell depletion were prepared from anti-murine CD4 (GK1.5), anti-murine CD8 (Tib 105), anti-murine CD45R (B220, RA3G2B), and anti-murine κ (187.1) hybridomas.

*Lymphocyte preparation*

Single cell suspensions of spleen and lymph nodes were prepared in RPMI 1640 (Mediatech) containing 5% (v/v) bovine calf serum (BCS), 5 × 10⁻⁵ M 2-ME, 100 U/ml penicillin, and 100 U/ml streptomycin (RPMI5% BCS). Erythrocytes in splenic suspensions were lysed by Geys solution.

Cell recovery from recipient mice was calculated from the percentage of CD4⁺, CD8⁺, and CD4⁺/CD8⁻ T cells obtained by flow cytometry and the absolute number of cells obtained. For each recipient, the spleen and eight lymph nodes (inguinal, brachial, axillary, and popliteal) were harvested.

To deplete T and B cells from wild-type spleen, splenic cell suspensions were incubated with anti-CD4 (GK1.5), anti-CD8 (Tib 105), anti-CD45R (B220, RA3G2B), and anti-κ (187.1). The cells were washed twice and incubated by rocking with BioMag goat anti-rat IgG-coated magnetic beads (Qiagen) for 45 min at 4°C. Ab-coated cells were removed by magnetic depletion. The remaining cells were washed and resuspended in RPMI5% BCS.

*Flow cytometry*

For direct staining, single cell suspensions (10⁷ cells/ml) were washed with cold (4°C) PBS containing 0.02% (v/v) sodium azide (Sigma-Aldrich) (PBS/azide). The cells were incubated with the appropriate Abs in PBS/azide containing 1% (v/v) BSA fraction V (Sigma-Aldrich) for 30 min at 4°C. After washing with cold PBS/azide, the cells were fixed with 1% (v/v) methanol-free formaldehyde (Ted Pella) in PBS/azide and stored at 4°C until analysis. Flow cytometry was performed on a Coulter Epics Elite Flow Cytometer (Coulter) or a BD LSRII (BD Biosciences) calibrated with DNA check beads (Coulter).

Staining for DNA-incorporated BrdU was performed using a modification of a previously described method (1). Single cell suspensions from BrdU-pulsed mice were stained for TCRβ, CD4, and CD8 expression using Abs in PBS containing 1% BSA (PBS/BSA) for 30 min at 4°C and then washed with cold (4°C) PBS. The cells were fixed for 30 min on ice after the addition of 350 μl of 70% ethanol (~20°C) while gently vortexing. The cells were washed twice with cold PBS, pelleted by spinning at 10,000 × g, and fixed with 350 μl of 1% methanol-free formaldehyde for 15 min on ice. The cells were permeabilized in 50 μl of PBS containing 1% methanol-free formaldehyde and 0.01% Tween 20 overnight at 4°C. After washing twice with cold PBS, the cells were incubated with 50 Kunitz units of DNase I (Sigma-Aldrich) in 0.15 M NaCl (pH 5) containing 4.2 mM MgCl₂ for 15 min at 37°C (1 ml of 50 μl/ml). The cells were washed twice with cold PBS/BSA and incubated with anti-BrdU FITC (BD Biosciences) in 100 μl of PBS/BSA for 30 min on ice. After washing twice with PBS/BSA, the cells were fixed in 1% methanol-free formaldehyde in PBS/BSA and stored at 4°C until analysis.

*Analysis of Fas-mediated cell death*

Combined lymph node and spleen cells isolated from recipient mice were resuspended in complete RPMI 1640 and cultured at 37°C for 3 h either with 250 ng/ml FLAG-tagged recombinant FasL (Alexis Biochemicals) cross-linked by 4 μg/ml anti-FLAG Ab (Sigma-Aldrich) or with anti-FLAG Ab only. Cells (0.6 × 10⁶) were stained for TCRβ, CD4, and CD8 expression using mAbs in PBS/BSA for 30 min at 4°C and then washed with cold (4°C) PBS. The cells were fixed with 350 μl of 1% methanol-free formaldehyde for 15 min on ice, washed with cold PBS, and pelleted by spinning at 10,000 × g. The cells were resuspended in 350 μl of cold 70% ethanol for 15 min and then washed twice with cold PBS. The TUNEL reaction was performed by incubating the cells in 50 μl of reaction mix containing 10 U of terminal deoxyribosyl transferase, 10 mM biotin-UTP, and 2.5 μM cobalt chloride in 1× terminal transferase reaction buffer (Boehringer Mannheim) for 1 h at 37°C. The cells were washed twice with cold PBS/BSA and incubated with streptavidin-TRI-COLOR in PBS/BSA for 20 min on ice. After washing twice with PBS/BSA, the cells were fixed in 1% methanol-free formaldehyde in PBS/BSA and stored at 4°C until analysis.

*Detection of FasL mRNA*

RNA was extracted from cells using Ultraspec (Biotecx Laboratories) or RNeasy kit (Qiagen) and were prepared according to the manufacturer’s instructions. RNA was treated with DNase using DNA-free (Ambion) according to the manufacturer’s instructions. RNA was treated with DNase using DNA-free (Ambion) according to the manufacturer’s instructions.

Abbreviations used in this paper: FasL, Fas ligand; BCS, bovine calf serum.
to the manufacturer’s instructions. Oligo-dT<sub>18</sub> or random hexamer priming and reverse transcriptase (Invitrogen) was used to prepare cDNA from RNA samples. Quantitative PCR was performed using a 7900HT Sequence Detection System (Applied Biosystems) at the Vermont Cancer Center DNA Analysis Facility. Primers for murine FasL were 5′-GCCAGCACCTGATAAT TACC-3′ and 5′-GGAGGACCGAGATGAAATGTG-3′. The amplified fragment of FasL contained the sequence bound by the fluorochrome-labeled primer: 5′-6-FAM (TGTGCCACATCTCTGGTAGACACGA) BHQ-1-3′ (Sigma Genosys). Amplification of FasL cDNA was normalized to the endogenous control cDNA for murine γ-actin. Primers for murine γ-actin were 5′-GACCGTACAGCTAGAAGATTA-3′ and 5′-AGGCCACCGATCCAGCTGAGT-3′. The amplified fragment contained the sequence bound by the fluorochrome-labeled primer 5′-6-FAM (CATTGCTCCCTCGAGGC) BHQ-1-3′. Validation experiments were performed to measure the efficiency of the target (FasL) and control (γ-actin) gene amplification over a range of three logs of sample cDNA concentration and showed a highly constant ratio of FasL/γ-actin amplification. All assays were run in duplicate.

### Results

**Fas-deficient lpr T cells phenotypically resemble homeostatically proliferating T cells**

Fas-deficient lpr mice manifest a pronounced age-dependent lymphadenopathy composed of CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as an unusual population of CD4<sup>+</sup> 8<sup>+</sup> TCR<sup>αβ</sup> T cells. The absence of any significant defect in thymic production or selection of T cells (24–30) and the lack of any pronounced delay in Ag-induced T cell deletion in vivo in lpr mice (31, 32) led us to consider that the marked increase in T cell number in lpr mice might result from unregulated proliferation during basal T cell homeostasis. Consistent with this view, an increased fraction of lpr T cells from 11-wk-old mice incorporated BrdU compared with fas<sup>+</sup> wild-type T cells over a 24-h period (Fig. 1 and Ref. 38). This was statistically significant for CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>+</sup> 8<sup>+</sup> T cells from both the lymph node and spleen. In addition, CD4<sup>+</sup> 8<sup>+</sup> T cells exhibited increased BrdU incorporation compared with CD4<sup>+</sup> and CD8<sup>+</sup> T cells. This was particularly elevated in lpr CD4<sup>+</sup> 8<sup>+</sup> T cells, where 15–18% of the cells synthesized DNA in a 24-h period. Similar results were obtained using 5-wk-old mice (data not shown). Thus, lpr mice contain an increased proportion of T cells that have spontaneously proliferated in vivo and this phenotype manifests at an early age. Because the mice were not exposed to any known Ag, we considered that this reflected an increase in the proportion of lpr T cells that had undergone basal levels of cell cycling.

We next compared the phenotype of freshly isolated lpr T cells to fas<sup>+</sup> wild-type T cells that had undergone homeostatic proliferation. The T cells that accumulate with age in lpr mice have a phenotype distinct from naive or Ag-stimulated T cells. Naive fas<sup>+</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells are CD26L<sup>−</sup>CD44<sup>low</sup>CD25<sup>−</sup> (Fig. 2). After Ag stimulation, T cells rapidly decrease CD62L expression and up-regulate CD44 and CD25 expression. In contrast, lpr CD4<sup>+</sup> and CD8<sup>+</sup> T cells are bimodal for CD62L expression, largely CD44<sup>high</sup> and lack CD25 expression (Fig. 2 and data not shown). In addition, an increased fraction of lpr CD8<sup>+</sup> T cells express CD122 (IL-2Rβ) compared with fas<sup>+</sup> CD8<sup>+</sup> T cells. This phenotype closely resembles that of T cells undergoing homeostatic expansion. Fas<sup>+</sup> or lpr lymph node cells were transferred into RAG-1-deficient recipient mice, and the phenotype of combined spleen and lymph node cells was analyzed at 14, 25, and 31 days after transfer. Fas<sup>+</sup> T cells undergoing homeostatic proliferation were bimodal for CD62L, CD44<sup>high</sup>, and lacked surface CD25 (Fig. 2 and data not shown). Homeostatically expanding CD8<sup>+</sup> T cells also express CD122 (Fig. 2B and Refs. 13 and 15). Lpr T cells undergoing homeostatic proliferation have a phenotype similar to that of homeostatically proliferating fas<sup>+</sup> T cells. These phenotypic parallels underscore the notion that the accumulation of T cells in lpr mice may reflect preservation of T cells undergoing homeostatic expansion.

**Fas<sup>+</sup> wild-type and Fas-deficient lpr T cells enter cell cycle at similar rates**

To examine the role of Fas in homeostatic proliferation, we adoptively transferred fas<sup>+</sup> wild-type or lpr T cells into lymphopenic RAG-1-deficient recipients. The increased incorporation of BrdU over 24 h in T cells of lymphocyte-replete lpr mice could result from either an increased fraction of the cells entering the cell cycle or a reduction in death within the cycling population. To distinguish between these two possibilities, we analyzed initiation of cell cycling after adoptive transfer of CFSE-labeled fas<sup>+</sup> or lpr lymph node cells into RAG-1-deficient recipients. As shown in Fig. 3A, the transferred cells had begun cycling by day 5, but the fraction of cycling cells was not different between recipient mice that received either fas<sup>+</sup> (23%) or lpr (26%) cells. This similarity in
cycling rate persisted during days 6 and 7. BrdU incorporation was used to determine whether similar rates of cycling persisted at later time points when the cells had proliferated beyond the number of cycles detectable by CFSE labeling. In mice that received BrdU from day 13 to day 14, there was still substantial cell cycling during this 24-h period, yet there was no statistical difference in the fraction of cycling T cells from fas/H11001 or lpr donor cells (Fig. 3B). BrdU labeling over days 24–25 revealed a slight increase in BrdU incorporation in lpr T cells, which became more pronounced between days 29 and 30. Although this pattern might reflect a sudden late increase in the cell cycle rate of lpr T cells, more likely it represents a retention of cycled lpr T cells that does not occur with fas/H11001 T cells. As such, this begins to resemble the 24-h BrdU incorporation of intact lpr mice shown in Fig. 1.

FIGURE 3. Similar cell cycle entry of fas+ and lpr T cells after transfer into lymphopenic recipients. A, Lymph node cells from 5- to 6-wk-old Thy1.1 B6 fas+ and B6 lpr mice were labeled with CFSE and transferred into RAG-1-deficient recipients. Shown is CFSE and TCR/H9252 expression on T cells from lymph nodes and spleens harvested from recipients on the days indicated. Number inserts represent the percentage of cells that have undergone one or more cell divisions. B, RAG-1-deficient recipients received four i.p. injections of BrdU (1 mg each) over the 24-h period from days 13–14, 24–25, or 29–30 postadoptive transfer. Lymph node and spleen cells from RAG-1-deficient mice that received fas+/H11001 or lpr lymph node cells were surface stained for CD4, CD8, and CD90.1 and were analyzed for BrdU incorporation by flow cytometry. Shown are the mean and SD for the fraction of BrdU+ cells in the CD4+ and CD8+ T cell subsets (n = 4 per donor strain). Differences between fas+ and lpr donor T cells at the indicated time point were not statistically significant, except for CD8+ T cells on days 29–30 (t test, p < 0.05).

Homeostatically proliferating wild-type T cells are sensitive to Fas-induced death

Because there was no detectable difference in the initial entry into cell cycling between adoptively transferred fas+ wild-type and lpr T cells during the early period of homeostatic proliferation, this suggested that the increased proportion of cycling T cells detected by BrdU incorporation in lymphocyte-replete lpr mice might result from decreased death of cycling cells compared with T cells from fas/H11001 mice. Therefore, we examined whether T cells undergoing homeostatic proliferation were sensitive to Fas-mediated cell death. Lymph node and spleen cells were harvested from RAG-1-deficient recipients that had received fas+/H11001 or lpr lymph node cells 20 days previously. Fas+ cells from both CD4+ and CD8+ populations cultured in the presence of

FIGURE 2. The phenotype of lpr T cells resembles T cells undergoing homeostatic proliferation. Shown is the surface expression of CD62L, CD44, and CD122 on B6 fas+ wild-type splenic T cells, B6 lpr splenic T cells, and combined lymph node and splenic T cells isolated from B6 RAG-1-deficient mice that received either fas+ or lpr lymph node cells. Donor T cells were identified by expression of TCRβ or CD90.1 (Thy1.1). B6 fas+ and B6 lpr mice were 17 wk of age. For T cells undergoing homeostatic proliferation, shown is the expression of CD62L and CD44 on day 14 posttransfer and of CD122 on day 31 posttransfer. Number inserts represent the percentage of positive cells. For CD44 expression, numbers in parentheses represent the median fluorescence intensity of the entire peak.
cross-linked FasL for 3 h contained 20–30% dead cells by TUNEL assay (Fig. 4A). There was a low level of spontaneous death in untreated cultures. The level of death in lpr T cells was not increased above background in the presence of cross-linked FasL. Thus, wild-type T cells undergoing homeostatic proliferation are sensitive to Fas-mediated death.

The cycling T cells detected by BrdU incorporation over 24 h in lymphocyte-replete mice (Fig. 1) suggested that a population of Fas-sensitive T cells might exist during normal lymphoid homeostasis. Freshly isolated fas+ splenocytes from lymphocyte-replete mice were thus cultured in the presence of cross-linked FasL. After 3 h, both CD4+ and CD8+ T cells from these lymphocyte-replete mice contained 20–35% apoptotic cells by TUNEL assay (Fig. 4B). Earlier studies in which Fas was cross-linked using anti-Fas Abs also reported low but not negligible levels of cell death in freshly isolated T cells (39). These data are consistent with the possibility that Fas-mediated death may be a constant feature during normal T cell homeostasis in wild-type mice.

These findings raised the question of where FasL was expressed during homeostatic proliferation. FasL mRNA was readily detectable in the spleen cells of mice undergoing homeostatic proliferation compared with lower levels in freshly isolated B6 fas+ splenocytes from lymphocyte-replete mice (Fig. 4C). The cells recovered from the lymphoid tissues of recipient mice were comprised of the homeostatically expanding donor T cells as well as RAG-1-deficient host cells, which included predominantly macrophages and NK cells. FasL mRNA was also very abundant in splenocytes from RAG-1-deficient mice that had not received transferred T cells. Thus, FasL expressed on either the donor T cells or host splenocytes might initiate Fas-mediated cell death.

As noted in Fig. 4C, FasL mRNA expression by RAG-1-deficient spleen cells was considerably increased compared with the levels in freshly isolated B6 fas+ wild-type splenocytes. This apparent difference in FasL expression in total spleen between the two strains of mice was a reflection of the cellular composition of the spleens. Due to the lack of T and B cells, RAG-1-deficient spleens are composed primarily of NK cells and macrophages. When wild-type spleen was depleted of T and B cells, the remaining cells were greatly enriched for macrophages and NK cells and expressed a substantially higher level of FasL mRNA than did total wild-type spleen (Fig. 4D). As such, the T and B cell-depleted spleen cells more closely resembled the cellular composition of RAG-1-deficient spleens. The presence of high levels of FasL on macrophages and NK cells supports the view that Fas-mediated cell death of homeostatically expanding T cells could occur in lymphocyte-replete mice.

**Lpr T cells accumulate to greater numbers during homeostatic proliferation**

The sensitivity of T cells undergoing homeostatic proliferation to Fas-mediated cell death suggested that T cells lacking Fas might accumulate to higher levels during homeostatic expansion. Thus, we compared the number of CD4+ and CD8+ T cells recovered on days 14, 25, and 31 after transfer of equal numbers of fas+ or lpr T cells to RAG-1-deficient recipients (Fig. 5). All recipient mice appeared healthy with no evidence of a wasting syndrome over the

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**FIGURE 4.** T cells undergoing homeostatic proliferation are sensitive to Fas-mediated cell death. A, Lymph node and spleen cells were isolated from RAG-1-deficient mice that had received wild-type fas+ or lpr lymph node cells 20 days previously. Cells were cultured in vitro with FLAG-tagged FasL cross-linked by anti-FLAG Ab (■) or with anti-FLAG Ab only (□). After 3 h, the cells were surface stained and analyzed for the presence of nicked DNA by TUNEL assay. The fraction of cells containing nicked DNA was calculated for each T cell subset. Shown are the mean and SD (n = 4 per donor strain). B, Freshly isolated splenocytes from B6 lymphocyte-replete fas+ mice were cultured with or without FasL as described in A and were analyzed by TUNEL assay. Shown are the mean and SD (n = 4) of the percentage of apoptotic T cells. C, Lymph node and spleen cells undergoing homeostatic proliferation express FasL mRNA. mRNA was isolated from combined lymph node and spleen cells obtained from RAG-1-deficient mice that received either fas+ or lpr lymph node cells 20 days previously, as well as from freshly isolated splenocytes from RAG-1-deficient and B6 lymphocyte-replete fas+ mice. cDNA was prepared and expression of fasL and β-actin mRNA was determined by real-time PCR. Shown is the relative expression of FasL mRNA compared with fasL mRNA in B6 lymphocyte-replete fas+ wild-type spleen cells. Data are representative of five mice per donor strain. D, T and B cell-depleted B6 fas+ spleen expressed a substantially higher level of FasL mRNA than did total spleen. cDNA was prepared from freshly isolated total spleen cells from B6 fas+ mice and from spleen cells depleted of T and B cells. The expression of fasL and control β-actin mRNA was determined by real-time PCR.
The expression of FasL mRNA by RAG-1-deficient splenocytes suggested that FasL expression by host cells could initiate Fas-mediated cell death of the proliferating donor T cells. Thus, we compared the number of CD4\(^+\) and CD8\(^+\) T cells recovered after transfer of equal numbers of fas\(^+\) wild-type, lpr, or FasL-mutant gld T cells to RAG-1-deficient recipients (Fig. 6). On day 31 posttransfer, recipients that received lpr cells had on average 4.6-fold more CD4\(^+\) and 13.7-fold more CD8\(^+\) T cells compared with mice that received fas\(^+\) cells, consistent with the data in Fig. 5 (p < 0.05). By comparison, mice that received gld T cells had an intermediate 2.9-fold increase in CD4\(^+\) and a 3.6-fold increase in CD8\(^+\) T cells compared with mice that received fas\(^+\) cells. Thus, FasL expression by nonlymphoid cells of the recipient contributed to the death of T cells during homeostatic expansion. In addition,

31-day test period. The total number of recovered T cells increased continually over this time and expanded to substantially higher levels with lpr T cells. On day 14 posttransfer, the number of CD4\(^+\) and CD8\(^+\) T cells recovered was no different between mice that had received fas\(^+\) or lpr cells. However, by day 25 recipients that received lpr cells had on average 2.4-fold more CD4\(^+\) and 3.4-fold more CD8\(^+\) T cells. By day 31, there was a further 2-fold increase in the number of CD4\(^+\) T cells compared with day 25, and the mice that received lpr T cells continued to have 2.5-fold more CD4\(^+\) T cells than did mice that received fas\(^+\) cells. The number of CD8\(^+\) T cells recovered from mice that received fas\(^+\) cells increased only minimally from day 25 to day 31, whereas the number of lpr CD8\(^+\) T cells increased 1.5-fold. This resulted in 4.4-fold more CD8\(^+\) T cells in mice that received lpr cells compared with fas\(^+\) cells on day 31 posttransfer. These differences in cell number between fas\(^+\) and lpr T cells were significant for both CD4\(^+\) and CD8\(^+\) T cell subsets on days 25 and 31 posttransfer (p < 0.05). Overall, the number of CD4\(^+\) and CD8\(^+\) T cells recovered from mice that received lpr cells increased 5.4-fold from day 14 to day 31 posttransfer, whereas the number of T cells recovered from mice that received fas\(^+\) cells increased only 2.2-fold.

The expression of FasL mRNA by RAG-1-deficient splenocytes suggested that FasL expression by host cells could initiate Fas-mediated cell death of the proliferating donor T cells. Thus, we compared the number of CD4\(^+\) and CD8\(^+\) T cells recovered after transfer of equal numbers of fas\(^+\) wild-type, lpr, or FasL-mutant gld T cells to RAG-1-deficient recipients (Fig. 6). On day 31 posttransfer, recipients that received lpr cells had on average 4.6-fold more CD4\(^+\) and 13.7-fold more CD8\(^+\) T cells compared with mice that received fas\(^+\) cells, consistent with the data in Fig. 5 (p < 0.05). By comparison, mice that received gld T cells had an intermediate 2.9-fold increase in CD4\(^+\) and a 3.6-fold increase in CD8\(^+\) T cells compared with mice that received fas\(^+\) cells. Thus, FasL expression by nonlymphoid cells of the recipient contributed to the death of T cells during homeostatic expansion. In addition,
although the differences in cell number between gls and fas+ donor T cells did not reach statistical significance, the increased number of gls T cells suggested that FasL expression by T cells might also contribute to the death of homeostatically expanding T cells.

Discussion

The current observations propose an explanation for the accumulation of T cells that occurs in the absence of Fas in humans and mice (23, 40, 41). T cells undergoing homeostatic proliferation express Fas and are sensitive to Fas-mediated cell death. Fas-deficient T cells that are transferred into a lymphopenic environment accumulate to significantly higher levels than do wild-type Fas+ T cells. This occurs despite equivalent rates of cell cycle entry between Fas+ and Fas-deficient lpr donor cells. This favors the view that the increased homeostatic expansion of Fas-deficient T cells is the result of diminished cell death and not increased proliferation. As such, the death receptor, Fas, plays a prominent role in the regulation of the expansion of peripheral T cells in response to self-peptide/MHC during T cell homeostasis.

The lpr genotype was originally identified over a decade ago as a retropon disruption of the fas gene (42), yet no adequate explanation of the age-dependent adenopathy and unique phenotype of the accumulating T cells has been elucidated. Little if any defect in thymic positive or negative selection has been identified in lpr mice (24–27). Although a few reports have shown a delay of deletion by exogenous superantigens or foreign Ags, there does not appear to be any accumulation of these Ag-reactive T cells in the periphery (28–30). Deletion of pre-existing peripheral T cells in vivo through activation by exogenous Ags or superantigens also occurs normally in lpr mice (31, 32). Moreover, the surface phenotype of the accumulating CD4+ and CD8+ T cells in lpr mice more closely parallels that of T cells undergoing homeostatic proliferation than those activated by foreign Ag. In agreement with these findings is the observation that a substantial proportion of T cells in lpr mice cycle in a 24-h period without foreign Ag stimulation. These remarkable similarities support the view that the pronounced adenopathy and phenotype of lpr T cells are more likely driven by homeostatic expansion than by foreign Ag activation. This is also consistent with earlier findings that lpr mice raised under germ-free and Ag-free conditions still develop lymphadenopathy (36).

Mice bearing defects in other death receptors or their ligands, such as TNFR and TRAIL, do not develop lymphadenopathy. Mice deficient for TNFR1 or TNFR2 exhibit increased susceptibility to bacterial infections but show no differences in lymphocyte numbers or subpopulations (43–45). TRAIL-deficient mice have increased susceptibility to autoimmune disease and tumor development but do not manifest adenopathy (46–48). This suggests that there is a nonredundant function of Fas in regulation of lymphocyte homeostasis.

Mice bearing mutations in other signaling molecules that disrupt traditional points of T cell death also do not develop adenopathy. Mice deficient in CD30 or CD40L have defects in thymic negative selection but do not accumulate lymphocytes (49–51). Mice lacking the Bcl-2 family members Bim or Bax/Bak exhibit impaired deletion of peripheral T cells activated by foreign Ags or superantigens, yet they manifest only a modest (2- to 4-fold) increase in lymph node size compared with the pronounced enlargement seen in lpr mice (21, 22). In addition, Bim- or Bax/Bak-deficient mice do not accumulate the CD4+8− T cells characteristic of lpr mice. Thus, paradoxically, dysregulation of the genes that control thymic selection or foreign Ag-induced deletion do not result in the progressive accumulation of T cells in peripheral lymphoid tissues to the degree that is seen in lpr mice.

Other mutations do lead to adenopathy, but the phenotype of these mice does not resemble that of lpr mice. For example, mice deficient for Cita-4 have a massive accumulation of activated lymphocytes that express CD25 and CD69 at an early age and undergo spontaneous proliferation in vitro but do not accumulate CD4+8− T cells (52, 53). The mice die at a young age with multiorgan mononuclear infiltrates. CD25 and CD69 are not found on lpr T cells or on cells undergoing homeostatic proliferation. Unlike the absence of Fas, loss of CTLA-4 removes a negative regulator of T cell stimulation that profoundly augments activation and the rate of cell cycle entry. IL-2-deficient mice also accumulate activated T cells that exhibit increased BrdU incorporation in vivo (54). These mice have lymphocytic infiltration of bone marrow, inflammatory changes in many organs, and severe anemia. IL-2Rα-deficient mice accumulate large numbers of CD44high T cells but not CD4+8− T cells between the ages of 4 and 6 wk (55). These T cells show no evidence of activation or increased cell cycling, and deletion of superantigen-reactive T cells is impaired. With age, IL-2Rα-deficient mice manifest autoimmunity with a predominance of anemia and inflammatory bowel disease. Beyond adenopathy, the phenotypes of the CTLA-4−, IL-2−, and IL-2Rα-deficient mice differ significantly from that seen in Fas-deficient mice. Lpr mice manifest an age-dependent lymphadenopathy predominantly of CD4+8− T cells, develop immune complex glomerulonephritis on certain strain backgrounds, and have little lymphocytic infiltration of nonlymphoid organs (23).

A recent report showed that selective inactivation of Fas in T cells in vivo led to an age-dependent severe lymphopenia and fatal wasting syndrome (56). This is reminiscent of earlier studies demonstrating that irradiated fas+ wild-type mice also developed a wasting syndrome after transfer of lpr bone marrow (57, 58). At 5 mo of age, mice with selective inactivation of Fas in T cells manifested a loss of splenic architecture, pronounced thymic atrophy, increased FasL expression on the Fas-deficient T cells, and a significant decline in T cell number. The authors attributed the wasting syndrome, which appears at 10 mo of age, to the interaction of the FasL-expressing, Fas-deficient T cells with Fas+ cells in the environment, leading to destruction of the lymphoid and nonlymphoid architecture. However, at 2 mo of age, before the appearance of these age-dependent features, the number of Fas-deficient T cells was actually 160% of that in control mice. Thus, at early time points, the phenotype of T cell conditionally Fas-deficient mice was consistent with the hypothesis that Fas regulates T cell homeostasis. The data from these studies also suggest that in addition to Fas inactivation in T cells, defective Fas expression in nonlymphoid cells may be required to develop lymphadenopathy as seen in lpr mice.

We and others (13, 17) have shown that the number of T cells recovered after their transfer into lymphopenic recipients is low compared with the high cell cycle rate. During a 24-h labeling period from day 24 to day 25 posttransfer, 20% of the fas+ wild-type or lpr donor-derived cells underwent cell cycling. However, by day 31, fas+ T cell numbers had not increased proportionally from those on day 25, despite their continued proliferation. Conceivably some of the lack of expected accumulation might result from migration of proliferating T cells to nonlymphoid organs or from diminished cell cycling. An initial analysis has not revealed any infiltration of homeostatically expanding T cells into livers (K.A. Fortner, unpublished observations), a site to which Ag-activated T cells frequently migrate (59). Alternatively, in the face of continued proliferation, the plateau phase of peripheral T cell expansion likely results from the onset of apoptosis. This is consistent with the finding that T cells undergoing homeostatic proliferation are sensitive to
FasL, which is expressed in lymphoid tissues during this process. We have observed that some, but not all, of the T cells undergoing homeostatic proliferation have down-regulated expression of CD62L, and this is more pronounced on CD4+ T cells compared with CD8+ T cells. Because the donor T cells used in these experiments were >90% CD62L+, down-modulation of surface CD62L likely occurred after T cell transfer into RAG-1-deficient recipients. The increased fraction of CD62L+ CD8+ T cells may reflect the actual loss of the CD62L+ population, consistent with the minimal increase in cell number of fas+ CD8+ T cells (Fig. 5).

The studies showing that T cells undergoing homeostatic expansion maintain CD62L expression used the transfer of OT-I TCR transgenic CD8+ T cells into irradiated B6 mice (9, 15). OT-I CD8+ T cells may be more representative of the subset of T cells within a polyclonal population that maintain CD62L expression during homeostatic expansion.

Because homeostatic proliferation is driven by self-Ags, the potential for autoimmunity disease clearly exists in situations where the expanding T cells are not constrained. In humans, infection and treatment-induced lymphopenia, such as during HIV infection and after chemotherapy, are strongly linked to autoimmune tendencies (60–62). Newborn mammals are moderately lymphopenic, and neonatal thymectomy prolongs this condition and also promotes autoimmune disease of various tissues (63). In NOD mice, lymphopenia-induced homeostatic proliferation contributes to the development of autoimmune diabetes (64). A premature reduction in thymic output and compensatory increases in the basal rate of peripheral T cell proliferation have been suggested to contribute to the pathogenesis of rheumatoid arthritis (65, 66). Even in the absence of lymphopenia, alterations in T cell homeostasis, as in the presence of Fas mutations, may contribute to autoimmune disease. Lpr mice manifest an autoimmune disease characterized by autoantibodies and glomerulonephritis (23). Similarly, humans bearing Fas mutations in the autoimmune lymphoproliferative syndrome also manifest autoimmune phenomena (40, 41).

A possible corollary to our findings is that the basal level of T cell cycling in nonlymphopenic wild-type mice may be higher than previously appreciated. If we assume that the absence of Fas in lpr mice abrogates only the death of T cells without enhancing cell cycling, then the increased spontaneous BrdU incorporation of T cells in lpr mice may more closely reflect the true rate of spontaneous T cell cycling in wild-type mice. We observed a 2-fold increase in BrdU incorporation during a 24-h period in the T cells of lymphocyte-replete lpr mice compared with fas+ wild-type mice (Fig. 1). Yet there was no observable difference in the rates of entry into cell cycle during the early phases of reconstitution as assessed by CFSE or BrdU between fas+ and lpr donor T cells in lymphopenic recipients. Thus, the difference in accumulating cell numbers likely lies in the increased death of wild-type T cells during homeostatic proliferation. Conceivably, normal homeostatic proliferation of T cells in lymphocyte-replete wild-type mice may progress at a considerably higher rate than previously considered. Consistent with this possibility, we observed that 20–25% of freshly isolated wild-type spleen T cells were sensitive to Fas-mediated cell death and that FasL mRNA was expressed by wild-type spleen cells. Thus, the T cell expansion and cell death observed in lymphopenia-induced homeostatic proliferation in RAG-1-deficient mice may merely reflect a greatly accelerated rate of the processes that normally occur in lymphocyte-replete wild-type mice. Collectively, these findings suggest that a primary function of Fas is the deletion of T cells that are cycling in response to self-peptide/MHC during normal lymphoid homeostasis.

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