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Fas-Dependent Elimination of Nonselected CD8 Cells and lpr Disease

Linda A. Trimble,* Kenya A. Prince,* Gary A. Pestano,* John Daley, † and Harvey Cantor2*‡

MHC/self peptide interactions with cognate coreceptor/TCR complexes are central to homeostasis of the T cell repertoire. Recent reports have also underlined the critical role of IL-15/IL-2 cytokines in regulating this homeostatic process. In this study, we investigate mechanisms that regulate potentially autoreactive CD8 cells that have escaped intrathymic selection. These cells, upon exit from the thymus, express high levels of CD44, B220, and the IL-15R/IL-2R, and undergo fas-dependent apoptosis. Defects in fas signaling allow increased IL-15/IL-2-dependent survival of these CD44/B220⁺ CD8⁺ as well as the double-negative T cells characteristic of lpr disease. The Journal of Immunology, 2002, 168: 4960–4967.

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trigent control of the peripheral T cell repertoire is required to protect against autoimmunity while allowing a broad spectrum of cells capable of reacting to potential microbial pathogens. Immature T cells are screened for functional TCRs as well as autoreactivity in the thymus before entry into peripheral lymphoid tissues. These intrathymic screening processes, involving death by neglect and positive and negative selection, lead to maturation of thymocytes with optimal affinity to self peptide MHC complexes (1, 2). However, this affinity-driven selection process is not foolproof. Emigrant T cells that do not engage thymic ligands in the periphery or lose the ability to recognize Ag/MHC complexes are purged from the peripheral T cell pool.

We have recently described a screening mechanism that is responsible for clearing such useless CD8 T cells in the periphery (3). The CD8 coreceptor apparently plays a critical role in this process (3, 4): in the absence of constant trimolecular interactions among the TCR/CD8 and self MHC, peripheral CD8 cells down-regulate the CD8 coreceptors to become CD4⁺ CD8⁻ TCR⁻ (double-negative (DN)) T cells and undergo fas-dependent programmed cell death (3). DN T cells unable to maintain CD8/MHC/TCR interactions obtain an abnormal activated phenotype characterized by the expression of high levels of CD44 and B220.

T cells that express this surface phenotype accumulate in lpr/gld diseases as well as in systemic lupus erythematosus and acquired lymphoproliferative syndrome (5–7). These diseases reflect genetic defects in fas (CD95) signaling and are characterized by progressive accumulation of DN TCRαβ⁺ cells that display surface markers of activated/memory cells (CD44high) and high levels of B220 (8). However, the origin and development of this subset are unclear. Expansion of this T cell subset in fas+/− mutant mice depends in part on thymic processing, because thymectomy within the first 4 days of life inhibits lymphadenopathy (9). Additional peripheral mechanisms also contribute to lymphoproliferation because removal of the thymus in young animals does not inhibit lymphocyte accumulation (10). Attempts to elucidate the mechanisms responsible for lymphocyte expansion in this disease (11–13) have suggested that the DN T cell subset may be derived from abnormally accumulating CD8 precursor cells in the periphery (14–17).

Analysis of CD8 proliferation has indicated the critical role of IL-2 and IL-15 cytokines for both cell proliferation and survival (reviewed in Refs. 18–20). IL-15 has been described as a survival cytokine that may be responsible for the slow turnover of memory cells after initial encounter with MHC/peptide Ag. In contrast, IL-2 may support an initial burst of proliferation during Ag activation and sensitize activated T cells to fas-mediated cell death. These findings indicate that MHC/peptide ligands work in concert with cytokines to regulate the life span of correctly selected functional T cells. However, the role of the MHC/peptide complexes, fas, and IL-2/IL-15 in peripheral elimination of autoreactive and useless cells has not been defined.

In this study, we examine the relative roles of the cytokines IL-2 and IL-15 in the generation and expansion of thymically derived, nonselected CD8 T cells that differentiate in the periphery to DN TCRαβ⁺ expressing cells. We contrasted the fate of nonselected T cells with negatively selected cells and positively selected cells using anti-HY TCR (specific for male Ag, HY) transgenic (tg) mice in the presence and absence of competent fas signaling. Our findings indicate that the progressive accumulation of DN T cells in the absence of fas is a peripheral rather than thymic event, leading to expansion of nonselected T cells that have slipped through selection and display the distinctive CD44highB220⁺ phenotype that defines the abnormal T cell subset in lpr disease.

Materials and Methods

Mice

C57BL/6 (B6) (H-2b), MRL (H-2p) mice with and without the fas+/− mutation were obtained from The Jackson Laboratory (Bar Harbor, ME), and B6 recombinase-activating gene (RAG)-2+/− (H-2b) mice expressing the anti-HY peptide TCR as a transgene were obtained from Taconic Farms (Germantown, NY). Mice were intercrossed to yield HY-Ag-positive (male) and HY-Ag-negative (female) progeny that were RAG-2+/+ or
RAG-2<sup>−/−</sup> and fas<sup>homo</sup> or fas<sup>hetero</sup> on the selecting (H-2<sup>b</sup>) and nonselecting (H-2<sup>k</sup>) MHC backgrounds. To assess the effects of activating Ag contributions to the thymic and peripheral selection processes, mice of either sex (presence or absence of the HY Ag) were analyzed. Mice were housed under specific pathogen-free conditions and fed autoclaved diets. These conditions provide minimal exposure to environmental Ags.

**Antibodies**

Abs unconjugated or conjugated to FITC, R-PE, CyChrome, or allophycocyanin used for flow cytometry and cell sorting were as follows: anti-mouse CD8 (53-6.7), CD4 (RM4-5), CD90.2/Thy-1.2 (53-2.1), CD44 (IM7), CD<sup>5</sup> (B6), CD<sup>4</sup> (B3H1), CD24/heat stable Ag (M1/69), CD8<sup>+</sup>/B220 (RA3-6B2), TCR<sup>+</sup> chain (H57-597), V<sub>β</sub>8.1, 8.2 TCR (MR5-2), H-2<sup>k</sup> (AF6-88.5), H-2<sup>k</sup> (BD PharMingen, San Diego CA), and anti-mouse CD8<sup>β</sup> (CT-CD8B) (Caltag Laboratories, Burlingame, CA). The clonotypic Ab, T3.70, which recognizes the TCR specific for the male Ag, HY, was kindly provided by H.-S. Teh (University of British Columbia, Vancouver, Canada). Five-color flow cytometry was conducted with streptavidin-7-amino-4-methylcoumarin-3-acetic acid (Coulter/Immuno-tech, Miami, FL) and anti-CD4-RED613 (H129.19; Life Technologies, Gaithersburg, MD), anti-B220-PE-Cy7 (RA3-6B2), and CD4 PE-Cy7 (CT-CD4) (Caltag Laboratories).

**FACS analyses**

Single cell suspensions of thymic and peripheral (spleen and lymph nodes) cells were prepared, as described (3, 21). Briefly, RBCs were lysed (ammonium chloride potassium lysing solution) and washed with medium (DMEM containing 5% FCS). For Ab staining, 10<sup>6</sup> cells in 100 μl medium were stained with appropriate fluorochrome-conjugated Abs for 20 min on ice. After washing with PBS, the cells were resuspended in 1% paraformaldehyde (in PBS) before flow cytometric analysis. Unless otherwise indicated, all Abs were purchased from BD PharMingen.

**Polymerase chain reaction**

Genomic DNA was screened by PCR conducted on proteinase-digested tails, essentially as described (22), to determine mutations in the fas and rag2 genes and to confirm the presence of the HY TCR transgene (V<sub>8.2</sub>). Oligonucleotides were synthesized (Amiot, Allston, MA; for PCR and use as follows: Fas1, 5’-GATTCCATTGTTGCTGTGTG-3’; Fas2, 5’-CTCTCAACTGGTGTGCAGA-3’; Fas3, 5’-CAGGGAATGTCGCAAGATG-3’ (23). RAG-2, Neo 3’-CCAAGCCTATGCTGGATACGGT; RAG-2-1, 5’-TTAATCCGACGGCTTCTACTF-3’; RAG-2-2, 5’-GCGCTATGCTTGCTGCA-3’ (communicated by F. W. Alt, Harvard Medical School, Boston, MA). V<sub>H</sub>8.2 (HY TCR), V<sub>H</sub>8.2A, 5’-ACAGTCAGTCTGTGTCAGA-3’; V<sub>H</sub>8.2, 5’-ACAGTGTTGTCAGA-3’ (communicated by H. von Boehmer, Dana-Farber Cancer Institute, Boston, MA).

**Adoptive transfers**

CD8<sup>−/−</sup> T cells were enriched from the lymph nodes of HY<sup>−/−</sup> Rag-2<sup>−/−</sup>, female H-2<sup>b</sup> mice using purified rat anti-mouse B220 (RA3-6B2), NK1.1 (PK136), MAC-1e chain (M1/70), and LY-6G/Gr-1 (RB6-8C5) and sheep anti-rat IgG Dynabeads (Dynal Biotech, Lake Success, NY), as per the manufacturer’s protocol. The resulting cells were >93% CD8<sup>−/−</sup> T cells by FACS analysis. A total of 4 × 10<sup>6</sup> CD8 T cells was injected i.v. into either H-2<sup>k</sup>- or H-2<sup>b</sup>-<sup>−/−</sup> B6 Rag-2<sup>−/−</sup> female mice. Two or 4 days later, the lymph nodes and spleens were harvested, and single cell suspensions were analyzed for T3.70, CD8, CD44, B220, and CD122 expression by flow cytometry.

**CD8 cell cycle analysis**

CD8<sup>+</sup> cells were isolated from the lymph nodes using purified rat anti-NK1.1 (PK136), MAC-1e chain (M1/70), LY-6G/Gr-1 (RB6-8C5), and sheep anti-rat IgG Dynabeads (Dynal Biotech, Lake Success, NY), as per the manufacturer’s protocol. The resulting CD8<sup>−/−</sup> T cells were cultured at 5 × 10<sup>5</sup> cells/well in round-bottom 96-well plates in complete T cell medium (DMEM containing 10% FCS (Sigma-Aldrich, St. Louis, MO), 10 μM penicillin-streptomycin (Life Technologies), 2 mM glutamine (Life Technologies), 10 mM HEPES buffer (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 5 × 10<sup>−3</sup> M 2-ME (Life Technologies)) supplemented with various concentrations of murine rIL-2 (BD PharMingen) or murine rIL-15 (Research Diagnostics, Flanders, NJ) for 40 h. The cell cycle phases were determined after staining with CD8 FITC, ethanol fixation, RNA digestion, and propidium iodide incorporation (Coulter). Percentages of cells in S/G2/M are reported as being in cycle and subdiploid cells as apoptotic.

**RTE analysis**

Thymocytes were labeled intrathymically, as described previously (24). Briefly, 10 μl FITC in PBS (1 mg/ml) was injected into one or both thymic lobes of 5- to 8-wk-old Rag-2<sup>−/−</sup> HY<sup>−/−</sup> male and female mice on H-2<sup>b</sup>-<sup>−/−</sup> or H-2<sup>k</sup>-<sup>−/−</sup> backgrounds. Approximately 40 h later, individual mice were sacrificed, and single cell suspensions were prepared from the lymph nodes and spleens. Respective cell samples were pooled, and FITC-positive cells in the live cell gate (forward scatter vs side scatter) were analyzed further for CD8, T3.70 TCR, and CD122 expression by flow cytometry.

**Results**

**Thymic nonselection is a unique process that is distinct from positive and negative selection.**

We have previously shown that CD8 cells in MHC class I (β<sub>2</sub>-microglobulin)-deficient mice down-regulate CD8 expression due to the inability to form trimolecular interactions among TCR-, CD8-, and MHC-bearing peptide molecules. This process was proposed to be initiated in the thymus and marked by remethylation at the CD8<sub>α</sub> gene locus (3). Thymocytes that fail to make this trimolecular interaction are neither positively nor negatively selected. To determine whether these nonselected or useless T cells normally escape from the thymus, we generated anti-HY TCR tg mice on a Rag<sup>−/−</sup> background that contain T cells unable to recognize their cognate or self peptide due to presentation by the wrong MHC (H-2<sup>k</sup> or H-2<sup>b</sup>). We confirmed earlier findings that positive selection of CD8 cells occurs in H-2<sup>b</sup> female mice (HY<sup>+</sup>), while negative selection occurs in male (HY<sup>−</sup>) mice (25-27) using male and female H-2<sup>b</sup>-<sup>−/−</sup> Rag-2<sup>−/−</sup> mice (Fig. 1). In H-2<sup>b</sup> animals, i.e., in which the TCR and coreceptor do not simultaneously coengage the presenting MHC (3, 28, 29), the numbers of thymocytes should be identical regardless of sex (30). Examination of thymocytes expressing the specific TCR in male or female mice with the wrong (H-2<sup>k</sup>)

![FIGURE 1](http://www.jimmunol.org/ Downloaded from http://www.jimmunol.org/)

"FIGURE 1. Thymocyte nonselection is distinct from positive and negative selection. Male and female mice H-2<sup>b</sup>-<sup>−/−</sup> Rag-2<sup>−/−</sup> (selecting) or H-2<sup>k</sup>-<sup>−/−</sup> Rag-2<sup>−/−</sup> (nonselecting) on MHC backgrounds were analyzed for CD4 and CD8 expression on thymocytes positive for the anti-HY TCR (clonotypic Ab, T3.70 reactive) by flow cytometry. The distribution of the CD4 (PE-conjugated) and CD8 (CyChrome) surface markers is shown on cell populations after gating on TCR<sup>+</sup>-reactive cells (T3.70 FITC). Data shown are for individual fas<sup>−</sup> mice representative of four to five animals analyzed from each group."
MHC showed no differences in the thymic cellularity of male or female nonselecting mice (Fig. 1). Furthermore, thymic nonselection differed from negative selection in that anti-HY TCR (T3.70)-expressing double-positive (DP) thymocytes survived deletion. Furthermore, nonselection was not accompanied by significant numbers of single-positive (SP) T3.70 CD8 cells in the thymus.

Nonselected CD8 T cells emigrate from the thymus

We determined the fate of nonselected thymocytes using intrathymic injection of FITC to identify recent thymic emigrants (RTEs) in the periphery of HY-specific TCR RAG-2−/− mice with the selecting or nonselecting MHC. More than 99% of FITC+ RTEs in the lymph node and spleen of all mice analyzed were TCR nonselected RTEs had proliferated during this 48-h interval, thereby confirming the thymic origin of nonselected T cells (Fig. 2). In addition, because fluorescent decay of FITC indicates cell division by the RTE because labeling (31, 32), this experiment indicated that nonselected RTEs had proliferated during this 48-h interval, because the majority of the cells were FITCbright (Fig. 2). Further analysis indicated that the majority of the cells (~80%) were DN (data not shown). In contrast, ~50% of RTEs from the positively selecting model retained high levels of the FITC fluorochrome and express high levels of the CD8 coreceptor (Fig. 2 and data not shown). We hypothesize that RTEs with the DN T cell phenotype observed in the positively selecting model may be in part due to nonselection occurring in conjunction with positive selection in the latter model. Similar experiments conducted with an alternative TCR tg mouse model, specific for the P14 lymphocytic choriomeningitis virus, resulted in >90% CD8+FITC+ RTEs in the lymphocytic choriomeningitis virus tg RAG-2−/− mice. This suggests that the efficiency of the nonselection process may also be influenced by Ag affinities, and is lower in the presence of TCR with high affinities for selecting Ags.

Nonselected CD8 cell emigrants coexpress CD122, CD44, and B220

We then asked whether nonselected proliferating RTEs persist in the periphery after failing to ligate either self or activating Ag. CD8 cells in the periphery of nonselecting MHC mice expressed high levels of CD44 and CD122 (Fig. 3A) similar to the activated surface phenotype of nonselected CD8 RTEs (data not shown). Peripheral CD8 cells from female H-2b/b mice (positively selected) did not express significant levels of either CD44 or CD122 (Fig. 3A).

Because RAG-2−/− mice lack the full repertoire of T and B lymphocytes that may regulate normal T cell homeostasis, it was important to determine the phenotype of nonselected T cells in RAG-2−/− mice. T cells from the nonselecting HY-tg model on the RAG-2−/− background also expressed high levels of CD122 and CD44, as well as the B220 molecule, which is normally restricted to B-lineage cells (Fig. 3B). In contrast, neither self-reactive CD8 T cells that had escaped negative selection, nor positively selected CD8 T cells from female H-2b/b mice expressed B220.

Deprivation of homeostatic or activating MHC/peptide interactions results in up-regulation of CD44, CD122, and B220 on CD8 cells

CD8 cells from H-2b/b anti-HY-tg female RAG-2−/− mice transferred into RAG-2−/− recipients that do not facilitate coligation of the TCR/CD8 coreceptor complex (H-2k+ RAG-2−/−) showed increased expression of CD44, CD122, and B220 accompanied by the down-regulation of CD8 expression by 4 days posttransfer (Fig. 4). The donor T cells were not detectable by day 7 (data not shown), consistent with our previous studies that have demonstrated this transition and a similarly rapid clearance of these cells (3). In contrast, CD8 cells transferred to H2b/b female RAG-2−/− hosts and analyzed over the same period retained a naive, CD44low, cell phenotype and CD8 expression (Fig. 4). DN TCRαβ+ T cells that express CD44 and B220 accumulate in Fas+/− mice (33, 34). Our observations that the nonselecting environment selectively induces cells with the same activated phenotype, in contrast to positively selected cells or negative selection escapees, suggest that DN cell generation as a result of thymic nonselection may play a role in lpr disease.

Defective fas signaling and thymic selection

Because fas can mediate apoptosis in peripheral CD8 cells that fail to coligate the TCR and CD8 coreceptor (3), we attempted to determine whether fas was important in thymic selection processes. The thymus in RAG-2−/−lpr mice expressing the nonselecting
H-2<sup>b</sup> MHC did not show significant alterations of thymocyte subset development in the absence of fas signaling (Fig. 5). In contrast, RAG-2<sup>−/−</sup>-<i>lpr</i> H-2<sup>b</sup> (male) mice displayed significant rescue of DP and of SP CD8 cells in the face of active negative selection (Fig. 5).

**Thymic nonselection enhances development of peripheral DN T cells in lpr mice**

The lack of a detectable effect of <i>fas</i> deficiency on intrathymic development of useless CD8 cells suggested that <i>fas</i>-dependent elimination of these cells in the peripheral lymphoid tissues may play a key role in the disposal of these cells. We asked whether DN T3.70<sup>+</sup>-nonselected cells would accumulate in the periphery in the absence of fas signaling. This was the case: the proportion of T3.70<sup>+</sup> cells increased by up to 20-fold higher than in positively selecting H-2<sup>b</sup> anti-HY · RAG-2<sup>−/−</sup>-<i>lpr</i> mice, and 20-fold higher than in negatively selecting <i>lpr</i> mice (Figs. 6 and 7).

Because peripheral accumulation of lymphocytes in HY-tg RAG-2<sup>−/−</sup>-<i>lpr</i> mice is apparently limited by the absence of cytokine support from accessory B cells or CD4 cells in RAG-2<sup>−/−</sup>-<i>lpr</i> mice (13, 32, 35, 36) and a reduction in the TCR repertoire introduced by the expression of the transgenes (37–39), we also analyzed T cells from RAG-2<sup>−/−</sup>-HY TCR<sup>+</sup> mice. RAG-2<sup>−/−</sup>-<i>lpr</i> mice expressing the HY TCR displayed similar thymic profiles to the RAG-2<sup>−/−</sup>- HY TCR<sup>+</sup> mice: fas signaling did not contribute to nonselection and positive selection, while there was a marked increase in the CD8 SP subset in H-2<sup>b</sup> <i>lpr</i> negatively selected male mice (Fig. 8). All groups of mice with defective fas signaling exhibited an age-dependent increase in T3.70<sup>+</sup> T cells by 35 wk (Fig. 9A). The total number of T3.70<sup>+</sup> T cells had increased by up to at least 10-fold in <i>fas<sup>−/−</sup></i> animals over <i>fas<sup>−/−</sup></i> counterparts of the same age (data not shown), but thymic emigrants from nonselecting <i>fas<sup>−/−</sup></i> mice underwent the most rapid increase in HY TCR DN T cells.

**FIGURE 5.** Effect of <i>fas</i> deficiency on thymic selection in RAG-2<sup>−/−</sup>-anti-HY TCR<sub>αβ</sub> mice. TCR tg H-2<sup>2b</sup> or H-2<sup>2k</sup> RAG-2<sup>−/−</sup> male and female mice that express <i>fas<sup>−/−</sup></i> (left panels) or <i>fas<sup>−/−</sup></i> (right panels) were analyzed by flow cytometry for CD4 and CD8 expression on T3.70<sup>+</sup> thymocytes. Data shown are for individual <i>fas<sup>−/−</sup></i> and <i>fas<sup>−/−</sup></i> mice representative of three to four animals analyzed in each group.

**FIGURE 6.** Subset representation of peripheral DN to CD8 T cells expressing the anti-HY TCR is selectively enhanced in the nonselecting (H-2<sup>b</sup>) MHC. The ratio of T3.70<sup>+</sup> DN:CD8 cells was determined for each RAG-2<sup>−/−</sup> selection model shown. Total numbers of the respective lymphocyte subsets were determined for the lymph nodes and spleen in the respective individual age-matched male or female <i>fas<sup>−/−</sup></i> HY-TCR<sup>+</sup> on the H-2<sup>b</sup> or H-2<sup>2k</sup> MHC. Data shown are averaged from at least three age-matched animals per group.

**FIGURE 7.** <i>Fas</i> deficiency affects peripheral T cell subset distribution in RAG-2<sup>−/−</sup>-anti-HY-tg mice. <i>Fas<sup>−/−</sup></i> and <i>Fas<sup>−/−</sup></i>, RAG-2<sup>−/−</sup> male and female mice on H-2<sup>2b</sup> or H-2<sup>2k</sup> MHC backgrounds were analyzed by flow cytometry for CD4 and CD8 expression on lymph node cells coexpressing the tg HY-TCR (T3.70<sup>+</sup>). Data shown are from individual mice and are representative of three to four animals (~6 wk of age) analyzed for each group. Samples were analyzed as described in Fig. 1.
cell numbers, as predicted from results with the identification of RTEs in RAG-2−/−-HY TCR tg mice. The proportion of DN T3.70+ peripheral T cells was at least 10-fold higher in nonselecting mice compared with negative or positive selecting mice. Thus, negatively selecting mice contained normal numbers of DN T cells, while CD8 cell numbers were increased by ~15-fold over fas wild-type (wt) age-matched controls (Fig. 9A), indicating that autoreactive T cells do not contribute to the abnormally expanding DN subset of T cells observed in lpr mice. In support of this hypothesis, 24 ± 5% of T3.70+ cells in RAG-2−/− HY-Tg H-2bb male mice with intact fas are CD8+ compared with 82 ± 7% of age-matched fasbr control mice (Fig. 7).

An alternative explanation for the accumulation of DN cells in lpr mice involves expansion secondary to activation of positively selected CD8 T cells through environmental Ags (40, 41) and inhibition of normal apoptosis by the lpr mutation (42). Although there is a slow rate of DN lymphoaccumulation in positively selected lpr mice (Fig. 9A), DN lpr cells comprised a relatively small percentage of the T3.70+ T cells at all ages tested (Fig. 9A) so that the percentage of T3.70+ DN cells was ~2- to 3-fold more than wt.

Because the absolute numbers of HY-TCR+ cells generated in each model were differentially affected by thymic selection, we determined the impact of the lpr mutation on the rate of accumulation of DN cells (Fig. 9B). The rate of DN-lpr T cell accumulation was highest in the nonselecting environment, while the positively selecting environment was at least three times slower than that observed for the nonselecting MHC, and the lpr mutation had no positive effect on the rate of DN T cell accumulation in a negatively selecting environment (H2b/b, HY−).

CD8 T cells in nonselecting lpr mice express CD122, CD44, and B220 and proliferate in response to CD122 signaling

Analysis of HY-specific CD8 T cells in selecting and nonselecting lpr environments showed a similar, but exaggerated phenotype compared with that of fas wt animals (Fig. 3B). CD122, CD44, and B220 were expressed by the large majority of CD8 T cells in nonselecting lpr mice (Fig. 10), but the IL-2/15R β-chain expression was increased in fasbr-nonselecting mice compared with faswt mice. Very few CD8 cells from the positively selecting environment expressed CD122, CD44, or B220.

In view of increased CD122 expression in nonselecting lpr mice, we asked about IL-2 and/or IL-15 responsiveness by nonselected CD8 cells. Our previous experiments have shown rapid proliferation is one part of the cleansing program induced in nonselected CD8 cells as they transition to the DN phenotype. In the presence of IL-2 and IL-15, CD8 cells from RAG-2−/−-nonselecting mice selectively entered the G2 M phase of the cell cycle (Fig. 11, A and B). These findings are significant given that the shift in the cell cycle is observed in the absence of other accessory cells or Ag presentation, suggesting that the accumulation of DN T cells in lpr disease may be cytokine rather than Ag driven. In addition, both IL-15 and IL-2 were capable of protecting both nonselected and autoreactive cells from apoptosis (Fig. 11, C and D).
Discussion

It is increasingly apparent that the composition and number of mature T lymphocytes are regulated by both intrathymic and peripheral mechanisms. The extent of negative selection reflects deletion of autoreactive T cell clones in the thymus that depends on apoptosis-induced via TCR-associated signals (43–45). Mature T cells are regulated by a complex interaction, including signals emanating from death complexes formed among members of the TNF and TNFR family of proteins (46, 47). However, the mechanisms that normally eliminate thymic emigrants that are useless, i.e., after incorrect thymic selection for which no ligands exist in the periphery, have not been elucidated. In this study, we traced the fate of such useless CD8 cells that emigrate to the periphery. T cell clones that do not undergo positive or negative selection in the thymus differentiate into peripheral DN T cells and normally undergo fas-dependent apoptosis. These findings delineate fas signaling as a last line of defense that inactivates and destroys this T cell subpopulation.

Our analysis depended on RAG-2−/− and RAG-2−/+ anti-HY-specific TCR tg mice that generate T cell clones that do not efficiently recognize β2-microglobulin-associated MHC class I products in the thymus or periphery. Clones emgerant from this process were defined as useless to the peripheral repertoire, because they do not participate in normal immune responses (3). Comparison of these nonselected T cells with either positively selected or negatively selected cells indicated that the process of nonselection is unique: nonselected cells do not expand as do positively selected cells (48), nor are they deleted by negative selection, as attested to by high numbers of DP T3.70+ thymocytes (Figs. 1 and 5). We have previously reported that peripheral T lymphocytes normally require continuous MHC/TCR/coreceptor (trimolecular) interactions to prevent spontaneous down-regulation of CD8, cellular activation, and fas-dependent apoptosis. We hypothesized that these useless clones would accumulate as DN T cells in the periphery of nonselecting H-2b lpr (fas-deficient) mice. These data (Fig. 2) extend our previous observations that naive CD8 cells transferred to MHC-deficient hosts (nonselection) undergo similar rapid proliferation before apoptotic cell death (3) and support our hypothesis that MHC/coreceptor signals provide a braking mechanism that normally inhibits spontaneous proliferation. Our findings in this study also underline a key role for fas signaling that prevents progressive accumulation of abnormal DN T cells and define the relative contributions of negative, positive, and failed selection to the accumulating peripheral lymphocyte subsets in lpr (and in gld) mice.

Thymic selection has been proposed to occur on a graded scale of MHC/peptide affinity (and avidity) for the TCR and coreceptor. Interactions of the highest affinity/avidity lead to death by negative selection (49), while interactions of intermediate avidity lead to signaling for survival or positive selection. Interaction of very low avidity (less than required for positive selection) results in death by neglect for the vast majority (>90%) of intrathymic interactions. If the concentration of peptide is high, fas (CD95) plays a role in negative selection; however, low concentrations of peptide induce negative selection in a fas-independent pathway (43). As described by Kishimoto and Sprent (43), this population of lymphocytes carries the restriction of self tolerance, while harboring the ability to react to foreign Ags. This selection process, however, has been questioned for its fidelity (50, 51), i.e., T cell deletion in the thymus is not complete, and the possible escape of low affinity T cells to the periphery is inevitable. These potentially autoreactive T cells that have escaped negative selection now fall under the regulation of peripheral tolerance (52) that prevents these cells from attacking self, thus providing a secondary mechanism for clearing potential autoreactive cell from the periphery T cell repertoire.

We reasoned that cells that fail to undergo positive/negative selection must exist at the threshold between positive selection and neglect. The anti-HY/H-2b T3.70 T cells analyzed in this work represent an enriched population of CD8 cells that have successfully evaded death by neglect and emigrated from the thymus. This subset of cells is normally cleansed from the peripheral repertoire by fas-dependent mechanisms and accumulates as a dedifferentiated DN T cell population in the absence of fas.

Earlier studies have noted that the introduction of transgenes encoding TCRs into lpr mice inhibits lymphadenopathy (37–39). Our examination of the periphery in the anti-HY-tg models used in this study is consistent with these findings, because the overall representation of DN lpr T cells was reduced compared with either H-2b/H-2b non-TCR tg lpr mice (data not shown). However, lymphoaccumulation was readily observed in TCR tg lpr mice compared with TCR tg fas wt litters. Expression of a functional rearranged TCRαβ in H-2b mice that interacts well with H-2b is likely to reduce the numbers of nonselected T cells that normally emigrate from the thymus of non-TCR tg mice. In contrast, in H-2b tg mice, peripheral lymphocytes expressing the tg TCR contain an enriched population of monoclonal T cells that did not undergo positive selection.

A significant number of thymocytes that undergo nonselection escape death in the thymus, as illustrated by examination of peripheral T cells from anti-HY TCR/H-2b MHC mice carrying RAG-2−/− or RAG-2−/+ genotypes (Fig. 3). Migrant nonselected T cells in the periphery are then normally subjected to an inactivating peripheral sequence of events that includes coreceptor down-regulation, spontaneous activation, and fas-dependent apoptosis (3). Although defective fas expression did not have an obvious impact on intrathymic development in mice expressing the nonselecting MHC (Fig. 1), these mice contained an abnormally large proportion of TCR− DN T cells in the periphery (Figs. 2 and

FIGURE 11. Nonselected CD8+ T cells are induced to G2M and are rescued from apoptosis by IL-2 and IL-15. CD8 T cells from the lymph nodes of RAG-2−/− HY TCR tg-selecting and -nonselecting mice were depleted of NK1.1+ NK cells by Dynabead depletion before incubating with murine IL-2 (A and C) or IL-15 (B and D) for 48 h. Cells were stained for CD8 expression before ethanol fixation and propidium iodide DNA staining. The cell cycle profile was determined by flow cytometry, and cells with >2 N DNA content were considered to be in cycle (A and B), while cells with <2 N DNA were considered apoptotic (C and D).

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7). These cells proliferate in the periphery, and in the presence of competent fas signaling are rapidly cleared. Our previous studies indicate that these cells undergo at least three rounds of division during this process (3). The expression of the class I-restricted TCR on CD4-lpr cells similarly resulted in an expanded nonselected T cell population in this lineage (data not shown). These cells are analogous to nonselected CD8 cells because they are unable to form the trimolecular interactions required between MHC, TCR, and the coreceptor. However, although nonselected CD4 T cells do not down-regulate their coreceptor, they also express high levels of B220 and accumulate in the absence of fas. Taken together, these data indicate that the periphery, rather than the thymus, is the major site of fas-dependent deletion of useless T cells.

Of interest also was the abrogation of lymphoaccumulation in nonselecting RAG-2-/- lpr mice. Two possible hypotheses are proposed to account for this phenomenon: 1) nonselection is enhanced by the expression of multiple TCRs on the surface of developing thymocytes even in the absence of a transgene (29, 53), and/or 2) help may normally be provided from B cells and/or CD4 T cells (38, 39, 55-57). Although a relatively large number of potentially autoreactive T cells with low or no expression CD8 coreceptor was generated in H-2b male anti-HY TCR tg mice (negatively selecting; data not shown), a fas defect was associated with a decreased (rather than increased) representation of the DN T cell population (data not shown), and the small population of DN cells in these mice did not express B220. These data indicate that, although CD8 thymocytes may normally down-modulate the coreceptor to escape negative selection, fas defects allow enhanced numbers of CD8 cells with elevated levels of the coreceptor to migrate to the periphery (Fig. 9A), where they do not serve as a major source of DN T cells and continue to maintain active expression of the CD8 coreceptor (Fig. 7). These data rule out a contribution by negative selection “escapees” to aspects of the phenotype observed in fas-/- associated diseases. Indeed, the significant impact of defective fas signaling on negative selection apparently results in increased emigration of negative selection escapees that seed the periphery in the lpr mutants. A consequence of fas defects that break peripheral tolerance mechanisms that normally clears or inactivates cells in the thymus may be autoreactive T cell responses (38, 39, 55-57).

**Fas**-/- mice that positively select anti-HY CD8 T cells also displayed sustained CD8 gene expression (Fig. 7), although a slow rate of DN T cell accumulation was apparent in these mice (Fig. 9). Because antigenic activation apparently does not result in CD8 down-modulation in lpr mice, it is unlikely that peripheral autoantigen or environmental Ag(s) drives the expansion noted in this work for the evolving DN T cells. We instead propose that the expansion of DN T cells with the hallmark high expression of CD44 and B220 in these mice may develop due to HY-specific thymocytes that fall into the nonselected window. According to this view, expansion of DN T cells with the hallmark high expression of CD44 and B220 in normal mice may develop due to 1) near threshold-level selection, 2) selection on thymic restricted Ags, or 3) induced down-regulation of peripheral MHC class I.

The quality control mechanism described in this study defines a selective role for fas in regulating the composition of the peripheral T cell repertoire. Defects in this mechanism are associated with defective fas, in which DN T cells accumulating in lpr disease are most likely generated from nonselected CD8 T cells, but not from negative selection escapees nor from positively selected T cells.

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**References**


