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The Molecular Signature of Murine T Cell Homeostatic Proliferation Reveals Both Inflammatory and Immune Inhibition Patterns

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Abstract

T lymphocyte homeostatic proliferation, driven by the engagement of T cell antigen receptor with self-peptide/major histocompatibility complexes, and signaling through the common γ -chain-containing cytokine receptors, is critical for the maintenance of the T cell compartment and is regulated by the Fas death receptor (Fas, CD95). In the absence of Fas, Fas-deficient lymphoproliferation spontaneous mutation (*Ipr*) mice accumulate homeostatically expanded T cells. The functional consequences of sequential rounds of homeostatic expansion are not well defined. We thus examined the gene expression profiles of murine wild-type and Fas-deficient *Ipr* CD8⁺ T cell subsets that have undergone different amounts of homeostatic proliferation as defined by their level of CD44 expression, and the CD4⁺CD8⁺TCR $\alpha\beta$ ⁺ T cell subset that results from extensive homeostatic expansion of CD8⁺ T cells. Our studies show that recurrent T cell homeostatic proliferation results in global gene expression changes, including the progressive upregulation of both cytolytic proteins such as Fas-Ligand and granzyme B as well as inhibitory proteins such as programmed cell death protein 1 (PD-1) and lymphocyte activating 3 (Lag3).

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Authorship Contribution

K.A.F. designed and performed research, analyzed and interpreted data, and wrote the manuscript. J.P.B. performed the bioinformatic analysis of the microarray data. J.W.A. designed, performed and analyzed the methylation studies. J.M.B. contributed to the methylation studies. J.P.B., J.W.A., and J.M.B. critically reviewed the manuscript. R.C.B. contributed to the experimental design and data interpretation and edited the manuscript.

Conflict of Interest Disclosure

The authors declare no competing financial interests.

These findings provide an explanation for how augmented T cell homeostatic expansion could lead to the frequently observed clinical paradox of simultaneous autoinflammatory and immunodeficiency syndromes and provide further insight into the regulatory programs that control chronically stimulated T cells.

Keywords

T lymphocytes; Fas (CD95); Homeostatic proliferation; CD8⁺ T cells; Cytolytic proteins; Inhibitory proteins

1. Introduction

The number of peripheral T cells is maintained by the emigration of naive T cells from the thymus and their subsequent homeostatic proliferation in response to self-peptide/major histocompatibility complex (MHC) and signaling through the common γ -chain-containing cytokine receptors including those for IL-7 and IL-15 [1–5]. Elevated rates of homeostatic expansion exist in naturally occurring states of relative lymphopenia, such as neonates and the elderly [6, 7] as well as in autoimmune disorders including rheumatoid arthritis and systemic lupus erythematosus [8, 9]. T cell populations in these environments frequently manifest diminished immune responses to infections or vaccines [10–17], suggesting a link between increased homeostatic proliferation and immune exhaustion. Paradoxically, lymphopenic states, such as following viral infections, chemotherapy, and bone marrow transplantation, can be associated with autoimmune manifestations [18–20]. The association of increased T cell homeostatic proliferation with both autoimmunity and reduced immune function suggests that persistent homeostatic proliferation could lead to changes in T cell function that promote both inflammation and immune suppression.

T cells undergoing homeostatic expansion can contribute to the memory T cell pool. In response to lymphopenia resulting from irradiation or genetic T cell deficiency, T cells undergo augmented homeostatic expansion and acquire a memory-like phenotype [21, 22]. These T cells manifest effector functions and provide protective immunity to bacterial infection [23, 24]. However, in other studies, lymphopenia-induced proliferation generated PD-1⁺ T cells with impaired function [25]. Even in the absence of lymphopenia, homeostatic proliferation occurs at a low but significant rate [26]. In young unimmunized mice, the peripheral T cell pool contains 10–20% memory-phenotype T cells and their numbers increase with age [23, 27–30]. Moreover, within the foreign antigen-specific CD8⁺ T cell pool, 10–30% of the cells have a pre-existing memory-phenotype in unimmunized conventional mice as well as in germ-free animals [23]. The presence of memory-phenotype T cells in germ-free mice supports a model that these T cells arise via homeostatic mechanisms. In addition to effector capabilities, memory-phenotype CD8⁺ T cells have been reported to exert a regulatory role [25, 31, 32], and hence, this T cell population may both restrain and enhance immune reactivity.

In order to maintain T cell numbers at a constant level, homeostatic proliferation must be balanced by an equivalent rate of cell death. We have shown that the Fas cell surface death receptor (Fas, CD95, apoptosis antigen 1 (Apo-1), TNF receptor superfamily member 6) has

a prominent role in the regulation of T cell homeostatic expansion [26]. T cells from Fas-deficient lymphoproliferation spontaneous mutation (*Ipr*) (*Fas^{lpr/lpr}*) mice accumulated to much greater levels than wild-type T cells during lymphopenia-induced proliferation in Rag1-deficient mice, despite equivalent rates of proliferation [26]. *Fas^{lpr/lpr}* mice thus manifest an age-dependent lymphadenopathy, even under germ-free conditions, which includes CD44^{high}CD4⁺ and CD44^{high}CD8⁺ T cells as well as polyclonal CD4⁺CD8⁺TCRαβ⁺ T cells that derive from CD8⁺ T cell precursors based on genetic studies [33–38]. On a genetically susceptible background, *Fas^{lpr/lpr}* mice also develop an autoimmune syndrome resembling human systemic lupus erythematosus. The same subsets of homeostatically expanding T cells, including CD4⁺CD8⁺TCRαβ⁺ T cells, also exist in wild-type mice, albeit at substantially lower numbers. Hence, Fas-deficiency does not give rise to a separate lineage of T cells that are unique to *Fas^{lpr/lpr}* mice, but rather, allows the extended survival of T cells undergoing homeostatic proliferation. As such, the T cells that accumulate in *Fas^{lpr/lpr}* mice may provide valuable insight into the gene expression profiles of homeostatically expanding CD8⁺ T cells and why it is important for Fas to limit their survival.

Here, we examined the gene expression profiles of both C57BL/6 wild-type and *Fas^{lpr/lpr}* CD8⁺ T cell subsets based on their progressive upregulation of CD44 expression during homeostatic proliferation. These T cell subpopulations represent a continuum in which CD8⁺ T cells progressively upregulate CD44 expression from low to intermediate to high with repeated cycles of homeostatic proliferation and then finally become CD4⁺CD8⁺.

2. Methods

2.1 Mice

Mice were bred and housed in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facilities of The University of Vermont. All mice in these studies were on a C57BL/6 background and were used between 10 and 13 weeks of age. Original breeding pairs of *B6.PL-Thy1^a/Cy* (B6.CD90.1, wild-type), B6.MRL-*Fas^{lpr}/J* (*Fas^{lpr/lpr}*), and B6.129S7-Rag1^{tm1Mom/J} (recombination-activating gene, RAG1-deficient) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Breeding of B6.CD90.1 mice with *Fas^{lpr/lpr}* mice generated mice homozygous for CD90.1. All animal studies were conducted in accordance with the policies of The University of Vermont's Animal Care and Use Committee.

For the *in vivo* proliferation studies, mice received four intraperitoneal injections of 1 mg 5-bromo-2'-deoxyuridine (BrdU) in sterile phosphate buffered saline (PBS) (Sigma) during the 24 h period prior to tissue harvest. Three injections were given on the day prior to tissue harvest and one injection on the day of sacrifice 1 h prior to tissue harvest.

2.2 Lymphocyte preparation

Single cell suspensions of pooled inguinal, brachial, axillary, cervical, and popliteal lymph nodes were prepared in RPMI 1640 (CellGro, Corning, Manassas, VA) containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 5% v/v bovine calf serum

(BCS)(HyClone, Logan, UT), 5×10^{-5} M β -mercaptoethanol (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 U/ml streptomycin (Life Technologies-Invitrogen)(RPMI/5% BCS).

To isolate T cell subsets by negative selection, lymph node cells were incubated with the appropriate antibodies (see below), washed, and then incubated by rocking with goat anti-rat and goat anti-mouse IgG-coated beads (Qiagen, Valencia, CA). Antibody-coated cells were removed by magnetic depletion. Cell suspensions were incubated with anti-MHC class II (3F12), anti-CD11b (M1/70), anti-NK1.1 (PK136), anti-kappa (187.1), and anti-CD4 (GK1.5). To obtain CD8⁺ T cells, anti-CD45R (B220, RA3GB2) was also added. To isolate CD4-CD8-TCR $\alpha\beta$ ⁺ T cells, anti-CD8 (Tib105) was also included.

Naïve CD8⁺ T cells were isolated using Miltenyi Biotec's naïve CD8 α ⁺ T cell isolation kit according to the manufacturer's instructions. Naïve CD8⁺ T cells were then labeled with 4 μ M 5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) in PBS containing 0.1% w/v bovine serum albumin (BSA)(Sigma) for 10 min at 37°C, washed three times with cold PBS/0.1 % BSA, and resuspended in PBS for adoptive transfer.

2.3 Flow cytometry

Single cell suspensions were incubated with Live/Dead Fixable Blue Stain, washed with cold PBS containing 1% w/v BSA (PBS/1%BSA), and then incubated with unconjugated rat IgG and hamster IgG (50 μ g/ml each) (MP Biochemicals). After washing, the cells were incubated with the appropriate antibodies in PBS/1% BSA, washed, and fixed with freshly made 1% v/v methanol-free formaldehyde (Ted Pella, Redding CA) in PBS/1%BSA. Flow cytometry was performed on an LSRII (BD Bioscience) and the data were analyzed using FloJo software (TreeStar, Ashland OR).

The following antibodies to murine cell surface proteins were purchased from BD Biosciences (San Jose, CA): Fluorescein isothiocyanate (FITC)-conjugated anti-TCR $\gamma\delta$ (GL3), FITC-conjugated anti-BrdU, FITC-conjugated anti-NK1.1 (PK136), allophycocyanin (APC)-conjugated anti-TCR β (H57-597), Pacific Blue-conjugated anti-CD45R (B220, RA3-6B2). The following antibodies were purchased from Life Technologies-Invitrogen (Grand Island, NY): phycoerythrin (PE)-conjugated anti-CD44 (IM7), PE-Texas Red-conjugated anti-CD4 (RM4-5), PE-Texas Red-conjugated anti-CD8 (5H10), PE Cyanine (Cy)5.5-conjugated anti-CD8 (5H10), PE Cy5.5-conjugated anti-CD4 (RM4-5), Pacific Orange-conjugated anti-CD45 (30-F11). The following antibodies were purchased from Biolegend (San Diego, CA): PE-conjugated anti-PD-1 (29F.1.A12), PE-conjugated anti-Lag3 (C9B7W), peridinin chlorophyll protein (PerCP) Cy5.5-conjugated anti-CD4 (RM4-5), PE-Cy7-conjugated anti-TCR β (H597-597), Alexa Fluor 647-conjugated anti-TCR $\gamma\delta$ (UC7-13D5), Alexa Fluor 647-conjugated anti-TCR β (H597-597), Alexa Fluor 700-conjugated anti-CD19 (6D5), APC-Cy7-conjugated anti-CD45R (B220, RA3-6B2), Pacific Blue-conjugated anti-CD19 (6D5), Pacific Blue-conjugated anti-CD44 (IM7). Live/Dead Fixable Blue and Near-IR Dead Cell Stains were purchased from Invitrogen. CD1d- α -galactosyl ceramide tetramer (PBS-57) was obtained from the NIH Tetramer Core Facility.

Staining for DNA-incorporated BrdU was performed as described previously [26]. In brief, cells from BrdU-pulsed mice were incubated with Live/Dead stain followed by rat and hamster IgG as described above. The cells were stained for expression of cell surface proteins, and then fixed with 70% ethanol (-20°C) followed by 1% v/v methanol-free formaldehyde in PBS. The cells were permeabilized in PBS containing 1% v/v methanol-free formaldehyde and 0.01% v/v Tween-20 overnight at 4°C . The cells were incubated with 50 Kunitz units of DNase I (Sigma) in 150 mM NaCl (pH=5) containing 4.2 mM MgCl_2 for 15 min at 37°C , incubated with anti-BrdU FITC (BD Biosciences), and fixed in 1% v/v formaldehyde in PBS/1% BSA.

2.4 Immunoblot analysis

Cells were lysed in buffer containing 0.5% Nonidet P-40, 20 mM Tris-HCl (pH=7.4), 150 mM NaCl, 2 mM sodium orthovanadate, 10% glycerol, and complete protease inhibitor (Roche Diagnostics, Indianapolis, IN). Protein concentration was determined by Bradford assay (Bio-rad, Hercules, CA). Protein lysates were separated by SDS-PAGE using 12.5% acrylamide gels and transferred to Immobilon-FL (Millipore, Billerica, MA) membranes. Membranes were blocked with 4% w/v milk in PBS and then incubated with primary antibody in PBS containing 4% milk and 0.1% v/v Tween-20 overnight at 4°C . After washing with PBS containing 0.1% Tween-20, membranes were incubated with fluorescent-conjugated species-specific secondary antibodies in PBS containing 4% milk, 0.1% Tween-20, and 0.01% SDS. Images were acquired with an Odyssey CLx using Image Studio software (LI-COR, Lincoln, NE). The fluorescence signals for each sample were background subtracted and normalized to α -tubulin using Image Studio software. The following antibodies were used for immunoblot analysis: anti-Fasligand (FasL) (R&D Systems, MAB5262), anti- α -tubulin (Cell Signaling Technology), anti-granzyme B (Cell Signaling Technology), IRDye 800CW goat anti-rat (LI-COR), IRDye 680RD goat anti-mouse IgG1 (LI-COR), IRDye 800CW goat anti-rabbit (LI-COR).

2.5 Assay of cytolytic activity

T cell suspensions were resuspended in RPMI 1640 supplemented with 25 mM Hepes, 2500 $\mu\text{g}/\text{ml}$ glucose, 10 $\mu\text{g}/\text{ml}$ folate, 110.04 $\mu\text{g}/\text{ml}$ pyruvate (Life Technologies-Gibco), 5×10^{-5} M β -mercaptoethanol, 292.3 $\mu\text{g}/\text{ml}$ glutamine, 100U/ml penicillin, 100U/ml streptomycin, and 5% v/v bovine calf serum, and plated at 37°C for 2 h. Jurkat T cell targets were incubated with ^{51}Cr for 1 h at 37°C , washed three times, and then mixed with the freshly isolated T cell effectors at an effector:target ratio of 10:1. After incubating at 37°C for 4 h, co-culture supernatants were removed and counted for gamma emission. Spontaneous release was determined from labeled targets in the absence of effector cells. Maximal Fas-mediated ^{51}Cr release was measured by culturing Jurkat T cells with 250 ng/ml FLAGTM-tagged recombinant FasL (Alexis Biochemicals) plus anti-FLAGTM antibody (Sigma) for 4 h. The percentage of ^{51}Cr release was calculated as: % cytotoxicity=[(experimental cpm-spontaneous cpm)/FasL cpm-spontaneous cpm].

2.6 Bisulfite Sequencing

Genomic DNA was prepared using the Quick gDNA Micro Prep Kit (Zymo Research) according to the manufacturer's instructions. Bisulfite sequencing was adapted from

Youngblood et al [39]. Briefly, genomic DNA was bisulfite treated using the EZ DNA Methylation Gold Kit (Zymo Research). Bisulfite-converted DNA was amplified via region specific primers and cloned using the TOPO TA cloning kit (Life Technologies). Individual colonies were selected from three biological replicates, sequenced, and significance determined by a Fischer's Exact Test.

2.7 Microarray sample preparation

Single cell suspensions were prepared from inguinal, brachial, axillary, cervical, and popliteal lymph nodes. Lymph node cells from five 10-week old female wild-type mice were pooled and stained with live/dead stain followed by antibodies for CD4, CD8, TCR β , CD44, CD19, TCR $\gamma\delta$, and CD1d tetramer (Natural Killer T (NKT) cells). The CD8⁺TCR $\alpha\beta$ ⁺ subset was sorted into CD44^{low}, CD44^{intermediate}, and CD44^{high} populations. CD4⁻CD8⁻TCR $\alpha\beta$ ⁺ T cells were identified as TCR β ⁺CD19⁻TCR $\gamma\delta$ ⁻CD1d tetramer⁻. For *Fas*^{lpr/lpr} samples, lymph node cells from three 10-week old female *Fas*^{lpr/lpr} mice were pooled and stained with live/dead stain followed by antibodies for CD4, CD8, TCR β , CD44, CD45R, and NK1.1. CD4⁻CD8⁻TCR $\alpha\beta$ ⁺ T cells were identified as TCR β ⁺CD44⁺CD45R⁺NK1.1⁻. All sorts were performed on a FACS Aria (BD Bioscience). Sort purity was >97% for all populations. Total RNA was prepared from sorted T cell subsets using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions.

2.8 Microarray and data processing

RNA was processed, amplified, labeled, and hybridized to Affymetrix GeneChip Mouse 430 2.0 in the Vermont Genetics Network Microarray Facility. The signal intensity for each probe on each chip was calculated from scanned images using GeneChip Operating Software (Affymetrix). Calculations were performed using the R language and environment for statistical computing and graphics [40] with Bioconductor packages [41]. Probe intensities were background corrected, normalized, and summarized using the Robust Multi-array Analysis (RMA) method as implemented in the *affy* package using the default settings. A probe set was retained if it was called "present" in at least three samples based on the *mas5* procedure in the *affy* package [42].

2.9 Heat map construction

The wild-type and *Fas*^{lpr/lpr} expression matrices were joined using the 26,738 probe sets detected in both data sets and the top 2⁻⁸ (105) probe sets with respect to variance were selected without regard for the fit to any statistical model. The heat map was constructed using the heatmap.2 function in the gplots package. The column dendrogram was constructed using the complete linkage option and the Euclidean distance between profiles.

2.10 Principal component analysis

Wild-type and *Fas*^{lpr/lpr} expression statistics were expressed relative to the CD8⁺ CD44^{low} profiles from the same experimental batch and the CD8⁺CD44^{low} samples were set to the origin. The wild-type and *Fas*^{lpr/lpr} expression matrices were then joined based on probe sets called present in both sets. Principal component scores and loadings were obtained using the *prcomp* procedure from the *R stats* package (uncentered, unscaled). To obtain a real-valued

measure of the contribution of each gene to the departure of the CD4⁻CD8⁻TCRαβ⁺ T cells with respect to the path from CD8⁺ CD44^{low} to CD44^{intermediate} to CD44^{high}, we used the displacement of the sample scores for the CD4⁻CD8⁻TCRαβ⁺ T cells along a line perpendicular to a line fit to our CD8⁺ T cell subsets.

2.11 Identification of patterns of gene expression across samples

To identify patterns of probe set expression across samples, the top 2⁻³ probe sets (3,343) with respect to overall variation, from the combined wild-type and *Fas^{lpr/lpr}* expression matrices (26,738 probe sets), were selected and each assigned to one of eight classes using the k-means algorithm. Each sample was expressed relative to the CD8⁺CD44^{low} (L) sample of the same batch and the profiles of genes were scaled across samples to have the same variance. We then used the *k-means* procedure in the R *stats* package with default options, specifying eight clusters.

3. Results

3.1 Surface levels of CD44 expression reflect progressive rounds of homeostatic proliferation

Surface levels of CD44 expression are upregulated following T cell receptor for antigen (TCR) activation and cell proliferation [43]. We considered that CD44 might be progressively upregulated *in vivo* during T cell homeostatic proliferation. Following adoptive transfer of CD44^{low} T cells into Rag1-deficient recipients, T cell homeostatic expansion was induced and CD44 expression was progressively upregulated with each cell cycle (Fig. 1A, B). Furthermore, spontaneous cell cycling of T cells from C57BL/6 wild-type and *Fas^{lpr/lpr}* mice *in vivo*, as measured by BrdU incorporation, showed that the fraction of CD8⁺ T cells that incorporated BrdU progressively increased with increasing levels of CD44 expression (Fig. 1C, D). These data are consistent with the view that CD44 is upregulated during homeostatic proliferation and serves as a biomarker of this process. Of interest, CD4⁻CD8⁻TCRαβ⁺CD44^{high} T cells manifested a substantial rate of BrdU incorporation (18%) in a 24 h period (Fig. 1C, D), yet they exhibited a reduced cell size as measured by forward scatter compared with CD8⁺CD44^{high} T cells, their likely precursor (Fig. 1E). Since pre-existing *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells do not proliferate either *in vivo* or *in vitro* (K. A. F. unpublished data)[33], these data suggested that cell cycling occurred in the precursor CD8⁺ T cells just prior to the transition to the CD4⁻CD8⁻ phenotype and that once converted, cell cycling was rapidly arrested.

3.2 Continued T cell homeostatic proliferation results in changes in gene expression

To determine the functional consequences of recurrent T cell homeostatic proliferation, we analyzed the gene expression profiles of CD8⁺ T cell subsets from both C57BL/6 wild-type and *Fas^{lpr/lpr}* mice based on their levels of CD44 expression (low, intermediate, and high) and CD4⁻CD8⁻TCRαβ⁺ T cells which express high levels of CD44 (Fig. 2A, B). We chose to perform our studies using C57BL/6 *Fas^{lpr/lpr}* mice, which manifest lymphadenopathy but limited renal pathology and autoantibody production, in order to analyze changes in T cell gene expression due to homeostatic proliferation in the absence of the autoinflammation and autoimmune disease that develops in *MRL-Fas^{lpr}* mice. A small subpopulation of

CD4⁻CD8⁻TCRαβ⁺ T cells that lacked staining for TCRγδ, CD19, and CD1d-α-galactosyl ceramide tetramer (which identifies NKT cells) was present in wild-type mice, although there were relatively few of these cells (~0.6% of TCRαβ⁺non-NKT cells) presumably due to Fas-mediated cell death (Fig. 2A). By contrast, CD4⁻CD8⁻TCRαβ⁺ T cells were abundant in *Fas^{lpr/lpr}* mice (Fig. 2B). The CD4⁻CD8⁻TCRαβ⁺ T cells in wild-type mice likely represent recently generated CD4⁻CD8⁻TCRαβ⁺ T cells since they are rapidly eliminated in a Fas-dependent manner, whereas *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells persist.

Three independent sorts were performed on the combined lymph node cells from 3-5 mice for each genotype and were highly consistent. The four T cell subsets were readily distinguishable based on their gene expression patterns as shown in the heat map and sample-to-sample distances of the dendrogram (Fig. 2C). The CD8⁺ T cell subsets from wild-type and *Fas^{lpr/lpr}* mice bearing similar CD44 expression manifested very similar gene expression profiles. This is consistent with the view that the increased number of *Fas^{lpr/lpr}* T cells results merely from the accumulation of T cells that would normally be eliminated by Fas-mediated cell death and are not an aberrant lineage.

Principal component analysis revealed that 87% of the variation among the samples was captured by the first three principal components (PC) (Fig. 2D). PC1 and PC2 defined a line from CD8⁺CD44^{low} to CD8⁺CD44^{intermediate} to CD8⁺CD44^{high} to wild-type CD4⁻CD8⁻TCRαβ⁺ T cells and also defined the gene expression that distinguished *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells from the other subsets (Fig. 2E). PC1 and PC3 identified the shared gene expression between wild-type and *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells and distinguished them from the CD8⁺ T cell subsets (Fig 2F). PC2 and PC3 revealed the differences between wild-type and *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells (Fig 2G). This principal component analysis is consistent with a model in which gene expression changed progressively with continued homeostatic proliferation of CD8⁺ T cells along a path from CD44^{low} to CD44^{intermediate} to CD44^{high} and finally to CD4⁻CD8⁻TCRαβ⁺ T cells. CD4⁻CD8⁻TCRαβ⁺ T cells from wild-type mice shared features most closely with CD8⁺CD44^{high} T cells, consistent with being recently-derived from CD8⁺ T cell precursors (Fig. 2E). Wild-type and *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells had substantially similar gene expression (Fig. 2F), which likely reflects their common lineage, despite some differences (Fig. 2E, G).

Mathematical modeling of the changes in the gene expression profiles of wild-type and *Fas^{lpr/lpr}* T cells using differential geometry further demonstrated the similarities between equivalent subsets in wild-type and *Fas^{lpr/lpr}* mice, including the CD4⁻CD8⁻TCRαβ⁺ T cells (Supplemental Fig. 1A). Evaluation of the genes that differ specifically between CD4⁻CD8⁻TCRαβ⁺ T cells and their respective CD8⁺ T cell subsets using Fisher's exact test showed there was a very strong association ($p < 10^{-15}$) between wild-type and *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells (Supplemental Fig. 1B). Collectively, these analyses underscore the fact that although wild-type CD4⁻CD8⁻TCRαβ⁺ T cells are present in only small numbers, they likely have a similar lineage derivation as *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells.

3.3 Gene expression patterns reveal substantial increases and decreases with recurrent homeostatic proliferation

To identify patterns of gene expression during progressive rounds of homeostatic proliferation, the most differentially expressed probe sets were assigned to one of eight clusters based on their gene expression pattern, and the consensus expression profile determined for each cluster (Fig. 3). A subset of genes either progressively increased or decreased expression in a step-wise manner (Fig. 3A, E) as CD8⁺ T cells progressed from CD44^{low} to CD44^{intermediate} to CD44^{high} to CD4⁺CD8⁺TCRαβ⁺ T cells. This likely reflects the continuous changes in expression as T cells progress through multiple cycles of homeostatic proliferation and supports the view that CD4⁺CD8⁺TCRαβ⁺ T cells are an extension of the CD8⁺ T cell subsets. In two patterns (Fig. 3B, F), gene expression was strongly upregulated only as the CD8⁺ T cells became CD44^{high} and continued in wild-type CD4⁺CD8⁺TCRαβ⁺ T cells, undergoing a further change in *Fas*^{lpr/lpr} CD4⁺CD8⁺TCRαβ⁺ T cells. This suggested that a subset of CD8⁺CD44^{high} T cells shares a phenotype with CD4⁺CD8⁺TCRαβ⁺ T cells. Four additional patterns (Fig. 3C, D, F, G) revealed that gene expression differed between wild-type and *Fas*^{lpr/lpr} CD4⁺CD8⁺TCRαβ⁺ T cells, indicating that gene expression is further altered with the extended survival of CD4⁺CD8⁺TCRαβ⁺ T cells in *Fas*^{lpr/lpr} mice. The final pattern (Fig. 3H) showed that a small subset of genes was upregulated selectively in wild-type CD4⁺CD8⁺TCRαβ⁺ T cells. The genes associated with these expression patterns are summarized in Supplemental Table 1 and a subset of genes is highlighted in Table 1.

Genes in each pattern were analyzed for highly represented biological processes using Gene Ontology enrichment analysis (Supplementary Table 2). Genes progressively increasing in expression (Fig. 3A) included genes involved in proliferation, death, cell motility/migration, signaling, and inflammatory response. Genes strongly upregulated in both CD8⁺CD44^{high} and CD4⁺CD8⁺TCRαβ⁺ T cells (Fig. 3B) were highly enriched for genes involved in cell cycle and also cell death. Genes upregulated selectively in *Fas*^{lpr/lpr} CD4⁺CD8⁺TCRαβ⁺ T cells (Fig. 3C) included further control of the cell cycle, such as cell cycle checkpoints and regulation of cell cycle arrest. In contrast, genes progressively decreasing in expression (Fig. 3E) function in the regulation of immune responses, signaling, and response to IFNγ. Genes downregulated in *Fas*^{lpr/lpr} CD4⁺CD8⁺TCRαβ⁺ T cells (Fig. 3F, G) were involved in immune effector processes and lymphocyte activation as well as cytokine production (Fig. 3F), and regulation of TCR signaling (Fig. 3G). Collectively this analysis supports a model that recurrent homeostatic proliferation could lead to changes in the functional capabilities and survival of T cells.

3.4 Recurrent T cell homeostatic proliferation results in changes in expression of genes that regulate cytolytic activity, proliferation, and survival

A number of genes whose expression changes during homeostatic proliferation have important implications for immune function. Expression of a large number of genes involved in transcription were progressively increased or decreased with recurrent homeostatic proliferation (Fig. 4, Table 1). Of particular interest, *Eomes* and *Tbx21*, that coordinately regulate T cell survival and expression of cytolytic molecules [44], and NFATc1, a critical regulator of cytokine and effector protein expression [45–47], were progressively

upregulated as CD8⁺ T cells increased CD44 expression (Fig. 4A, B, Table 1). Genes regulating cell cycling, including *E2f2*, the cyclins *Ccnb2* and *Ccna2*, and the cell cycle inhibitors *Wee1* and *Jdp2*, were progressively upregulated as CD8⁺ T cells transitioned from CD44^{low} to CD44^{high} and finally to CD4⁺CD8⁺ (Fig. 4C, D, Table 1). Expression of *Wee1* and *Jdp2* were particularly increased in *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells, suggesting that further cell cycling is blocked in these T cells. Expression of the effector caspase, *Casp3*, was also progressively upregulated, potentially priming the cells for death (Fig. 4E). Furthermore, *Il7ra* expression was profoundly downmodulated in *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ cells (Fig. 4F), which may further limit their expansion.

Changes in expression of genes involved in the effector function of CD8⁺ T cells gave insight into the consequences of continued homeostatic expansion. Strikingly, the effector molecules *FasL*, *Gzmb*, and *Ifng* were upregulated during homeostatic proliferation (Fig. 4G-I, Table 1). The activating receptor *Klrk1* (NKG2D) which enhances CD8⁺ T cell cytotoxicity was also increased in both wild-type and *Fas^{lpr/lpr}* CD8⁺CD44^{high} and wild-type CD4⁺CD8⁺TCRαβ⁺ T cells, but downregulated in *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells (Fig. 4J). The costimulatory receptor *Cd160* was expressed in all T cell subsets except *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells (Fig. 4K). The inhibitory molecules *Lag3* (lymphocyte activating 3, CD223), *Pdcd1* (programmed cell death protein 1, PD-1, CD279), *Pdcd1lg2* (PD-1-ligand 2), and *Il10* were progressively upregulated as CD8⁺ T cells transitioned from CD44^{low} to CD44^{high} and finally to CD4⁺CD8⁺ in both wild-type and *Fas^{lpr/lpr}* mice (Fig. 4L-O, Table 1). The upregulation of this suite of genes suggested that CD8⁺ T cells undergoing chronic homeostatic proliferation acquire both cytolytic function and potential immune inhibitory function. Furthermore, wild-type CD4⁺CD8⁺TCRαβ⁺ T cells manifest elevated levels of the chemokine receptors *Ccr2* and *Ccr5* and the integrins *Itga1*, *Itga4*, and *Itgav* which function in recruitment and migration into non-lymphoid tissues (Table 1).

The profound upregulation of *FasL* and *Gzmb* gene expression in both *Fas^{lpr/lpr}* and wild-type CD4⁺CD8⁺TCRαβ⁺ T cells suggested that these T cells might be capable of antigen-independent cytolytic activity. Elevated expression of FasL and Granzyme B protein by *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells was confirmed by western analysis (Fig. 5A, B). In addition, freshly isolated *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells induced cytolysis of Jurkat T cell targets at a level five times that of wild-type CD8⁺ T cells (Fig. 5C). Thus, *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells are functionally capable of killing Fas-susceptible target cells without additional activation.

3.5 PD-1 is upregulated on both wild-type and *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells and regulated by gene methylation

Consistent with the upregulation of *Pdcd1* and *Lag3* gene expression (Fig. 4L, M), cell surface protein expression of PD-1 and Lag3 increased progressively from CD8⁺CD44^{low} to CD44^{high} (Fig. 6A–C). PD-1 expression was moderately increased on wild-type CD4⁺CD8⁺TCRαβ⁺ T cells and was strongly upregulated on *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells (Fig. 6A, B). Wild-type CD4⁺CD8⁺TCRαβ⁺ T cells could be subdivided into two subpopulations based on their CD44 and TCRαβ expression (Fig. 6A). The CD4⁺CD8⁺TCRαβ⁺ T cells expressing a slightly higher level of CD44 and reduced TCRαβ,

a phenotype similar to *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells, manifested increased PD-1 expression compared with the subset bearing less CD44, suggesting that the T cells that had undergone the most extensive homeostatic proliferation upregulated PD-1. Of note, a small subset of CD8⁺ CD44^{high} T cells had also upregulated PD-1 in both wild-type and *Fas^{lpr/lpr}* mice, consistent with the model that a subset of CD8⁺ CD44^{high} T cells shares a phenotype with CD4⁻CD8⁻TCRαβ⁺ T cells, and thus may be the most immediate precursor.

Expression of PD-1 by T cells is known to be epigenetically regulated [39]. We therefore examined the extent of DNA methylation in the *Pdcd1* transcriptional regulatory conserved region B (CR-B) and CR-C by bisulfite sequencing of genomic DNA from CD8⁺ and CD4⁻CD8⁻TCRαβ⁺ T cells from *Fas^{lpr/lpr}* mice. *Fas^{lpr/lpr}* CD8⁺ T cells manifested a highly methylated CR-B region and partially demethylated CR-C region, similar to that reported for naïve T cells (Fig. 6D) [39]. In contrast, *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells had a partially demethylated CR-B region and an almost completely demethylated CR-C region, consistent with their elevated expression of *Pdcd1*. While the methylation state of CR-B bears similarities to that reported for virus-specific memory CD8⁺ T cells, CR-C more closely resembles that of CD8⁺ T cells continually exposed to antigen during chronic infections [39].

4. Discussion

The current findings reveal that among the global changes in gene expression as CD8⁺ T cells undergo sequential rounds of homeostatic proliferation are molecules that could provoke either an autoinflammatory process (*Fasl* and *Gzmb*) or immune inhibition (*Pdcd1*, *Lag3*, *Ill10* and *Pdcd112*). These findings underscore the importance of regulating homeostatically expanding T cells. Our studies examined the gene expression profiles of CD8⁺ T cell subpopulations representing a continuum of homeostatic proliferation, following which a portion of the CD8⁺ T cells become CD4⁻CD8⁻ [26]. The small number of CD4⁻CD8⁻TCRαβ⁺ T cells in wild-type mice are probably recently derived from their CD8⁺ precursors since they are promptly eliminated by Fas [26]. In *Fas^{lpr/lpr}* mice, CD8⁺CD44^{high} and CD4⁻CD8⁻TCRαβ⁺ T cells accumulate and represent subpopulations that have likely undergone more extensive homeostatic proliferation. Thus, the absence of Fas does not give rise to T cell subsets that are unique to *Fas^{lpr/lpr}* mice, but rather allows the accumulation of T cells that normally arise in wild-type mice but are quickly eliminated by Fas. As such, gene expression analysis of these T cell subsets provides insight into the regulation and function of T cells undergoing chronic TCR stimulation.

Our findings provide insight into why it is critical to limit the accumulation of homeostatically expanding T cells. CD8⁺CD44^{high} and CD4⁻CD8⁻TCRαβ⁺ T cells from both wild-type and *Fas^{lpr/lpr}* mice manifest increased levels of *Ifng*, *Fasl*, *Gzmb*, and *Gzmk* gene expression. CD8⁺CD44^{high} and wild-type CD4⁻CD8⁻TCRαβ⁺ T cells also express the costimulatory receptors *Klrk1* (NKG2D) and *Cd160* that have been shown to enhance cytolytic responses [48, 49], and yet have only a modest increase in the inhibitory receptors *Pdcd1* and *Lag3*. Thus, CD8⁺CD44^{high} and recently derived CD4⁻CD8⁻TCRαβ⁺ T cells may be particularly potent mediators of inflammatory damage, without yet manifesting inhibition by PD-1 and Lag3. In the absence of Fas-mediated cell death, further gene

expression changes occur in *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells, including strongly increased expression of *Pdcd1* and *Lag3* as well as *Ill10* and *Pdcd112*. This suggests upregulation of a further regulatory program in *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells to control their function, since they continue to express high levels of mRNA for *Ifng*, *FasL*, *Gzmb*, and *Gzmk*. As wild-type mice express Fas on most tissues, the progressive upregulation of FasL could be particularly deleterious in wild-type mice. Consistent with this view are earlier findings that deletion of Fas selectively in T cells resulted in their increased FasL expression and an inflammatory fibrotic lung condition that was prevented with anti-FasL blocking antibody [50]. Moreover, Autoimmune Lymphoproliferation Syndrome (ALPS) patients [51, 52], who have inherited genetic defects in proteins that mediate lymphocyte apoptosis including Fas, manifest elevated levels of plasma FasL and IL-10 [53, 54].

Our studies also offer an explanation for the activated phenotype and distinctive cell cycle status of *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells. Previous genetic and functional studies have shown that *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells arise from CD8⁺ T cell precursors as a consequence of persistent homeostatic proliferation in conjunction with reduced cell death [35–38, 55] and not as a consequence of a defect in thymic negative selection nor the contraction of T cell expansion following acute infection [56–60]. Consistent with their origin from CD8⁺ T cells receiving chronic TCR stimulation, *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells have an activated phenotype that is characterized by high levels of active Fyn as well as the constitutive phosphorylation of a number of TCR signaling proteins including CD3ζ [61–63]. Yet, when examined *ex vivo*, *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells are not actively cycling [38, 64], and they do not proliferate following adoptive transfer into Rag1-deficient mice (K. A. F. unpublished data). This implies that their proliferation likely occurred immediately prior to the transition from CD8⁺ precursor to CD4⁻CD8⁻TCRαβ⁺ T cell and that this transition is rapidly followed by cell cycle arrest. A number of proteins could contribute to the cessation of cell cycling including reduced expression of IL-7Rα and increased expression of the cell cycle inhibitors Wee1 and Jdp2 as well as the inhibitory proteins PD-1 and Lag3. Lag3 limits T cell homeostatic proliferation and mice deficient for Lag3 have a two-fold increase in T cells compared with wild-type controls [65, 66]. Other proteins that may impact the proliferation and survival of CD4⁻CD8⁻TCRαβ⁺ T cells are the signaling lymphocyte activation molecule (SLAM) family members which act as important immunomodulatory receptors [67], as SLAMF7 expression is increased in this subset (Table I). Consistent with this, *Fas^{lpr/lpr}* mice bearing a mutation in the downstream signaling molecule for SLAM proteins, SLAM-associated protein (SAP), do not develop adenopathy or autoimmune disease [68]. The *SLAM* locus has also been linked with susceptibility to systemic lupus erythematosus [69, 70].

Progressive homeostatic proliferation may generate a subpopulation of CD8⁺ T cells, identified by high levels of CD44, PD-1, FasL, and IL-10 that could also function as immunosuppressive CD8⁺ T regulatory cells. This phenotype also provides an explanation for the previous observations showing that *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells are hyporesponsive to TCR stimulation *in vitro* and yet can suppress allogeneic skin grafts and are cytolytic *in vitro* [33, 71]. Thus, despite their hyporesponsiveness, they are not functionally inert. Of note in this regard, CD4⁺ T regulatory cells, like CD4⁻CD8⁻TCRαβ⁺

T cells, also manifest a high rate of homeostatic proliferation (K. A. F. unpublished observations) and express many of the same effector and inhibitory proteins as CD4⁺CD8⁺TCR $\alpha\beta$ ⁺ T cells [72]. Hence, our observations regarding the gene expression changes in homeostatically expanding CD8⁺ T cells may also apply to CD4⁺ T cells and, potentially to B cells, as it was recently observed that immunosuppressive IL-10-producing B cells preferentially repopulate the B cell pool following depletion with anti-CD20 Rituximab [73].

Our studies reveal that *Fas^{lpr/lpr}* CD4⁺CD8⁺TCR $\alpha\beta$ ⁺ T cells share features with the gene expression profile reported for antigen-specific exhausted T cells in chronic LCMV infection (Supplemental Fig. 2)[74, 75]. During chronic infections, persistent antigen stimulation results in global transcriptional changes including the progressive upregulation of a number of inflammatory (FasL, granzyme B) and inhibitory (PD-1, Lag3) proteins and reduced CD122 and CD127, leading to the loss of effector functions [76]. Homeostatic proliferation is similarly driven by chronic TCR stimulation from low affinity interactions with self-peptide/MHC combined with IL-7 and IL-15 in the absence of inflammation [1–5, 77–79]. Thus, *Fas^{lpr/lpr}* CD4⁺CD8⁺TCR $\alpha\beta$ ⁺ T cells may provide insight into a common gene expression profile used to regulate chronically stimulated T cells. In this regard, Fas also contributes to T cell deletion during chronic viral infection [57, 80, 81]. This suggests that chronic TCR stimulation leads to a unique cellular state in which the additional death signal provided by Fas is needed to eliminate unwanted, potentially inflammatory T cells.

Homeostatic proliferation makes a substantial contribution to both the neonatal immune system and maintenance of the T cell compartment with age [30, 82]. The functional changes that can result from persistent homeostatic expansion may provide a partial explanation for the reduced immune response to vaccination and increased susceptibility to infection observed in newborns and the elderly. Consistent with this, T cells in aged individuals have an increased portion of CD44^{high} T cells and inhibitory protein expression including PD-1 [83–85]. Augmented homeostatic proliferation may also be an underlying mechanism in the development of certain autoinflammatory syndromes. Autoinflammatory sequelae are associated with treatment-induced lymphopenia following chemotherapy and bone marrow transplants as well as following chronic viral infections [19, 20, 86]. Rapid T cell proliferation has also been observed in HIV-infected individuals, who can manifest a variety of autoinflammatory syndromes, such as psoriasis and psoriatic arthritis [18, 87]. Moreover, elevated rates of homeostatic expansion have been reported with autoimmune disorders including rheumatoid arthritis and systemic lupus erythematosus [8, 9, 88], and an increase in CD4⁺CD8⁺TCR $\alpha\beta$ ⁺ T cells has been observed in patients with systemic lupus erythematosus [9]. Our findings thus provide new insight into the process by which augmented T cell homeostatic expansion could lead to the two paradoxical clinical immunological scenarios of either immune deficiency complicated by autoimmune manifestations or, conversely, autoimmune disease in association with increased susceptibility to infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	allophycocyanin
BCS	bovine calf serum
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CFSE	5-(and 6-)-carboxyfluorescein diacetate succinimidyl ester
CR	conserved region
Cy	Cyanine
Fas	Fas cell surface death receptor, CD95, apoptosis antigen 1 (Apo-1)
FasL	Fas-ligand
FITC	Fluorescein isothiocyanate
FLAGTM	FLAG tag octapeptide DYKDDDDK
Hepes	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
Lag3	lymphocyte activating 3, CD223
Lpr	lymphoproliferation spontaneous mutation
MHC	major histocompatibility complex
NKT cell	natural killer T cell
PBS	phosphate buffered saline
PC	principal component
PD-1	programmed cell death protein 1, CD279
PE	phycoerythrin
PerCP	peridinin chlorophyll protein

RAG	recombination-activating gene
TCR	T cell receptor for antigen
w/v	weight to volume ratio (%)
v/v	volume to volume ratio (%)

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Highlights

CD8⁺ T cells undergoing homeostatic proliferation progressively upregulate cytolytic proteins

Homeostatically expanding CD8⁺ T cells also progressively increase inhibitory proteins

The findings could explain the clinical immunology paradox of simultaneous autoinflammatory and immunodeficient states

Figure 1. Increasing CD44 expression reflects T cell homeostatic proliferation

(A, B) CD44^{low}CD8⁺ T cells were isolated by positive selection from age and sex-matched 7-week old C57BL/6 wild-type and Fas-deficient (*Fas^{lpr/lpr}*) mice, labeled with CFSE, and then adoptively transferred to Rag-1-deficient recipients. Four days post-transfer, the donor CD8⁺ T cells were analyzed for proliferation by CFSE dilution and CD44 expression. (C, D) C57BL/6 wild-type and *Fas^{lpr/lpr}* mice (11 weeks old) received four intraperitoneal injections of BrdU over 24 h. Lymph node cells were stained for surface expression of CD4, CD8, CD44, CD45R (B220), and TCRβ, and analyzed for BrdU incorporation by flow

cytometry. The proportion of CD4⁻CD8⁻TCRαβ⁺ T cells in the *Fas^{lpr/lpr}* lymph nodes was 9.9±4.1% (mean±SD) (n=5). (C) Shown are representative histograms of BrdU incorporation for *Fas^{lpr/lpr}* CD8⁺ T cells subsets based on CD44 expression and *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells. (D) The mean and standard deviation for the percentage of BrdU⁺ cells in each T cell subset (n=5). The percentage of BrdU⁺ T cells in each T cell subset was significantly different from each of the other T cell subsets (ANOVA, Tukey post-test, p<0.05) except for the following pairs: WT-L:WT-I, WT-L:Lpr-L, WT-I:Lpr-L, WT-I:Lpr-I, and Lpr-L:Lpr-I where L=CD8⁺CD44^{low}, I=CD8⁺CD44^{intermediate}, H=CD8⁺CD44^{high}, and DN=CD4⁻CD8⁻TCRαβ⁺ T cells. (E) The mean forward scatter for CD8⁺CD44^{low}, CD8⁺CD44^{intermediate}, CD8⁺CD44^{high}, and CD4⁻CD8⁻TCRαβ⁺ T cells from C57BL/6 wild-type and *Fas^{lpr/lpr}* mice (n=3, 11 weeks old). Lines connect the T cell subsets from individual mice. CD4⁻CD8⁻TCRαβ⁺ T cells are significantly smaller than their respective CD8⁺CD44^{high} T cells (p<0.05, repeated measure ANOVA with multiple comparison post test). The proportion of CD4⁻CD8⁻TCRαβ⁺ T cells in the *Fas^{lpr/lpr}* lymph nodes was 27.4±8.0% (mean±SD).

Figure 2. Equivalent subsets of wild-type and *Fas*^{lpr/lpr} T cells manifest very similar gene expression profiles

(A) Freshly isolated lymph node cells from five 10-week old C57BL/6 wild-type mice were pooled and stained for CD4, CD8, TCR β , CD44, CD19, TCR $\gamma\delta$, and NKT tetramer. The CD8⁺TCR $\alpha\beta$ ⁺ subset was sorted into CD44^{low}, CD44^{intermediate}, and CD44^{high} populations. CD4⁻CD8⁻TCR $\alpha\beta$ ⁺ T cells were identified as TCR β ⁺ CD19⁻ TCR $\gamma\delta$ ⁻ NKT tetramer⁻. (B) Freshly isolated lymph node cells from three 10-week old C57BL/6 *Fas^{lpr/lpr}* mice were pooled and stained for CD4, CD8, TCR β , CD44, CD45R (B220), and NK1.1. The CD8⁺TCR $\alpha\beta$ ⁺ subset was sorted into CD44^{low}, CD44^{intermediate}, and CD44^{high} populations.

CD4⁻CD8⁻TCRαβ⁺ T cells were identified as TCRβ⁺, CD44⁺, CD45R⁺, and NK1.1⁻. The proportion of CD4⁻CD8⁻TCRαβ⁺ T cells in the *Fas^{lpr/lpr}* lymph nodes was 7.2±3.1% (mean ±SD). Sorted populations were of >97% purity upon reanalysis. RNA was extracted for microarray analysis using Affymetrix chips. (C) Heat map and dendrogram profile of three independent experiments (A, B, C) for wild-type and *Fas^{lpr/lpr}* CD8⁺CD44^{low} (Low), CD8⁺CD44^{intermediate} (Int), CD8⁺CD44^{high} (High), and CD4⁻CD8⁻TCRαβ⁺ (DN) T cells. Sample order was determined by the variation between samples as shown in the dendrogram. (D) Histogram of the first ten principal components showing that 87% of the variability was contained within the first three principal components. (E) Plot of principal components (PC) 1 and 2 suggests a progression of gene expression from CD8⁺CD44^{low} to CD8⁺CD44^{intermediate} to CD8⁺CD44^{high} to wild-type CD4⁻CD8⁻TCRαβ⁺ T cells and a significant departure of the *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells from the CD8⁺ T cell subsets. The CD8⁺CD44^{low} subset for each sample set was used as a reference point and centered at (0,0) and hence is not shown. I=CD8⁺CD44^{intermediate}, H=CD8⁺CD44^{high}, and DN=CD4⁻CD8⁻TCRαβ⁺ T cells. Open symbols represent wild-type T cells and closed symbols represent *Fas^{lpr/lpr}* T cells (n=3 for each subset). (F) PC1 and PC3 reveal the shared gene expression changes of wild-type and *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells compared with the collective CD8⁺ T cell subsets. (G) PC2 and PC3 show differences between wild-type and *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells.

Figure 3. Gene expression patterns reveal striking changes in gene expression as CD8⁺ T cells undergo progressive rounds of homeostatic proliferation

The most highly differentially expressed probe sets (3,343) were segregated into eight clusters defined by their gene expression patterns. Shown are the plots of the consensus expression profile for each cluster across the CD8⁺CD44^{low} (L), CD8⁺CD44^{intermediate} (I), CD8⁺CD44^{high} (H), and CD4[−]CD8[−]TCRαβ⁺ T cells (DN). The scores of the CD8⁺CD44^{low} T cell subsets were set at 0 as a reference point. Open circles represent C57BL/6 wild-type T cells and closed circles represent C57BL/6 *Fas*^{*lpr/lpr*} T cells (n=3 for each subset). (A, E) Two consensus profiles showed a step-wise progression, up or down, of gene expression from CD8⁺CD44^{low} to CD8⁺CD44^{intermediate} to CD8⁺CD44^{high} to CD4[−]CD8[−]TCRαβ⁺ T cells. (B, F) Gene expression was upregulated as CD8⁺ T cells became CD44^{high} and then underwent further change, up or down, in *Fas*^{*lpr/lpr*} CD4[−]CD8[−]TCRαβ⁺ T cells. (C, D, G) Gene expression profiles changed dramatically between wild-type and *Fas*^{*lpr/lpr*} CD4[−]CD8[−]TCRαβ⁺ T cells. (H) A small subset of genes was upregulated selectively in wild-type CD4[−]CD8[−]TCRαβ⁺ T cells. Numbers are the number of genes in each of the eight clusters.

Figure 4. Progressive upregulation of effector and inhibitory gene expression with continued homeostatic proliferation

The relative change in expression of each gene in CD8⁺CD44^{intermediate}, CD8⁺CD44^{high}, and CD4⁺CD8⁺TCRαβ⁺ T cells from C57BL/6 wild-type and *Fas^{Lpr/lpr}* mice was calculated compared with expression of their respective CD8⁺CD44^{low} T cell subset for each of the three independent sample sets. Expression in each CD8⁺CD44^{low} T cell subset was set to 1. Shown is the mean and standard deviation of the fold-change in expression of (A) *Eomes*, (B) *Tbx21* (Tbet), (C) *Wee1*, (D) *Jdp2*, (E) *Casp3*, (F) *Il7r*, (G) *FasL*, (H) *Gzmb*, (I) *Ifng* (J) *Klrl1* (Nkg2d), (K) *Cd160*, (L) *Lag3*, (M) *Pdcd1* (PD-1), (N) *Pdcd1lg2* (PD-L2), and (O) *Il10*.

III0. L= CD8⁺CD44^{low}, I=CD8⁺CD44^{intermediate}, H=CD8⁺CD44^{high}, and DN=CD4⁻CD8⁻TCRαβ⁺ T cells.

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Figure 5. *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells manifest spontaneous cytolytic activity
 (A, B) C57BL/6 *Fas^{lpr/lpr}* CD8⁺ and CD4⁻CD8⁻TCRαβ⁺ T cells and wild-type CD8⁺ T cells were isolated from lymph nodes by negative selection and whole cell lysates were analyzed by Western blot for (A) FasL and (B) Granzyme B. Shown in the upper panels are representative membranes for FasL, granzyme B, and tubulin loading control. Bar graphs below are the normalized western blot fluorescence for FasL and granzyme B (n=3 for each subset). Cells were obtained from 10-12 week old mice. The proportion of CD4⁻CD8⁻TCRαβ⁺ T cells in the *Fas^{lpr/lpr}* lymph nodes was 44.4±12.2% (mean±SD). (C) C57BL/6 *Fas^{lpr/lpr}* CD4⁻CD8⁻TCRαβ⁺ T cells and wild-type CD8⁺ T cells were isolated from lymph nodes of 10-12 week old mice by negative selection. The proportion of CD4⁻CD8⁻TCRαβ⁺ T cells in the *Fas^{lpr/lpr}* lymph nodes was 28.3±3.6% (mean±SD). Freshly isolated T cells were cocultured with ⁵¹Cr-labeled Jurkat T cells at an effector:target ratio of 10:1, and ⁵¹Cr released into the supernatant was measured after 4 h. Specific lysis was calculated by first subtracting the cpm from spontaneous release by the target cells alone and then the percentage of maximal lysis was determined based on lysis of Jurkat T cells with cross-linked soluble FasL. Shown are the mean and standard deviation of three independent experiments.

Figure 6. Increased expression of inhibitory proteins, PD-1 and Lag3, with recurrent homeostatic proliferation

C57BL/6 wild-type (A) and *Fas^{lpr/lpr}* (B, C) lymph node cells from 11-12 week old mice were stained for surface expression of CD4, CD8, CD44, CD45R (B220), and TCRβ, and analyzed by flow cytometry for expression of (A, B) PD-1 and (C) Lag3. Shown below each representative histogram is a graph of the mean and standard deviation (n=3) of the mean fluorescence intensity (MFI) of each protein by T cell subset. The proportion of CD4⁺CD8⁺TCRβ⁺ T cells in the *Fas^{lpr/lpr}* lymph nodes was 27.6±7.6% (mean±SD). (D) Methylation status of *Pcdcl* conserved region B (CR-B) and CR-C in genomic DNA from

13-week old C57BL/6 *Fas^{lpr/lpr}* CD8⁺ and CD4⁻CD8⁻TCRαβ⁺ T cells was determined using bisulfite sequencing. The proportion of CD4⁻CD8⁻TCRαβ⁺ T cells in the *Fas^{lpr/lpr}* lymph nodes was 27.5±1.7% (mean±SD). Filled circles indicate methylated cytosines. Open circles indicate nonmethylated cytosines. Each row represents an individual clone picked for sequencing. Clones were selected from three independent T cell subset isolations. Columns are CpG sites in CR-C (at -1280, -1158, -1099, -1069, -986) and CR-B (at -778, -672, -667, -636, -612, -535, -496, -491, and -465).

Table 1
Gene expression of wild-type and *Fas^{lpr/lpr}* CD4⁺CD8⁺TCRαβ⁺ T cells compared with their combined CD8⁺ T cell subsets.

Symbol	Entrez GeneID	UID	Pattern	DNmHIL				DNmHIL			
				FoldChange	p-value	FDR	Wild-type	FoldChange	p-value	FDR	Wild-type
Alr	11622	1422631_at	Fig. 3H	3.544	1.97E-07	1.91E-05	1.91E-05	-4.388	4.89E-07	1.93E-05	1.93E-05
Bcl2	12043	1443837_x_at	Fig. 3G	0.390	4.76E-02	1.04E-01	1.04E-01	-1.478	2.85E-05	3.12E-04	3.12E-04
Bcl2	12043	1437122_at	Fig. 3G	0.219	3.40E-03	1.21E-02	1.21E-02	-1.480	5.62E-07	2.10E-05	2.10E-05
Bcl2	12043	1440770_at	Fig. 3G	0.131	5.65E-01	6.84E-01	6.84E-01	-1.366	5.11E-04	2.86E-03	2.86E-03
Casp3	12367	1449839_at	Fig. 3A	1.401	1.19E-06	5.13E-05	5.13E-05	3.347	2.35E-08	3.37E-06	3.37E-06
Casp3	12367	1426165_a_at	Fig. 3A	1.030	3.85E-04	2.19E-03	2.19E-03	3.476	5.41E-07	2.06E-05	2.06E-05
Ccna2	12428	1417911_at	Fig. 3B	-0.473	2.64E-03	9.89E-03	9.89E-03	1.988	5.63E-05	5.22E-04	5.22E-04
Ccna2	12428	1417910_at	Fig. 3B	-0.628	4.42E-03	1.50E-02	1.50E-02	1.800	1.76E-04	1.25E-03	1.25E-03
Ccnb2	12442	1450920_at	Fig. 3B	-0.723	6.21E-03	1.98E-02	1.98E-02	2.762	3.74E-06	7.33E-05	7.33E-05
Ccr2	12772	1421187_at	Fig. 3F	2.960	3.59E-05	3.92E-04	3.92E-04	-0.259	4.66E-01	5.99E-01	5.99E-01
Ccr2	12772	1421188_at	Fig. 3F	2.342	1.48E-04	1.06E-03	1.06E-03	0.599	2.66E-02	6.75E-02	6.75E-02
Ccr2	12772	1421186_at	Fig. 3B	4.513	6.71E-07	3.68E-05	3.68E-05	1.441	3.42E-03	1.30E-02	1.30E-02
Ccr5	12774	1424727_at	Fig. 3B	3.673	1.46E-06	5.68E-05	5.68E-05	3.449	2.73E-05	3.03E-04	3.03E-04
Ccr5	12774	1422259_a_at	Fig. 3B	1.883	2.73E-05	3.25E-04	3.25E-04	2.993	7.38E-07	2.54E-05	2.54E-05
Ccr5	12774	1422260_x_at	Fig. 3B	1.083	1.60E-03	6.63E-03	6.63E-03	2.112	6.20E-06	1.04E-04	1.04E-04
Cd160	54215	1420066_s_at	Fig. 3G	1.106	5.07E-07	3.19E-05	3.19E-05	-3.918	5.95E-07	2.18E-05	2.18E-05
Cd160	54215	1420396_at	Fig. 3G	1.058	3.84E-07	2.74E-05	2.74E-05	-2.941	6.55E-09	1.80E-06	1.80E-06
Cd160	54215	1420067_at	Fig. 3G	1.045	8.09E-07	4.09E-05	4.09E-05	-3.649	5.02E-09	1.65E-06	1.65E-06
Cd160	54215	1456847_at	Fig. 3G	0.581	7.58E-05	6.57E-04	6.57E-04	-2.275	7.44E-06	1.18E-04	1.18E-04
Cd2	12481	1418770_at	Fig. 3G	-0.012	6.75E-01	7.73E-01	7.73E-01	-2.356	7.56E-09	1.88E-06	1.88E-06
Cd44	12505	1452483_a_at	Fig. 3B	3.347	1.22E-08	6.42E-06	6.42E-06	2.804	7.89E-07	2.63E-05	2.63E-05
Cd44	12505	1434376_at	Fig. 3B	3.112	7.45E-08	1.21E-05	1.21E-05	2.750	6.51E-07	2.33E-05	2.33E-05
Cd44	12505	1423760_at	Fig. 3B	2.850	1.78E-07	1.80E-05	1.80E-05	2.532	6.03E-07	2.20E-05	2.20E-05
Cd8a	12525	1444078_at	Fig. 3G	-4.139	2.30E-08	7.69E-06	7.69E-06	-7.589	5.14E-10	5.51E-07	5.51E-07
Cd8a	12525	1440164_x_at	Fig. 3E	-3.344	3.39E-09	4.32E-06	4.32E-06	-4.113	3.55E-07	1.58E-05	1.58E-05
Cd8a	12525	1425335_at	Fig. 3E	-3.819	4.44E-08	9.40E-06	9.40E-06	-6.050	1.16E-10	2.49E-07	2.49E-07
Cd8a	12525	1440811_x_at	Fig. 3E	-4.753	3.41E-11	1.54E-06	1.54E-06	-6.985	8.90E-11	2.49E-07	2.49E-07

Symbol	Entrez GenID	UID	Pattern	DNmHIL FoldChange Wild-type	DNmHIL p-value Wild-type	DNmHIL FDR Wild-type	DNmHIL FoldChange Fas ^{lpr/lpr}	DNmHIL p-value Fas ^{lpr/lpr}	DNmHIL FDR Fas ^{lpr/lpr}
Cd8a	12525	1451673_at	Fig. 3E	-5.336	2.55E-08	8.17E-06	-7.865	5.73E-12	5.17E-08
Cxcr3	12766	1449925_at	Fig. 3B	1.683	7.08E-08	1.18E-05	0.963	3.17E-04	1.97E-03
E2f2	242705	1436434_at	Fig. 3C	-1.347	1.99E-05	2.65E-04	2.124	1.80E-07	1.06E-05
Eomes	13813	1426001_at	Fig. 3B	1.791	5.68E-07	3.34E-05	2.390	4.74E-08	4.97E-06
Eomes	13813	1435172_at	Fig. 3B	1.644	1.92E-07	1.88E-05	2.059	1.70E-07	1.03E-05
FasI	14103	1449235_at	Fig. 3B	3.688	1.86E-08	7.02E-06	5.023	2.06E-07	1.16E-05
FasI	14103	1418803_a_at	Fig. 3B	3.288	4.21E-08	9.22E-06	4.923	5.05E-07	1.98E-05
Gzma	14938	1417898_a_at	Fig. 3B	2.015	2.48E-04	1.57E-03	-0.215	3.88E-01	5.26E-01
Gzmb	14939	1419060_at	Fig. 3B	2.054	4.10E-05	4.28E-04	3.525	5.00E-07	1.96E-05
Gzmc	14940	1421256_at	Fig. 3A	3.102	1.67E-06	6.11E-05	1.067	1.57E-02	4.43E-02
Gzmk	14945	1422280_at	Fig. 3B	1.764	4.89E-05	4.83E-04	5.083	6.02E-07	2.20E-05
Gzmm	16904	1449501_a_at	Fig. 3F	-0.130	3.04E-01	4.37E-01	-2.006	2.51E-06	5.58E-05
Ifng	15978	1425947_at	Fig. 3B	2.665	3.55E-07	2.65E-05	3.099	5.28E-06	9.28E-05
Il10	16153	1450330_at	Fig. 3C	1.181	1.21E-04	9.18E-04	4.832	2.95E-06	6.23E-05
Il2rb	16185	1417546_at	Fig. 3F	1.583	5.43E-07	3.28E-05	-0.372	2.52E-03	1.01E-02
Il2rb	16185	1448759_at	Fig. 3F	1.370	3.67E-08	8.65E-06	-0.378	7.03E-04	3.66E-03
Il7r	16197	1448576_at	Fig. 3G	0.139	4.27E-02	9.51E-02	-7.303	5.00E-09	1.65E-06
Il7r	16197	1448575_at	Fig. 3G	0.032	3.89E-01	5.24E-01	-7.719	3.76E-13	1.43E-08
Igfa1	109700	1439713_at	Fig. 3F	4.952	1.16E-07	1.54E-05	-0.562	2.58E-02	6.60E-02
Igfa4	16401	1427615_at	Fig. 3H	2.082	1.35E-06	5.46E-05	1.215	2.49E-05	2.82E-04
Igfa4	16401	1456498_at	Fig. 3D	1.019	6.23E-05	5.72E-04	1.032	6.33E-04	3.38E-03
Igfa4	16401	1421194_at	Fig. 3D	0.691	1.70E-05	2.41E-04	1.526	1.29E-05	1.75E-04
Igav	16410	1432678_at	Fig. 3A	2.597	3.58E-06	9.34E-05	2.914	7.67E-08	6.59E-06
Igav	16410	1432296_a_at	Fig. 3A	1.593	4.32E-05	4.43E-04	2.438	1.42E-05	1.87E-04
Igav	16410	1421198_at	Fig. 3C	0.748	6.07E-04	3.11E-03	2.645	8.10E-07	2.68E-05
Jdp2	81703	1450350_a_at	Fig. 3B	3.281	3.38E-08	8.65E-06	5.714	3.74E-08	4.34E-06
Klrg1	50928	1420788_at	Fig. 3F	0.688	3.49E-03	1.23E-02	-1.026	4.06E-04	2.39E-03
Klrl1	27007	1450495_a_at	Fig. 3F	5.568	1.53E-08	6.46E-06	-1.855	4.28E-05	4.23E-04
Lag3	16768	1449911_at	Fig. 3B	0.647	9.07E-04	4.24E-03	5.189	1.15E-07	8.10E-06
Nfatc1	18018	1447084_at	Fig. 3A	1.548	2.21E-06	7.05E-05	1.848	4.13E-06	7.84E-05

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Symbol	Entrez GenID	UID	Pattern	DNmHIL FoldChange Wild-type	DNmHIL p-value Wild-type	DNmHIL FDR Wild-type	DNmHIL FoldChange Fas ^{lpr/lpr}	DNmHIL p-value Fas ^{lpr/lpr}	DNmHIL FDR Fas ^{lpr/lpr}
Nfacc1	18018	1447085_s_at	Fig. 3A	1.501	8.84E-07	4.32E-05	1.767	2.47E-06	5.52E-05
Nfaic1	18018	1425761_a_at	Fig. 3A	0.828	1.19E-05	1.91E-04	2.369	2.00E-07	1.14E-05
Pdcd1	18566	1449835_at	Fig. 3A	1.251	4.69E-04	2.56E-03	4.343	1.30E-07	8.70E-06
Pdcd1lg2	58205	1450290_at	Fig. 3B	1.168	1.25E-05	1.97E-04	2.726	5.81E-06	9.92E-05
Prdm1	12142	1420425_at	Fig. 3F	0.828	2.29E-03	8.81E-03	0.357	3.54E-01	4.92E-01
Slamf7	75345	1453472_a_at	Fig. 3B	2.393	4.46E-07	2.95E-05	5.158	1.50E-07	9.53E-06
Socs3	12702	1416576_at	Fig. 3H	1.238	1.15E-07	1.53E-05	-2.250	4.40E-06	8.19E-05
Socs3	12702	1456212_x_at	Fig. 3G	1.146	1.88E-05	2.57E-04	-2.357	1.61E-06	4.10E-05
Socs3	12702	1455899_x_at	Fig. 3G	1.125	1.51E-05	2.22E-04	-2.443	4.12E-07	1.72E-05
Tbx21	57765	1449361_at	Fig. 3B	2.394	2.75E-07	2.31E-05	0.186	3.40E-01	4.79E-01
Thy1	21838	1423135_at	Fig. 3G	-1.133	1.83E-05	2.52E-04	-7.347	2.57E-10	3.67E-07
Wee1	22390	1416774_at	Fig. 3A	0.336	5.35E-04	2.83E-03	2.334	7.07E-06	1.14E-04
Wee1	22390	1416773_at	Fig. 3B	1.275	6.44E-07	3.59E-05	1.970	4.52E-08	4.90E-06

Shown are the fold-change, p-value, and False Discovery Rate (FDR) for wild-type and Fas^{lpr/lpr} CD4⁻CD8⁻TCRαβ⁺ T cells compared with their respective combined CD8⁺ T cell subsets (DNmHIL). L=CD8⁺CD44^{low}, I=CD8⁺CD44^{intermediate}, H=CD8⁺CD44^{high}, and DN=CD4⁻CD8⁻TCRαβ⁺ T cells. Patterns correspond to the gene patterns shown in Fig 4: Fig. 4A, progressive increase; Fig. 4B, increased at H, then further increase in Fas^{lpr/lpr} DN; Fig. 4C, increased in Fas^{lpr/lpr} DN; Fig. 4D, increased in Fas^{lpr/lpr} DN; Fig. 4E, progressive decrease; Fig. 4F, increased at H, then decreased in Fas^{lpr/lpr} DN; Fig. 4G, decreased in Fas^{lpr/lpr} DN; Fig. 4H, increased in wild-type DN.