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Fas (CD95/APO-1) limits the expansion of T lymphocytes in an environment of limited T-cell antigen receptor/MHC contacts

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Abstract

Fas-deficient mice (*Fas*$^{lpr/lpr}$) and humans have profoundly dysregulated T lymphocyte homeostasis, which manifests as an accumulation of CD4$^+$ and CD8$^+$ T cells as well as an unusual population of CD4$^-$CD8$^-$TCRαβ$^+$ T cells. To date, no unifying model has explained both the increased T-cell numbers and the origin of the CD4$^-$CD8$^-$TCRαβ$^+$ T cells. As *Fas*$^{lpr/lpr}$ mice raised in a germ-free environment still manifest lymphadenopathy, we considered that this process is primarily driven by recurrent low-avidity TCR signaling in response to self-peptide/MHC as occurs during homeostatic proliferation. In these studies, we developed two independent systems to decrease the number of self-peptide/MHC contacts. First, expression of MHC class I was reduced in OT-I TCR transgenic mice. Although OT-I *Fas*$^{lpr/lpr}$ mice did not develop lymphadenopathy characteristic of *Fas*$^{lpr/lpr}$ mice, in the absence of MHC class I, OT-I *Fas*$^{lpr/lpr}$ T cells accumulated as both CD8$^+$ and CD4$^-$CD8$^-$ T cells. In the second system, re-expression of β_2m limited to thymic cortical epithelial cells of *Fas*$^{lpr/lpr}$ β_2m-deficient mice yielded a model in which polyclonal CD8$^+$ thymocytes entered a peripheral environment devoid of MHC class I. These mice accumulated significantly greater numbers of CD4$^-$CD8$^-$TCRαβ$^+$ T cells than conventional *Fas*$^{lpr/lpr}$ mice. Thus, Fas shapes the peripheral T-cell repertoire by regulating the survival of a subset of T cells proliferating in response to limited self-peptide/MHC contacts.

Keywords: apoptosis, *Fas*$^{lpr/lpr}$ mice, homeostasis, lymphopenia

Introduction

The number of T cells in the peripheral lymphoid compartment is remarkably stable over time despite the continual export of thymocytes to the periphery where they undergo low-level homeostatic proliferation. The daily production of new T cells from the thymus combined with peripheral T-cell expansion would result in a continual increase in T-cell numbers if they were not balanced by the active removal of existing T cells.

The survival and homeostatic expansion of peripheral T cells require the engagement of TCR with self-peptide/MHC and cytokine receptor-mediated signals (1–7). Experimentally, homeostatic proliferation of T cells has been examined by transferring small numbers of T cells into irradiated or genetically lymphopenic mice. However, only a portion of polyclonal T cells proliferate following transfer to lymphopenic hosts (1, 8). This suggests that considerable heterogeneity exists in the capacity to undergo homeostatic proliferation. Further studies have suggested that the proliferative capacity of a specific T cell is determined by the TCR affinity for self-peptide/MHC complexes as well as the avidity or density of self-peptide/MHC complexes (9, 10). This is supported by studies using TCR transgenic T cells whose proliferation rate varies considerably among T cells expressing different TCR (1, 2, 7,11–13). For example, OT-I CD8$^+$ T cells proliferated robustly following adoptive transfer into syngeneic lymphopenic recipients, whereas proliferation of H-Y CD8$^+$ T cells was minimal (1, 2, 14). In addition, some TCRs have been shown to interact with a broad spectrum of self-peptide/MHC complexes, including both MHC class I and class II, thereby enhancing the number of complexes that can provide proliferative signals (15–19).

The factors that limit the homeostatic expansion of peripheral T cells have not been well characterized. It has been

suggested that T-cell expansion in lymphopenic recipients may be restricted by competition for limiting resources, including IL-7 and access to antigen-presenting cells bearing appropriate self-peptide/MHC (1,20–22). However, a significant portion of transferred T cells continues to proliferate as measured by 5-bromo-2-deoxyuridine (BrdU) incorporation even after stable numbers of T cells have been achieved (23, 24). This suggests that it is not entirely limitations of proliferation but also active cell death that prevents further increase in T-cell number.

The death receptor, Fas, has a prominent role in the regulation of the expansion of peripheral T cells in response to self-peptide/MHC during T-cell homeostasis. We have previously observed that T cells undergoing lymphopenia-induced proliferation express Fas and are sensitive to Fas-mediated cell death (23). Following transfer into lymphopenic hosts, Fas-deficient T cells accumulated to substantially greater numbers compared with wild-type Fas^+ T cells, despite equivalent rates of cell cycle entry (23). Thus, proliferation to self-antigen/MHC, like foreign antigen-driven proliferation, is regulated by active cell death.

Fas-deficient *lpr* ($Fas^{lpr/lpr}$) mice manifest a profound age-dependent lymphadenopathy that includes both $CD44^{high}$ $CD4^+$ and $CD44^{high}$ $CD8^+$ T cells as well as an unusual population of polyclonal $CD4^-CD8^-TCR\alpha\beta^+$ T cells that express the B-cell isoform of CD45, CD45R (B220), and lack NK1.1 (25). Although the $Fas^{lpr/lpr}$ genotype was originally identified over 18 years ago as a retroposon disruption of the *fas* gene (26), the source and explanation for the age-dependent lymphadenopathy in $Fas^{lpr/lpr}$ mice have remained en enigma for many years. Little, if any, significant defect in thymic negative selection has been identified in $Fas^{lpr/lpr}$ mice based on deletion by endogenous or exogenous superantigens (27–32). Whereas Fas regulates antigen-mediated deletion *in vitro*, most studies support the view that there is little delay in deletion of $Fas^{lpr/lpr}$ T cells in response to antigens administered as exogenous proteins or following acute infections (33–35). However, Fas does contribute to the deletion of T cells during chronic infections (33, 36). The phenotype of $Fas^{lpr/lpr}$ T cells differs from that of T cells stimulated by exogenous or cognate antigen and bears a striking resemblance to T cells undergoing lymphopenia-induced proliferation, which are also $CD44^{high}CD25^-CD69^-$ (13, 37). Collectively, these observations support the view that the pronounced lymphadenopathy and phenotype of $Fas^{lpr/lpr}$ T cells are more likely driven by self-antigens during homeostatic proliferation than by foreign antigens. This is consistent with earlier findings that $Fas^{lpr/lpr}$ mice raised under germ-free and antigen-free conditions still develop lymphadenopathy (38) and that a substantial proportion of T cells in $Fas^{lpr/lpr}$ mice cycle in a 24-h period even in the absence of foreign antigen stimulation (23).

Little is known regarding what distinguishes the subset of T cells that is preserved in the absence of Fas. The accumulating $Fas^{lpr/lpr}$ T cells are clearly polyclonal and studies of their TCR-Vβ repertoire have revealed only subtle shifts from wild-type T cells (25, 39). However, $Fas^{lpr/lpr}$ T cells bearing monoclonal TCRαβ transgenes do not accumulate with age, suggesting that not all TCR specificities are preserved in the absence of Fas (40–43) (K. A. Fortner, unpublished observations). Thus, it is not clear whether the accumulating T cells in $Fas^{lpr/lpr}$ mice represent a selected repertoire. $Fas^{lpr/lpr}$ $CD4^-CD8^-TCR\alpha\beta^+$ T cells likely derive from $CD8^+$ T-cell precursors based on studies showing demethylation of the CD8α gene in $Fas^{lpr/lpr}$ $CD4^-CD8^-TCR\alpha\beta^+$ T cells (44). Consistent with this origin, β_2m-deficient ($\beta_2m^{o/o}$) $Fas^{lpr/lpr}$ mice, which are unable to positively select $CD8^+$ T cells, are nearly devoid of $CD4^-CD8^-TCR\alpha\beta^+$ T cells (45–47). In addition, lymphopenia-induced proliferation of $CD8^+$ T cells, but not $CD4^+$ T cells, generated $CD4^-CD8^-TCR\alpha\beta^+$ T cells (48). However, since $Fas^{lpr/lpr}$ mice accumulate $CD8^+$ T cells as well as $CD4^-CD8^-TCR\alpha\beta^+$ T cells, not all $CD8^+$ T cells may be precursors of $CD4^-CD8^-TCR\alpha\beta^+$ T cells.

TCR avidity to self-peptide/MHC is a critical factor that determines whether a T cell survives and proliferates. We considered the possibility that TCR avidity might also influence susceptibility to Fas-mediated cell death. If the TCR stimulation by self-peptide/MHC that drives homeostatic proliferation is a lower avidity interaction than occurs with exogenous antigen, then we reasoned that the absence of Fas preferentially favors the accumulation of T cells encountering limited MHC/peptide contacts. To test this model, we developed two systems to reduce the TCR contacts of $CD8^+$ T cells in Fas-deficient environments. First, we analyzed T-cell subsets in OT-I and OT-I $Fas^{lpr/lpr}$ mice in the presence or absence of MHC class I. OT-I $Fas^{lpr/lpr}$ mice did not manifest lymphadenopathy nor accumulation of $CD4^-CD8^-TCR\alpha\beta^+$ T cells. However, in a peripheral environment lacking MHC class I, OT-I $Fas^{lpr/lpr}$ T cells accumulated as both $CD8^+$ and $CD4^-CD8^-V\alpha2^+$ T cells. In the second system, we examined polyclonal $CD8^+$ T-cell responses using $Fas^{lpr/lpr}\beta_2m^{o/o}$ in which β_2m was re-expressed in thymic epithelium. In these mice, $CD8^+$ thymocytes entered a peripheral environment devoid of MHC class I. In this case, $CD4^-CD8^-TCR\alpha\beta^+$ T cells accumulated to a level considerably higher than conventional $Fas^{lpr/lpr}$ mice. These data support the model that Fas limits the expansion of T cells cycling in response to limited TCR interactions.

Methods

Mice

Mice were bred and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facilities of the University of Vermont College of Medicine and the Ludwig Institute for Cancer Research. Original breeding pairs of C57BL/6J, B6.MRL-Fas^{lpr}/J ($Fas^{lpr/lpr}$), B6.129S7-$Rag1^{tm1Mom}$/J [*Rag1*-deficient ($Rag1^{o/o}$)], B6.PL-$Thy1^a$/CyJ (CD90.1), B6.129P2-β_2m^{tm1Unc}/J ($\beta_2m^{o/o}$) and B6.CB17-$Prkdc^{scid}$/*SzJ* (*scid*) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). OT-I mice bear a transgenic TCR that recognizes chicken ovalbumin peptide 257–264 (SIINFEKL) restricted to MHC class I $H\text{-}2K^b$ and were kindly provided by Drs Francis Carbone and Michael Bevan (49). OT-I mice were maintained by breeding transgenic TCR heterozygotes with wild-type C57BL/6 mice. Offspring were screened for clonotype TCR expression on peripheral blood lymphocytes (PBL) by flow cytometry using

anti-Vα2 mAb. Breeding of OT-I mice with C57BL/6 *Fas*$^{lpr/lpr}$ mice generated OT-I *Fas*$^{lpr/lpr}$ mice. Offspring were screened for the *lpr* mutation by PCR as described previously (46) and for clonotype TCR expression on PBL. Breeding of CD90.1 mice with OT-I and OT-I *Fas*$^{lpr/lpr}$ mice generated CD90.1 OT-I and CD90.1 OT-I *Fas*$^{lpr/lpr}$ mice. Breeding of *scid* mice to $\beta_2m^{o/o}$ mice generated $\beta_2m^{o/o}$*scid* mice. Offspring were screened for the *scid* and β_2m mutations by PCR according to the protocols from the Jackson Laboratory Genotyping Lab (Bar Harbor, MA, USA). OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}$ $\beta_2m^{o/o}$ mice were generated by breeding, respectively, OT-I and OT-I *Fas*$^{lpr/lpr}$ mice to $\beta_2m^{o/o}$ mice. K14-β_2m transgenic mice express β_2m under the control of the human keratin 14 (K14) promoter and are maintained on a $\beta_2m^{o/o}$ background (50). K14-β_2m *Fas*$^{lpr/lpr}$$\beta_2m^{o/o}$ mice were derived by breeding *Fas*$^{lpr/lpr}$$\beta_2m^{o/o}$ mice with K14-β_2m $\beta_2m^{o/o}$ mice. Offspring were screened for the *lpr* mutation by PCR and for the K14-β_2m transgene by analyzing PBL for CD8$^+$ T cells using flow cytometry. All animal studies were conducted in accordance with the policies of the University of Vermont's Animal Care and Use Committee and the Veterinary Services of the Canton of Vaud.

Adoptive transfer of lymphocytes

Lymph node cells (5×10^6) from 5-week-old CD90.1 OT-I mice or the equivalent number of total CD8$^+$ T cells from age- and sex-matched CD90.1 OT-I *Fas*$^{lpr/lpr}$ mice were transferred intravenously via the tail vein into *Rag1*$^{o/o}$ mice.

To assess entry into cell cycle following adoptive transfer, donor cells were labeled with 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen/Molecular Probes, Carlsbad, CA, USA) prior to transfer. Lymph node cells were washed with PBS containing 0.1% bovine serum albumin (BSA) (PBS/0.1 % BSA), resuspended at 10^7 cells ml^{-1} and incubated with 4 μM CFSE for 10 min at 37°C. Labeling was stopped by addition of ice cold PBS/0.1 % BSA. The cells were washed three times with PBS/0.1 % BSA and resuspended in PBS for adoptive transfer.

To measure *in vivo* proliferation, mice received four intraperitoneal injections of 1 mg BrdU (100 μl of 10 mg ml^{-1} BrdU in sterile PBS) (Sigma, St Louis, MO, USA) during the 24 h prior to tissue harvest. Three injections were given on the day prior to tissue harvest and one injection on the day of sacrifice 1 hour prior to tissue harvest.

Reagents and antibodies

The following mAbs to murine cell surface molecules were purchased from Invitrogen/Caltag Laboratories (Carlsbad, CA, USA): PE-conjugated CD45R (B220), FITC-conjugated CD44, PE-conjugated CD44, TRI-COLOR-conjugated CD4, TRI-COLOR-conjugated CD8α, PE-Cy5.5-conjugated CD4, PE-Cy5.5-conjugated CD8α, PE-Texas-Red-conjugated CD8α and PE-Texas-Red-conjugated CD4. The following antibodies were purchased from BD Biosciences (San Jose, CA, USA): FITC-conjugated TCRβ, PE-conjugated TCRβ, allophycocyanin-conjugated TCRβ, FITC-conjugated Vα2, PE-conjugated Vα2, allophycocyanin-conjugated Vα2, FITC-conjugated CD90.1, PE-conjugated CD5, allophycocyanin-Cy7-conjugated CD11b, FITC-conjugated Vα3.2, FITC-conjugated Vα11.1/11.2, FITC-conjugated Vα8.3 and FITC-conjugated anti-BrdU. Lyophilized rat IgG and hamster IgG (ICN/Cappel, Costa Mesa, CA, USA) were resuspended in PBS and stored at −80°C.

Lymphocyte preparation

Single-cell suspensions of spleen and lymph nodes were prepared in RPMI 1640 (Mediatech, Inc., Herndon, VA, USA) containing 25 mM Hepes, 5% (v/v) bovine calf serum (BCS), 5×10^{-5} M β-mercaptoethanol, 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin (RPMI/5% BCS). Erythrocytes in splenic suspensions were lysed with Geys solution.

Cell recovery was calculated from the percentage of CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$TCRαβ$^+$ T cells obtained by flow cytometry and the absolute number of cells obtained. For each *Rag1*$^{o/o}$ recipient, the spleen and eight lymph nodes (inguinal, brachial, axillary and popliteal) were harvested.

Flow cytometry

For direct staining, single-cell suspensions (10^7 cells ml^{-1}) were washed with cold (4°C) PBS containing 0.02% (w/v)sodium azide (Sigma) (PBS/azide). The cells were incubated with the appropriate antibodies in PBS containing 1% (w/v) BSA fraction V (PBS/1% BSA) (Sigma) for 30 min at 4°C. After washing with cold PBS/azide, the cells were fixed with 1% (v/v) methanol-free formaldehyde (Ted Pella Inc., Redding, CA, USA) in PBS/azide and stored at 4°C until analysis. Flow cytometry was performed on a Coulter Epics Elite Flow Cytometer (Coulter Corp., Hialeah, FL, USA) or a LSRII (BD Biosciences) calibrated with DNA check beads.

Staining for DNA-incorporated BrdU was performed using a modification of a previously described method (23, 51). Single-cell suspensions from BrdU-pulsed mice were stained for TCRβ, CD4 and CD8 expression using antibodies in PBS/1% BSA for 30 min at 4°C and then washed with cold (4°C) PBS. The cells were fixed for 30 min on ice following the addition of 350 μl 70% ethanol (−20°C) while gently vortexing. The cells were washed twice with cold PBS, pelleted by spinning at 10 000 × *g* and fixed with 350 μl 1% methanol-free formaldehyde for 15 min on ice. The cells were permeablized in 500 μl PBS containing 1% methanol-free formaldehyde and 0.01% Tween 20 overnight at 4°C. After washing twice with cold PBS, the cells were incubated with 50 Kunitz units of DNase I (Sigma) in 0.15 M NaCl (pH = 5) containing 4.2 mM $MgCl_2$ for 15 min at 37°C (1 ml of 50 U ml^{-1}). The cells were washed twice with cold PBS/1% BSA and incubated with anti-BrdU FITC (BD Biosciences) in 100 μl PBS/1% BSA for 30 min on ice. After washing twice with PBS/1% BSA, the cells were fixed in 1% methanol-free formaldehyde in PBS/1% BSA and stored at 4°C until analysis.

Apoptotic cell death was examined by terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) (23). Briefly, cells were incubated with unconjugated rat and hamster IgG, stained for expression of cell surface molecules and then fixed with 1% methanol-free

formaldehyde followed by 70% ethanol. After washing, the TUNEL reaction was performed by incubating cells in 50 μl of reaction mix containing 10 U of terminal deoxribosyl transferase (TdT), 2.5 mM cobalt chloride in 1× TdT buffer (Roche, Indianapolis, IN, USA) and 0.2 pmol μl^{-1} of FITC-dUTP (Roche) in sterile distilled water for 1 h at 37°C. The cells were washed twice and fixed in 1% methanol-free formaldehyde. Murine thymocytes were included as a positive control for apoptotic cells.

Results

OT-I Fas$^{lpr/lpr}$ mice manifest an increased CD4$^+$:CD8$^+$ T-cell ratio with age

OT-I mice bear a transgenic TCR (Vα2 and Vβ5) that recognizes chicken ovalbumin peptide 257–264 SIINFEKL restricted to MHC class I H-2K^b and, as such, T lymphocytes preferentially select on class I to become CD8$^+$ T cells (49). OT-I mice bred onto the Fas-deficient (*Fas$^{lpr/lpr}$*) background did not manifest the age-dependent lymphadenopathy characteristic of non-TCR transgenic *Fas$^{lpr/lpr}$* mice. Lymph node T cells of OT-I and OT-I *Fas$^{lpr/lpr}$* mice contained predominantly CD8$^+$ T cells, and nearly, all the CD8$^+$ T cells expressed the clonotypic Vα2 TCR (Fig. 1A). However, CD8$^+$ Vα2$^+$ T cells from OT-I *Fas$^{lpr/lpr}$* mice contained a higher proportion of CD44^{high} cells relative to OT-I mice. In addition, both OT-I and OT-I *Fas$^{lpr/lpr}$* lymph nodes also contained a small percentage of CD4$^+$ T cells of which approximately 40% expressed Vα2, while the remainder expressed an endogenous Vα. Interestingly, an increased proportion of CD4$^+$ T cells expressed high levels of CD44 compared with CD8$^+$ T cells in both OT-I and OT-I *Fas$^{lpr/lpr}$* mice (Fig. 1A). Since the mice

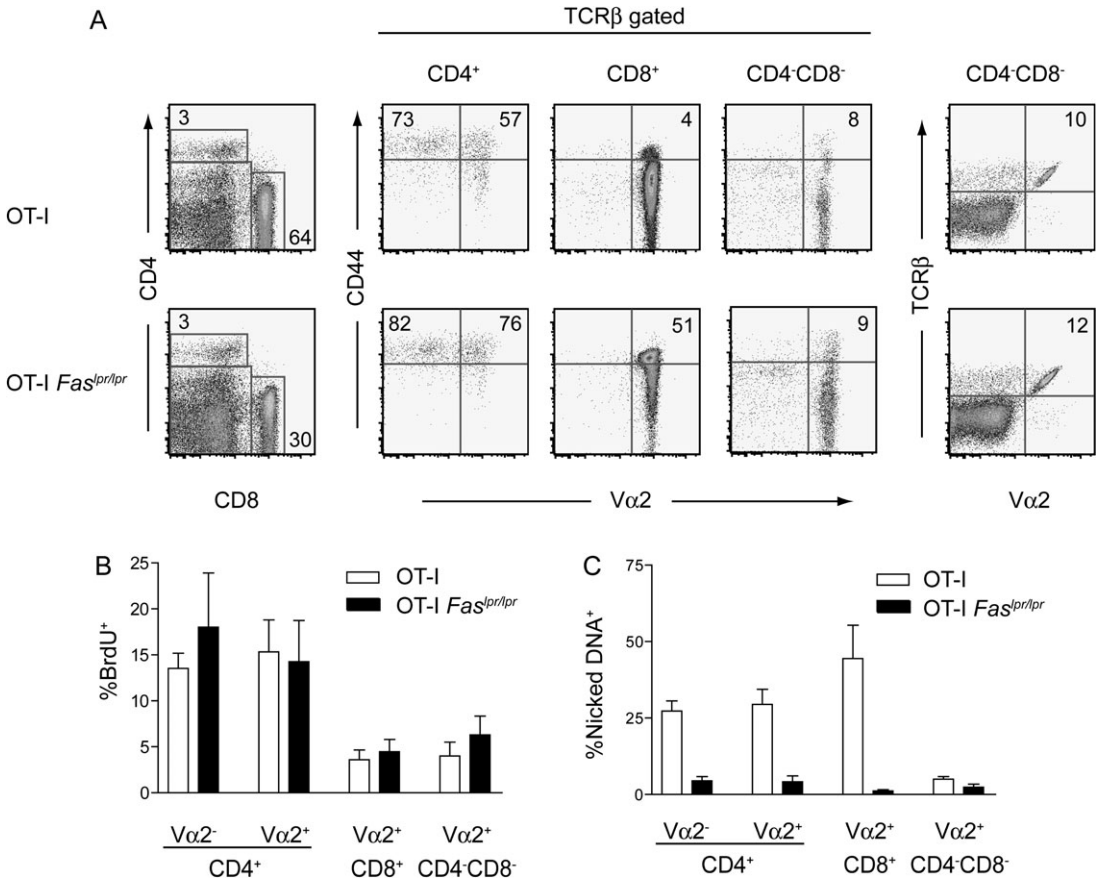

Fig. 1. OT-I *Fas$^{lpr/lpr}$* mice have an increased proportion of CD8$^+$CD44^{high} T lymphocytes compared with OT-I mice. (A) Shown is the surface expression of CD44 and Vα2 on CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ lymph node TCRβ$^+$ subsets from 13-week-old OT-I and OT-I *Fas$^{lpr/lpr}$* mice (representative of five mice per genotype). Number inserts represent the percentage of positive cells. (B) Age- and sex-matched OT-I (open bar) and OT-I *Fas$^{lpr/lpr}$* (solid bar) mice received four intraperitoneal injections of BrdU (1 mg each) over 24 h. Lymph node cells were stained for surface expression of CD4, CD8 and Vα2 and analyzed for BrdU incorporation by flow cytometry. Shown are the mean and standard deviation for the percentage of BrdU$^+$ cells in each T-cell subset (n = 3 per strain). The percentages of BrdU$^+$ cells between OT-I and OT-I *Fas$^{lpr/lpr}$* T cells were not statistically different for the CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ T-cell subsets [analysis of variance (ANOVA), Tukey post-test, $P > 0.05$]. However, the percentage of BrdU$^+$CD4$^+$ T cells was statistically increased compared with BrdU$^+$CD8$^+$ T cells in both OT-I and OT-I *Fas$^{lpr/lpr}$* mice (ANOVA, Tukey post-test, $P < 0.05$). (C) Single-cell suspensions of lymph node cells from age- and sex-matched OT-I (open bar) and OT-I *Fas$^{lpr/lpr}$* (solid bar) mice were cultured for 2.5 h either with or without FLAG-tagged FasL cross-linked by anti-FLAG antibody. The cells were then stained for expression of CD4, CD8, TCRβ and Vα2 and analyzed for the presence of nicked DNA by TUNEL staining. Shown are the mean and standard deviation of the proportion of apoptotic cells in each T-cell subset (n = 4–6 per group). The percentages of apoptotic T cells between OT-I and OT-I *Fas$^{lpr/lpr}$* T cells were statistically significant for the CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ T-cell subsets (ANOVA, Tukey post-test, $P < 0.05$). These data are representative of two experiments.

were not intentionally challenged with any antigen, this suggested that OT-I CD4$^+$ T cells might undergo a high rate of cell cycling in response to self-antigen/MHC. BrdU incorporation was used to determine the fraction of T cells that underwent cell cycling *in vivo* during a 24-h labeling period in the absence of any known foreign antigen. As anticipated, an increased proportion of CD4$^+$ T cells (15%) incorporated BrdU compared with CD8$^+$ T cells (4%) in both OT-I and OT-I *Fas*$^{lpr/lpr}$ mice (Fig. 1B). BrdU incorporation was also similar between Vα2$^+$ and Vα2$^-$ CD4$^+$ T cells and between OT-I and OT-I *Fas*$^{lpr/lpr}$ CD4$^+$ T cells. We have previously shown that T cells cycling in response to stimulation by self-peptide/MHC are sensitive to Fas-mediated cell death (23). To measure cell death, freshly isolated lymph node T cells were cultured in the presence of cross-linked Fas ligand and then analyzed by TUNEL staining. On average, 30% of OT-I CD4$^+$ T cells and 45% of OT-I CD8$^+$ T cells were undergoing apoptosis (Fig. 1C). There was a low level of spontaneous death in untreated cultures and this was not increased in OT-I *Fas*$^{lpr/lpr}$ T cells (data not shown). Thus, OT-I CD8$^+$ T cells manifested decreased cell cycling and increased sensitivity to Fas-mediated cells death compared with OT-I CD4$^+$ T cells.

There were, however, differences in the composition of the T-cell compartment between OT-I and OT-I *Fas*$^{lpr/lpr}$ mice with age that provided insight into the nature of the T cells that accumulate in *Fas*$^{lpr/lpr}$ mice. The increased BrdU incorporation of OT-I and OT-I *Fas*$^{lpr/lpr}$ CD4$^+$ T cells suggested that the proportion of CD4$^+$ T cells in OT-I mice might change with age. The number of CD8$^+$ and CD4$^+$ T cells in the lymph nodes of OT-I and OT-I *Fas*$^{lpr/lpr}$ mice was similar at 5 weeks of age (Fig. 2A and B). However, by 13 weeks of age, lymph nodes from OT-I *Fas*$^{lpr/lpr}$ mice contained a decreased number of CD8$^+$ T cells and an increased number of CD4$^+$ T cells. This resulted in a 4.6-fold increase in the number of CD4$^+$ T cells relative to CD8$^+$ T cells in OT-I *Fas*$^{lpr/lpr}$ mice from 5 (1:37) to 13 weeks (1:8) (Fig. 2D). In contrast, the number of CD4$^+$ and CD8$^+$ T cells in OT-I mice remained constant with age. Moreover, 13-week-old OT-I *Fas*$^{lpr/lpr}$ mice had a modest increase in the number of CD4$^-$CD8$^-$Vα2$^+$ T cells (Fig. 2C). The increase in the proportion of CD4$^+$ to CD8$^+$ T cells in OT-I *Fas*$^{lpr/lpr}$ mice suggested

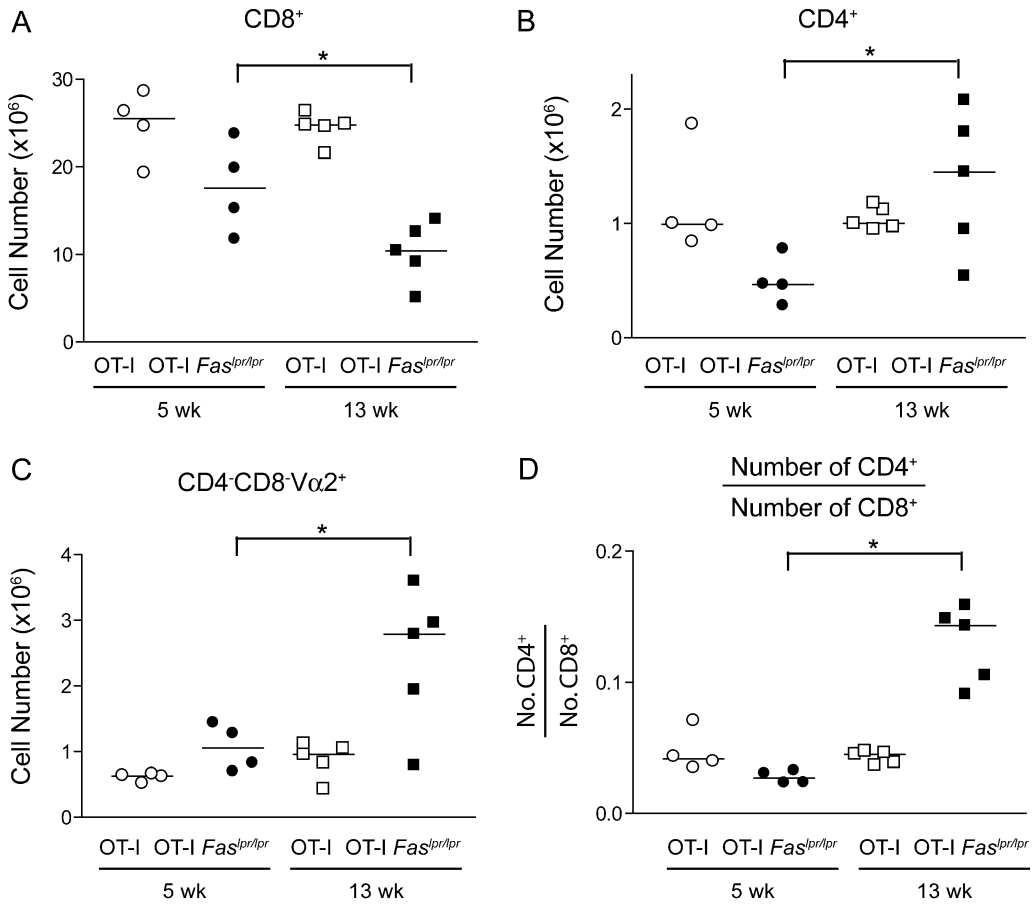

Fig. 2. The composition of the lymph node T-cell compartment in OT-I *Fas*$^{lpr/lpr}$ mice changes with age. The absolute numbers of (A) CD8$^+$, (B) CD4$^+$ and (C) CD4$^-$CD8$^-$Vα2$^+$ lymph node T cells recovered from OT-I (open symbols) and OT-I *Fas*$^{lpr/lpr}$ mice (solid symbols) at 5 and 13 weeks of age. Lymph node cells were stained for CD4, CD8, TCRβ and Vα2 and analyzed by flow cytometry. Cell numbers were calculated based on the percent positive cells. Each symbol represents an individual mouse analyzed (n = 4–5 per group). Horizontal lines indicate the median number for each T-cell subset. Differences in cell number between 5- and 13-week-old OT-I *Fas*$^{lpr/lpr}$ mice were significant for the CD8$^+$, CD4$^+$ and CD4$^-$CD8$^-$Vα2$^+$ T-cell subsets [analysis of variance (ANOVA), Tukey post-test, *$P < 0.05$]. Differences in cell number between 13-week-old OT-I and OT-I *Fas*$^{lpr/lpr}$ mice were significant for the CD8$^+$ and CD4$^-$CD8$^-$Vα2$^+$ T-cell subsets but not the CD4$^+$ T-cell subset (ANOVA, Tukey post-test, $P < 0.05$). (D) The absolute number of CD4$^+$ T cells divided by the absolute number of CD8$^+$ T cells.

that the loss of Fas led to increased survival of OT-I CD4$^+$ T cells relative to OT-I CD8$^+$ T cells.

OT-I CD4$^+$ T cells preferentially expand during lymphopenia-induced proliferation

The high rate of proliferation of OT-I CD4$^+$ T cells in the absence of administered antigen suggested that this subset either underwent rapid proliferation to self-antigen or had an intrinsically increased survival rate compared with OT-I CD8$^+$ T cells. To further explore these possibilities, we used the experimental model of lymphopenia-induced proliferation by adoptively transferring OT-I and OT-I *Fas$^{lpr/lpr}$* lymph node cells into syngeneic *Rag1$^{o/o}$* hosts and analyzed the number and phenotype of the donor T cells analyzed 14 days after transfer. The ratio of CD4$^+$:CD8$^+$ T cells recovered from the recipients on day 14 post-transfer revealed a dramatic increase in CD4$^+$ T cells (Fig. 3A). Donor OT-I T cells contained a CD4$^+$:CD8$^+$ ratio of only 1:13, whereas the OT-I T cells that had undergone 14 days of lymphopenia-induced proliferation manifested a CD4$^+$:CD8$^+$ T-cell ratio that had increased substantially to 1:1.5. Similarly, the ratio of CD4$^+$:CD8$^+$ T cells in the donor OT-I *Fas$^{lpr/lpr}$* inoculum was 1:17, whereas in the T cells recovered from the *Rag1$^{o/o}$* hosts, it was 1:1.1. This represented an average 8.6-fold increase in CD4$^+$ T cells compared with CD8$^+$ T cells using OT-I donor T cells and an average 15.7-fold increase using OT-I *Fas$^{lpr/lpr}$* donor T cells.

The preferential expansion of OT-I CD4$^+$ T cells could result from a more rapid entry of CD4$^+$ T cells into the cell cycle. We therefore analyzed the initiation of cell cycling after adoptive transfer of CFSE-labeled OT-I and OT-I *Fas$^{lpr/lpr}$* lymph node cells into *Rag1$^{o/o}$* recipients. On day 3 post-transfer, 30% of CD4$^+$ T cells had undergone at least one cell cycle, while 65% of CD8$^+$ T cells had cycled from both OT-I and OT-I *Fas$^{lpr/lpr}$* mice (Fig. 3B). Thus, OT-I CD4$^+$ T cells entered the cell cycle considerably less rapidly than OT-I CD8$^+$ T cells. BrdU incorporation was used to determine if this pattern of cell cycling continued at later time points when the cells had proliferated beyond the number of cycles detectable by CFSE labeling. In mice that received BrdU from days 13 to 14 post-transfer, on average, 40% of the CD4$^+$ T cells from both OT-I and OT-I *Fas$^{lpr/lpr}$* mice were BrdU$^+$ following the 24-h labeling period (Fig. 3C). By contrast, only about 12% of the CD8$^+$ T cells were BrdU$^+$ following the same 24-h period. This pattern of BrdU incorporation paralleled that seen in intact OT-I and OT-I *Fas$^{lpr/lpr}$* mice (Fig. 1B). Although the disparate cycling results from CFSE versus BrdU analysis could result from a sudden increase in the rate of cell cycle entry by OT-I CD4$^+$ T cells at later time points, it more likely represented an increased survival of the OT-I CD4$^+$ T-cell subset compared with OT-I CD8$^+$ T cells. Hence, despite the increased selection of OT-I CD8$^+$ T cells during thymic development, there was nonetheless a greater homeostatic expansion and accumulation of OT-I *Fas$^{lpr/lpr}$* CD4$^+$ T cells.

CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells express low levels of surface CD5

In nearly all cases, expression of a TCR transgene in *Fas$^{lpr/lpr}$* mice eliminates both the lymphadenopathy and the accumulation of CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells (40–42) (K. A. Fortner,

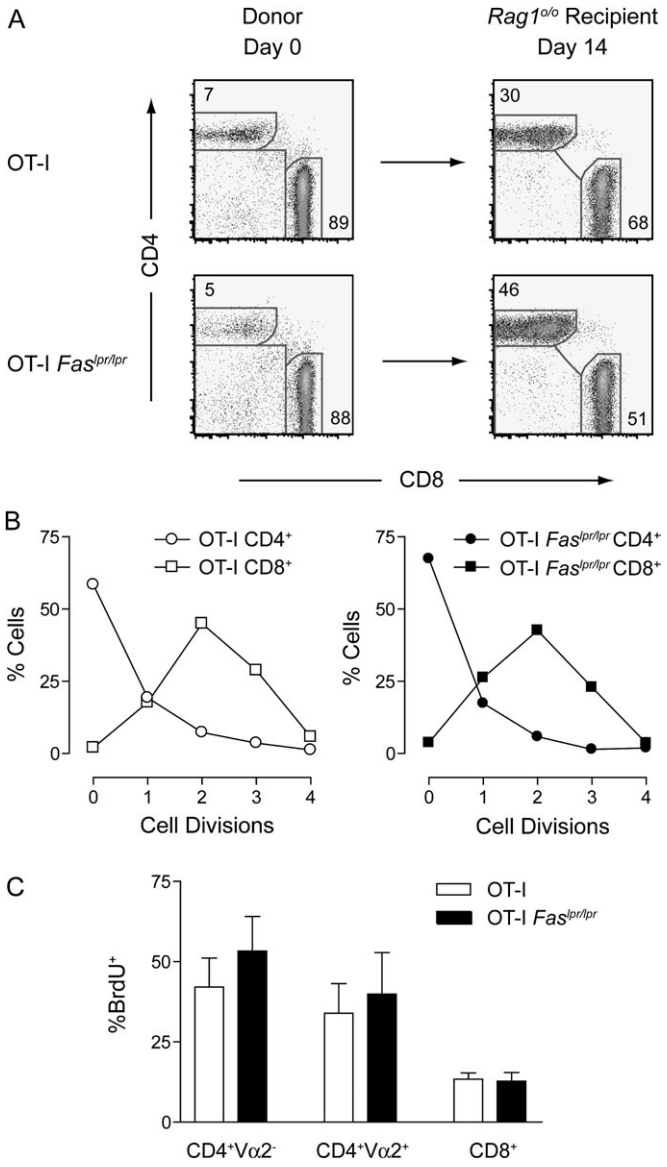

Fig. 3. The proportion of OT-I CD4$^+$ T cells increases compared with OT-I CD8$^+$ T cells following lymphopenia-induced proliferation. (A) Equal numbers of lymph node cells from 5-week-old CD90.1$^+$ (Thy1.1) OT-I and OT-I *Fas$^{lpr/lpr}$* mice were transferred to CD90.2$^+$ (Thy1.2) *Rag1$^{o/o}$* recipients. Shown is the surface expression of CD4 and CD8 on TCRβ^+ cells from the donor lymph nodes and of the T cells recovered from recipient mice on day 14 post-transfer (representative of four mice per recipient group). Number inserts represent the percentage of positive cells. (B) Lymph node cells from 5-week-old OT-I and OT-I *Fas$^{lpr/lpr}$* mice were labeled with CFSE and transferred into *Rag1$^{o/o}$* recipients. On day 3 post-transfer, lymph node and spleen cells were stained for CD4, CD8, Vα2 and TCRβ and analyzed for CFSE by flow cytometry. Shown is the percent of CD4$^+$ TCRβ^+ and CD8$^+$ TCRβ^+ T cells in each cell cycle as determined by CFSE dilution. Data are representative of two independent experiments. (C) *Rag1$^{o/o}$* recipients received four intraperitoneal injections of BrdU (1 mg each) over the 24-h period from day 13 to 14 post-adoptive transfer. Lymph node and spleen cells from *Rag1$^{o/o}$* mice that received OT-I (open bar) or OT-I *Fas$^{lpr/lpr}$* (solid bar) lymph node cells were surfaced stained for CD4, CD8, Vα2 and TCRβ and analyzed for BrdU incorporation by flow cytometry. Shown is the mean and standard deviation for the fraction of BrdU$^+$ cells in the CD4$^+$ TCRβ^+ and CD8$^+$ TCRβ^+ T-cell subsets (n = 4 per donor strain). Differences between OT-I and OT-I *Fas$^{lpr/lpr}$* donor T cells were not statistically significant by t-test ($P > 0.05$).

unpublished observations). OT-I *Fas*$^{lpr/lpr}$ lymph nodes, however, contained a modestly increased proportion of CD4$^-$CD8$^-$Vα2$^+$ T cells compared with OT-I lymph node cells (Fig. 2C), but these did not accumulate with age as typically seen in *Fas*$^{lpr/lpr}$ mice lacking a TCR transgene. If, as suggested, *Fas*$^{lpr/lpr}$ CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells derive from a CD8$^+$ precursor (44–48), clearly not all CD8$^+$ T cells default to becoming CD4$^-$CD8$^-$ as *Fas*$^{lpr/lpr}$ mice also accumulate CD8$^+$ T cells.

Conceivably, the nature of the TCR signal may determine the phenotypic fate of CD8$^+$ T cells in *Fas*$^{lpr/lpr}$ mice. In order to examine the relative avidity of the TCR interaction with self-peptide/MHC of CD8$^+$ and CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells, we analyzed the cell surface expression of CD5. CD5 is a negative regulator of T-cell signaling and the level of its cell surface expression is directly proportional to the avidity of the interaction of TCR with self-MHC/peptide (52–54). CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells from *Fas*$^{lpr/lpr}$ mice expressed substantially lower levels of surface CD5 compared with *Fas*$^{lpr/lpr}$ CD8$^+$ T cells (Fig. 4A). This suggested that CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells might derive from a subset of CD8$^+$ T cells that received low-avidity TCR signals. In contrast, compared with the majority of non-TCR transgenic *Fas*$^{lpr/lpr}$ polyclonal CD8$^+$ T cells, OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells expressed higher levels of surface CD5, reflecting a relatively high-avidity TCR signal (Fig. 4A). There was no difference in CD5 expression between OT-I and OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells (Fig. 4B). Thus, the TCR avidity of OT-I CD8$^+$ T cells might be generally too high to allow either their conversion to CD4$^-$CD8$^-$Vα2$^+$ T cells or their accumulation as CD8$^+$ T cells in *Fas*$^{lpr/lpr}$ mice.

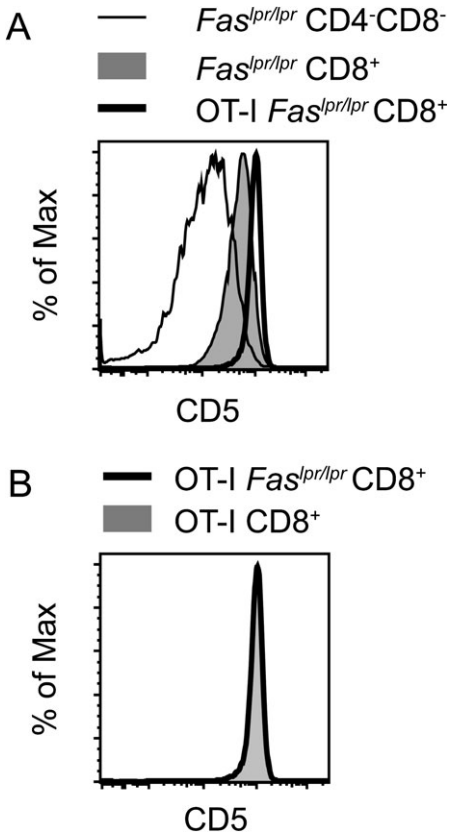

Fig. 4. Surface expression of CD5 is reduced on *Fas*$^{lpr/lpr}$ CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells. Lymph node cells from B6 *Fas*$^{lpr/lpr}$, OT-I *Fas*$^{lpr/lpr}$ and OT-I mice were stained for CD5, CD4, CD8, Vα2 and TCRβ. (A) Shown is the expression of CD5 on *Fas*$^{lpr/lpr}$ CD8$^+$ T cells (shaded area), *Fas*$^{lpr/lpr}$ CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells (thin line) and OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells (thick line). (B) Similar expression of CD5 on OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells (thick line) and OT-I CD8$^+$ T cells (shaded area).

OT-I CD8$^+$ and CD4$^-$CD8$^-$ T cells accumulate in a β_2m-deficient environment

To further explore the hypothesis that OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells selectively accumulate under conditions of reduced TCR signals, we adoptively transferred OT-I or OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells into syngeneic *scid* or MHC class I-deficient $\beta_2m^{o/o}$*scid* mice. Since increased cell numbers could result from more rapid proliferation, we first analyzed the initiation of cell cycling following adoptive transfer of CFSE-labeled lymph node cells. Entry into cell cycle was equivalent on day 3 between OT-I and OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells in *scid* mice (Fig. 5A). Their cell cycle rates were also similar in $\beta_2m^{o/o}$*scid* mice, albeit substantially delayed compared with cycling in *scid* recipients. However, the number of OT-I CD8$^+$ T cells recovered on day 25 following the transfer of equal numbers of OT-I and OT-I *Fas*$^{lpr/lpr}$ T cells differed depending upon the genotype of the recipient mice. The number of OT-I CD8$^+$ T cells recovered from $\beta_2m^{o/o}$*scid* recipients was on average 52% of the number recovered from *scid* mice (Fig. 5B). By contrast, the number of OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells recovered from $\beta_2m^{o/o}$*scid* mice was 75% of that recovered from *scid* mice. Hence, an increased proportion of OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells accumulated compared with OT-I CD8$^+$ T cells in a $\beta_2m^{o/o}$ environment containing few, if any, MHC contacts.

These results using a single inoculum of OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells into $\beta_2m^{o/o}$*scid* recipients suggested that OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ T cells might accumulate with time in an environment of chronic low to negligible TCR signaling. We therefore generated an experimental model in which OT-I and OT-I *Fas*$^{lpr/lpr}$ mice were crossed onto a $\beta_2m^{o/o}$ background. OT-I $\beta_2m^{o/o}$ mice provided a condition in which T cells bearing the OT-I TCR enter a peripheral environment essentially devoid of MHC class I/self-peptide signals. Given the natural tendency of the OT-I TCR to select to CD8$^+$ T cells, a small population of CD8$^+$ Vα2$^+$ single positive thymocytes was still produced in $\beta_2m^{o/o}$ mice (data not shown). As expected, the lymph nodes from young OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice contained few CD8$^+$ T cells and these CD8$^+$ T cells expressed almost exclusively the Vα2 transgene (Fig. 6A). Both OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ lymph nodes also contained a population of CD4$^-$CD8$^-$ T cells, nearly all of which expressed the transgenic Vα2. Reflecting their diminished MHC contacts, CD8$^+$ T cells from lymph nodes of OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice expressed greatly reduced levels of CD5 compared with CD8$^+$ T cells from either OT-I *Fas*$^{lpr/lpr}$ mice (Fig. 6B) or *Fas*$^{lpr/lpr}$ mice (Fig. 6C). In fact, the low level of CD5 surface expression on OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ CD8$^+$ T cells was comparable to CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells from *Fas*$^{lpr/lpr}$ mice (Fig. 6C). There was no difference in the CD5 expression on

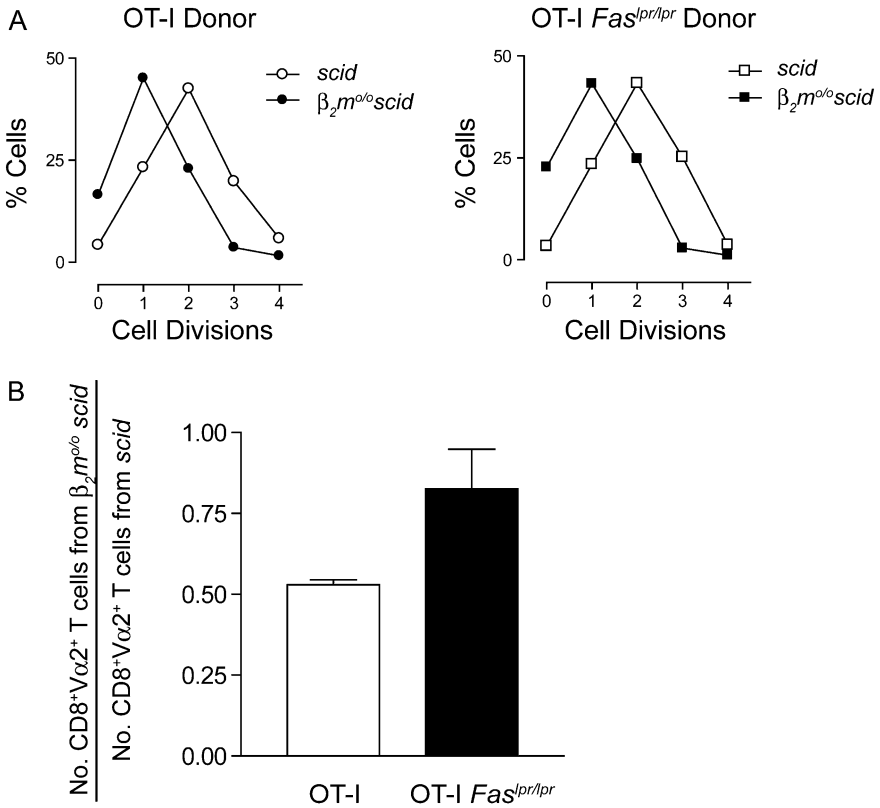

Fig. 5. An increased proportion of OT-I *Fas*$^{lpr/lpr}$ CD8$^+$ Vα2$^+$ T cells is recovered from $\beta_2m^{o/o}$*scid* recipients compared with OT-I CD8$^+$ Vα2$^+$ T cells. (A) Lymph node cells from 5-week-old CD90.1$^+$ OT-I and OT-I *Fas*$^{lpr/lpr}$ mice were labeled with CFSE and transferred into CD90.2$^+$ *scid* or $\beta_2m^{o/o}$*scid* recipients. On day 3 post-transfer, lymph node and spleen cells were stained for CD4, CD8, Vα2 and TCRβ and analyzed for CFSE by flow cytometry. Shown is the percent of CD8$^+$ Vα2$^+$ T cells in each cell cycle as determined by CFSE dilution. Data are representative of two independent experiments. (B) CD90.2$^+$ *scid* or $\beta_2m^{o/o}$*scid* recipients received a single equal inoculum of CD90.1$^+$ OT-I or OT-I *Fas*$^{lpr/lpr}$ lymph node cells. On day 24 or 25 post-transfer, lymph node and spleen cells from mice that received OT-I (open bar) or OT-I *Fas*$^{lpr/lpr}$ (solid bar) lymph node cells were surfaced stained for CD4, CD8, Vα2 and TCRβ and analyzed by flow cytometry. Cell numbers were calculated based on the percent positive cells. Shown are the mean and SEM (n = 3 experiments) for the absolute number of CD8$^+$Vα2$^+$ T cells recovered from $\beta_2m^{o/o}$*scid* recipients (five mice per group) divided by the absolute number of CD8$^+$Vα2$^+$ T cells recovered from *scid* recipients (five mice per group).

CD8$^+$ T cells from OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice (Fig. 6D). Thus, the absence of Fas did not directly alter surface CD5 expression.

In this $\beta_2m^{o/o}$ environment of chronic low to negligible TCR signaling, we postulated that OT-I CD8$^+$ T cells lacking Fas might accumulate to higher levels and exhibit a greater frequency of conversion to CD4$^-$CD8$^-$TCRαβ$^+$ T cells. Thus, we compared the number of T cells from OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice with age. The CD8$^+$ Vα2$^+$ T cell numbers were comparable between OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice until approximately 125 days of age (Fig. 7A). From this point, the number of CD8$^+$ Vα2$^+$ T cells increased with age in OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice while remaining constant in OT-I $\beta_2m^{o/o}$ mice and were 8.3-fold higher by 200 days of age.

The number of CD4$^-$CD8$^-$Vα2$^+$ T cells in OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice also increased with age and was 3.7-fold higher than found in OT-I $\beta_2m^{o/o}$ mice at 200 days of age (Fig. 7B). These CD4$^-$CD8$^-$Vα2$^+$ T cells did not express a second TCRVα as defined by mAb to Vα3.2, Vα11.1/11.2 and Vα8.3 (data not shown). Given the relatively few CD8$^+$ T cells produced in OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice, there was a high ratio of CD4$^-$CD8$^-$Vα2$^+$ T cells to CD8$^+$ T cells (7.8:1) in OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice compared with OT-I *Fas*$^{lpr/lpr}$ mice (0.3:1). Thus, the limited MHC contact experienced by OT-I CD8$^+$ T cells in a $\beta_2m^{o/o}$ environment likely favored the transition of CD8$^+$ T cells to CD4$^-$CD8$^-$ T cells as well as the accumulation of both CD8$^+$ and CD4$^-$CD8$^-$ T cells in the absence of Fas.

To assess whether the increased accumulation of CD8$^+$ and CD4$^-$CD8$^-$ T cells in OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice reflected augmented cell cycling, we analyzed *in vivo* proliferation by BrdU incorporation over a 24-h labeling period. The CD8$^+$Vα2$^+$ and CD4$^-$CD8$^-$Vα2$^+$ T-cell subsets manifested equivalent levels of BrdU incorporation between OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice (Fig. 7C). To assess the sensitivity of these T cells to Fas-mediated cell death, lymph node cells from OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice were cultured in the presence of cross-linked Fas ligand and analyzed by TUNEL staining. CD8$^+$Vα2$^+$ T cells from OT-I $\beta_2m^{o/o}$ mice but not from OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice contained a high proportion of apoptotic cells (Fig. 7D). Since there was no difference in cycling of these subsets between OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice, the accumulation of CD8$^+$Vα2$^+$

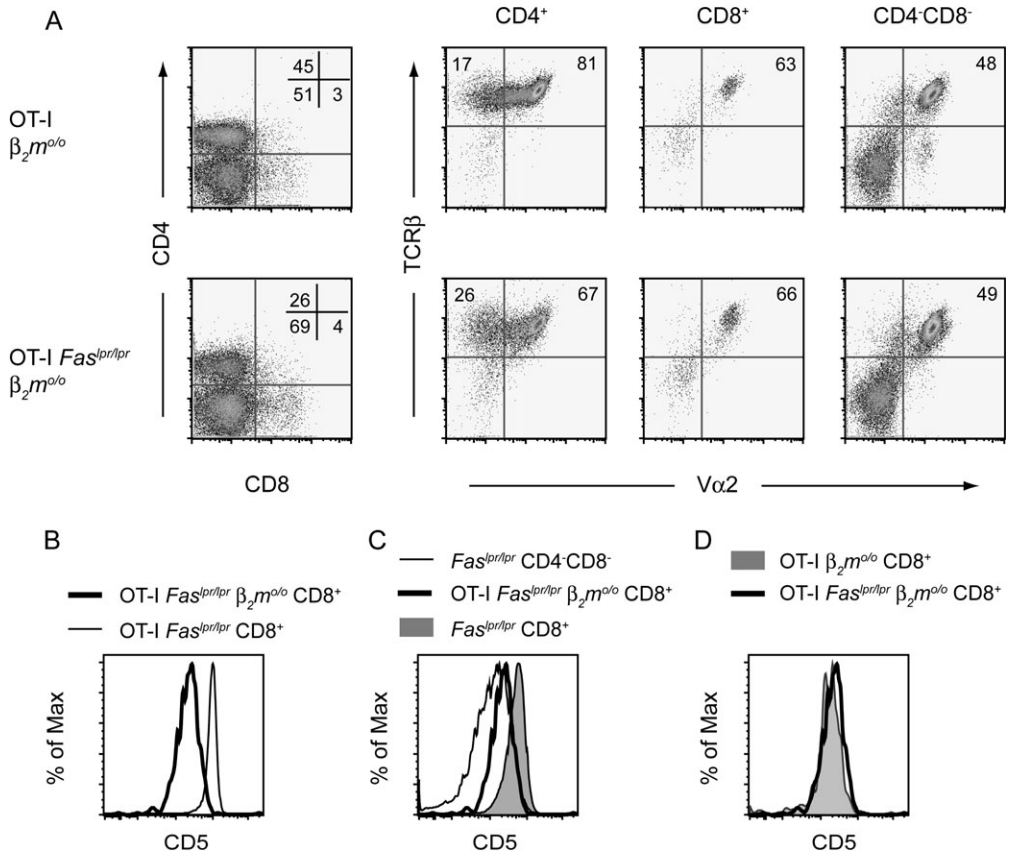

Fig. 6. Reduced CD5 expression on OT-I CD8$^+$ T cells in a $\beta_2m^{o/o}$ environment. (A) Lymph nodes were harvested from 125-day-old OT-I $\beta_2m^{o/o}$ and OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice (n = 4 mice per strain) and the cells were stained for CD4, CD8, Vα2 and TCRβ. Shown is the surface expression of TCRβ and Vα2 on the CD4$^+$, CD8$^+$ and CD4$^-$CD8$^-$ lymph node subsets. Number inserts represent the percentage of positive cells (B, C and D). Lymph node cells from B6 $Fas^{lpr/lpr}$, OT-I $Fas^{lpr/lpr}$ and OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice were stained for CD5, CD4, CD8, Vα2 and TCRβ. (B) Shown is the expression of CD5 on OT-I $Fas^{lpr/lpr}$ $\beta_2m^{o/o}$ CD8$^+$ T cells (thick line) and OT-I $Fas^{lpr/lpr}$ CD8$^+$ T cells (thin line). (C) The expression of CD5 on $Fas^{lpr/lpr}$ CD8$^+$ T cells (shaded area), $Fas^{lpr/lpr}$ CD4$^-$CD8$^-$TCRαβ$^+$ T cells (thin line) and OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ CD8$^+$ T cells (thick line). (D) Similar expression of CD5 on OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ CD8$^+$ T cells (thick line) and OT-I $\beta_2m^{o/o}$ CD8$^+$ T cells (shaded area).

and CD4$^-$CD8$^-$Vα2$^+$ T cells in OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice likely resulted from diminished cell death within these T-cell subsets. Consistent with their increased survival, the CD8$^+$Vα2$^+$ subset from OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice was also enriched for cells that expressed high levels of CD44 with age (Fig. 7E and F), which likely reflects the retention of cycling T cells within this subset in the absence of Fas.

Polyclonal CD8$^+$ and CD4$^-$CD8$^-$TCRαβ$^+$ T cells accumulate in a peripheral environment lacking MHC class I

The accumulation of monoclonal CD4$^-$CD8$^-$Vα2$^+$ T cells in OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice suggested that polyclonal CD8$^+$ T cells might also manifest an increased rate of conversion to CD4$^-$CD8$^-$TCRαβ$^+$ T cells in an environment of chronic low to negligible TCR signaling. To examine generality of this process, we developed a mouse model in which polyclonal CD8$^+$ T cells underwent normal positive selection in the thymus but then entered a peripheral environment devoid of MHC class I expression. Transgenic expression of β_2m under the control of the human keratin K14 promoter (K14-β_2m) in β_2m-deficient mice selectively restored MHC class I expression to thymic cortical epithelial cells and resulted in the generation of normal numbers of mature CD8$^+$ T cells in both the thymus and the periphery (50). K14-β_2m $\beta_2m^{o/o}$ mice were bred to $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice to generate K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice. At 6 months of age, both K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ and $Fas^{lpr/lpr}$ mice had increased numbers of CD8$^+$ T cells compared with wild-type mice (Fig. 8A). This accumulation was somewhat more pronounced in K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice, which had on average 2-fold more CD8$^+$ T cells compared with conventional $Fas^{lpr/lpr}$ mice. Of particular interest was that K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice accumulated significantly 8-fold more CD4$^-$CD8$^-$TCRαβ$^+$ T cells compared with $Fas^{lpr/lpr}$ mice and 50-fold more than $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice (Fig. 8B). The CD4$^-$CD8$^-$TCRαβ$^+$ T cells from K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice were phenotypically indistinguishable from $Fas^{lpr/lpr}$ mice based on their increased proportion of TCR Vβ8.3 usage and elevated expression of CD44 (data not shown). Despite only a modest increase of CD8$^+$ T cells in K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice, they manifested a substantially higher ratio of CD4$^-$CD8$^-$TCRαβ$^+$ T cells to CD8$^+$ T cells (5.5:1) compared with $Fas^{lpr/lpr}$ mice (1.3:1) (Fig. 8C). These data suggested that there is a higher rate of conversation of CD8$^+$ T cells to CD4$^-$CD8$^-$TCRαβ$^+$

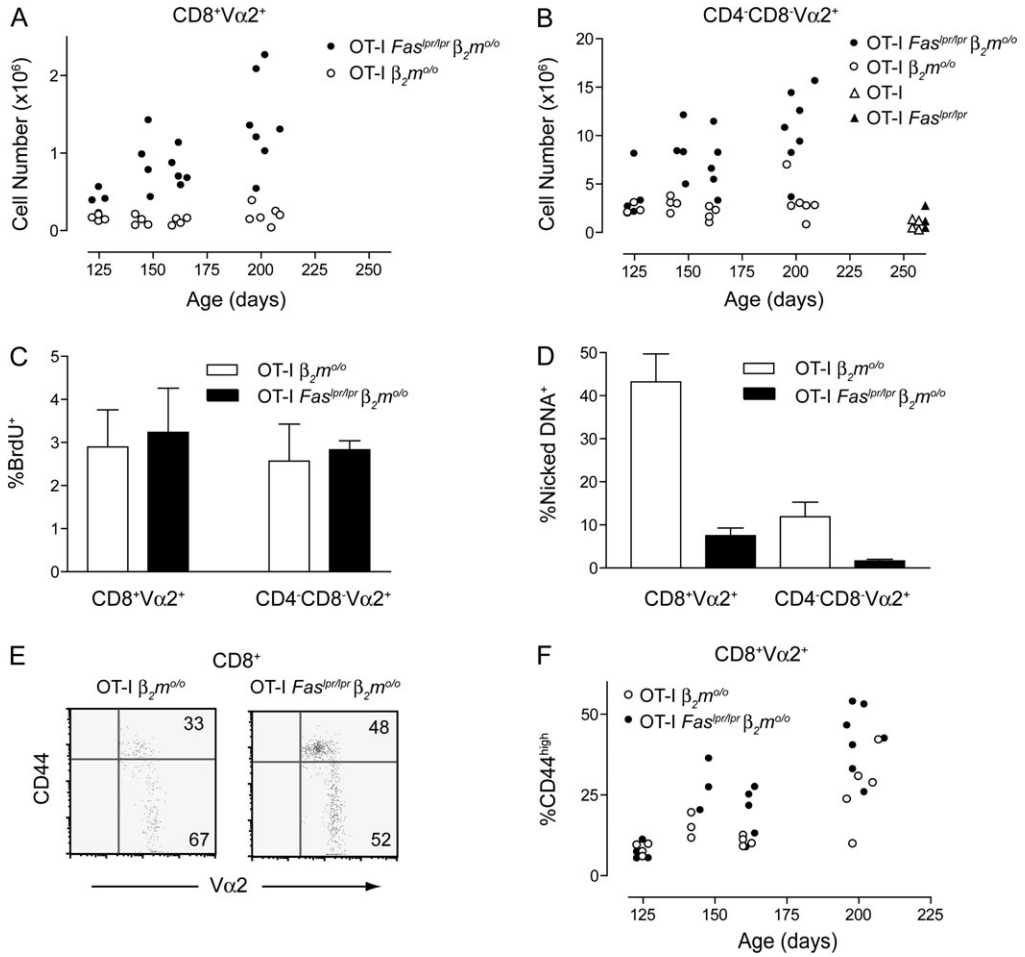

Fig. 7. With age, OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice have increased numbers of CD8$^+$ and CD4$^-$CD8$^-$Vα2$^+$ T cells compared with OT-I $\beta_2m^{o/o}$ mice. Axillary, brachial, inguinal and popliteal lymph nodes were harvested from OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice at the ages indicated. The cells were stained for CD4, CD8, Vα2 and TCRβ and the cell numbers calculated based on the percent of positive cells in each subset. Shown are the absolute numbers of (A) CD8$^+$Vα2$^+$ T cells and (B) CD4$^-$CD8$^-$Vα2$^+$ T cells. Each symbol represents an individual mouse analyzed (n = 4–7 per group). Differences in cell number between OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice were significant for the CD8$^+$Vα2$^+$ and CD4$^-$CD8$^-$Vα2$^+$ T-cell subsets [analysis of variance (ANOVA), $P < 0.05$]. (C) OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice received four intraperitoneal injections of BrdU (1 mg each) over the 24-h period. OT-I $\beta_2m^{o/o}$ (open bar) or OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ (solid bar) lymph node cells were surfaced stained for CD4, CD8, TCRβ and Vα2 and analyzed for BrdU incorporation by flow cytometry. Shown is the mean and standard deviation for the fraction of BrdU$^+$ cells in the CD8$^+$Vα2$^+$ and CD4$^-$CD8$^-$Vα2$^+$ T cell subsets (n = 3 mice per donor strain). Differences between OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ T cells were not statistically significant (t-test, $P > 0.05$). (D) Single-cell suspensions of lymph node cells from age- and sex-matched mice were cultured for 2.5 h either with or without FLAG-tagged FasL cross-linked by anti-FLAG antibody. The cells were then stained for expression of CD4, CD8, TCRβ and Vα2 and analyzed for the presence of nicked DNA by TUNEL staining. Shown are the mean and standard deviation of the percentage of apoptotic cells in each T-cell subset (n = 4–5 per group). Differences in the percentages of apoptotic T cells between OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ T cells were statistically significant for the CD8$^+$ and CD4$^-$CD8$^-$ T-cell subsets (ANOVA, Tukey post-test, $P > 0.05$). These data are representative of two experiments. (E) Lymph node cells from 200-day-old OT-I $\beta_2m^{o/o}$ mice (n = 5) and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice (n = 7) were stained for CD4, CD8, Vα2 and CD44. Shown is a representative profile of the expression of Vα2 and CD44 on CD8$^+$ T cells. Number inserts represent the percentage of positive cells. (F) Lymph nodes were harvested from OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice at the ages indicated and the cells stained for CD4, CD8, Vα2 and CD44. Shown is the percentage of CD44^{high} cells in the CD8$^+$Vα2$^+$ subset. Differences in the percentage of CD44^{high} cells between OT-I $\beta_2m^{o/o}$ and OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice were significant (ANOVA, $P < 0.05$).

T cells in K14-β_2m *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice than in conventional *Fas*$^{lpr/lpr}$ mice.

Discussion

The current observations propose that the accumulation of T cells in the absence of the death receptor Fas in humans and mice, including the unusual CD4$^-$CD8$^-$TCR$\alpha\beta^+$ subset, results from diminished TCR interactions with self-antigen/MHC. These studies also provide a unifying model that explains both the source of the accumulating T cells and the origin of the CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells in *Fas*$^{lpr/lpr}$ mice. Here, we reduced the number of TCR interactions with self-antigen/MHC for both a monoclonal CD8$^+$ TCR as well as polyclonal CD8$^+$ TCRs to show that Fas-deficient CD8$^+$ and CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells accumulate preferentially in a peripheral environment that provides only a low to negligible level of self-peptide/MHC class I. Thus, our findings support

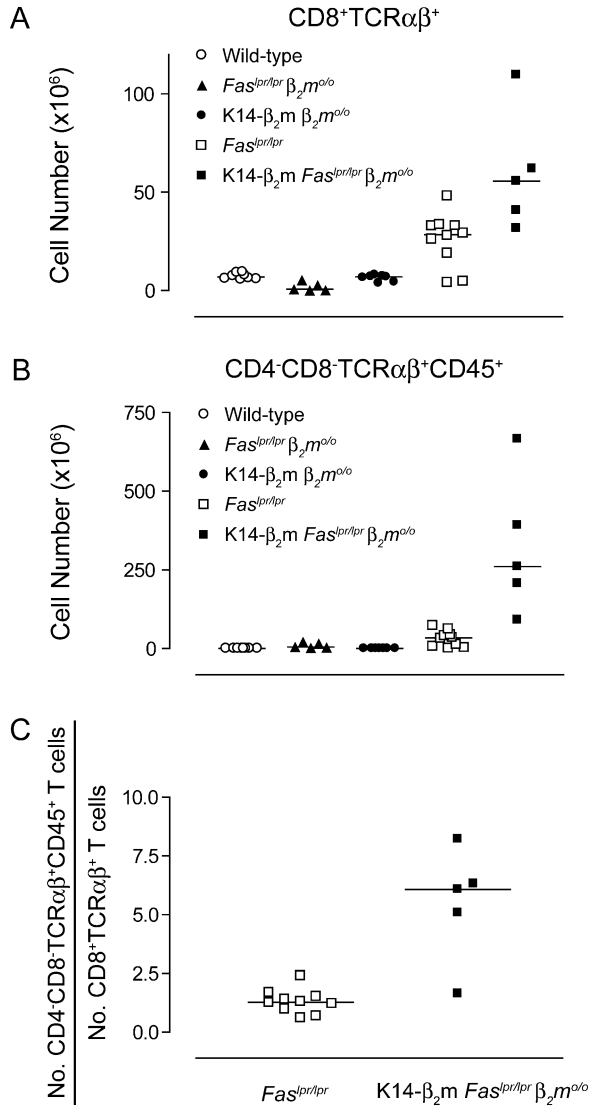

Fig. 8. K14-β_2m *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice had significantly increased numbers of CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells compared with *Fas*$^{lpr/lpr}$ mice. Axillary, brachial, inguinal and cervical lymph nodes were harvested from K14-β_2m *Fas*$^{lpr/lpr}\beta_2m^{o/o}$, *Fas*$^{lpr/lpr}$, *Fas*$^{lpr/lpr}\beta_2m^{o/o}$, K14-β_2m $\beta_2m^{o/o}$ and wild-type mice at 6 months of age. The cells were stained for CD4, CD8, TCRβ and CD45R and the cell numbers calculated based on the percent of positive cells in each subset. Shown are the absolute numbers of (A) CD8$^+$ TCRβ^+ T cells and (B) CD4$^-$CD8$^-$TCR$\alpha\beta^+$CD45R$^+$ T cells. Each symbol represents an individual mouse analyzed (n = 5–10 per group). Horizontal lines indicate the median number for each T-cell subset. Differences in cell number between K14-β_2m *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ and *Fas*$^{lpr/lpr}$ mice were significant for the CD8$^+$ TCRβ^+ and CD4$^-$CD8$^-$TCR$\alpha\beta^+$CD45R$^+$ T-cell subsets (analysis of variance, $P < 0.05$). (C) The absolute number of CD4$^-$CD8$^-$TCR$\alpha\beta^+$CD45R$^+$ T cells divided by the absolute number of CD8$^+$ T cells.

a model in which Fas shapes the peripheral T-cell repertoire by selectively limiting the survival of T cells cycling in response to reduced TCR interactions.

The lymphadenopathy and phenotype of T cells from *Fas*$^{lpr/lpr}$ mice are more likely driven by the expansion of peripheral T cells in response to self-peptide/MHC during T-cell homeostasis than by foreign antigen activation. Most studies observe that there is little delay in deletion of *Fas*$^{lpr/lpr}$ T cells compared with wild-type Fas$^+$ T cells *in vivo* following activation by a single dose of antigen or following acute infection (33–35, 55). However, Fas does contribute to the deletion of T cells during chronic infections (33). Yet no increase in lymphadenopathy was reported in any of the studies using chronic infection models. We have observed that Fas-deficient T cells undergoing lymphopenia-induced proliferation accumulate to higher numbers compared with wild-type Fas$^+$ T cells despite equivalent rates of cell cycle entry (23). In addition, consistent with genetic evidence suggesting that *Fas*$^{lpr/lpr}$ polyclonal CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells derive from CD8$^+$ T-cell precursors (44–47), lymphopenia-induced proliferation of *Fas*$^{lpr/lpr}$ CD8$^+$ T cells, but not CD4$^+$ T cells, yielded CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells (48). Expression of Fas ligand by both the T cells themselves as well as lymphoid stromal tissue contributes to deletion of T cells during homeostatic proliferation (23). Collectively, these studies support the view that the death receptor Fas limits the homeostatic expansion of T cells cycling in response to recurrent stimulation by self-peptide/MHC.

Little is known regarding features that distinguish the subset of T cells that accumulate in *Fas*$^{lpr/lpr}$ mice or the specific subset of CD8$^+$ T cells that are the precursors of CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells. The parallels between T cells from *Fas*$^{lpr/lpr}$ mice and T cells undergoing lymphopenia-induced proliferation, combined with the view that homeostatic proliferation is driven by recurrent TCR interactions with self-peptide/MHC that have a decreased avidity compared with cognate antigen, suggest that *Fas*$^{lpr/lpr}$ mice might selectively accumulate T cells that receive recurrent low-avidity TCR signals. *Fas*$^{lpr/lpr}$ CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells express lower levels of surface CD5 than the majority of CD8$^+$ T cells. This is consistent with the view that CD4$^-$CD8$^-$TCR$\alpha\beta^+$ T cells derive from CD8$^+$ T cells that received a low-avidity TCR signal. CD5 is a negative regulator of T-cell activation and its surface expression is directly proportional to the TCR/MHC avidity (52–54). The reduction in the density of TCR self-peptide/MHC interactions in OT-I *Fas*$^{lpr/lpr}\beta_2m^{o/o}$ mice also resulted in decreased CD5 expression on the accumulating CD8$^+$ and CD4$^-$CD8$^-$ T cells. Thus, T cells receiving low-avidity TCR interactions with self-peptide/MHC appear to be selectively preserved in the absence of Fas.

OT-I *Fas*$^{lpr/lpr}$ mice do not accumulate either CD8$^+$ or CD4$^-$CD8$^-$Vα2$^+$ T cells with age. Since CD4$^+$ and CD8$^+$ T cells from both OT-I or OT-I *Fas*$^{lpr/lpr}$ mice have the capacity to undergo homeostatic proliferation in response to self-MHC/peptide, this suggested that the lack of T-cell lymphadenopathy in OT-I *Fas*$^{lpr/lpr}$ mice, which express β_2m and MHC class I, might reflect the fact that insufficient numbers of T cells in OT-I mice receive the precise signal that leads to their accumulation in the absence of Fas. Consistent with this view, OT-I CD8$^+$ T cells express high levels of surface CD5 compared with polyclonal CD8$^+$ T cells, indicating that OT-I CD8$^+$ T cells have a high-avidity interaction with self-peptide/MHC. This is also consistent with more rapid homeostatic proliferation of OT-I CD8$^+$ T cells compared with wild-type polyclonal CD8$^+$ T cells in *Rag1*$^{o/o}$ mice (K. A. Fortner, unpublished observations). Thus, if Fas preserves T cells

receiving only a limited number of TCR contacts with self-peptide/MHC, the interaction of the OT-I TCR with self-peptide/MHC may be of too high avidity to permit these $CD8^+$ T cells to become $CD4^-CD8^-V\alpha2^+$ T cells or to be preserved in the absence of Fas. However, expression of the OT-I TCR on a $\beta_2m^{o/o}$ background greatly diminished the number of self-peptide/MHC class I complexes and resulted in the accumulation of OT-I $CD8^+$ T cells in the absence of Fas despite their greatly reduced proliferation. Furthermore, the proportion of $CD4^-CD8^-V\alpha2^+$ T cells is also greatly enhanced in OT-I $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice compared with either OT-I $Fas^{lpr/lpr}$ or OT-I $\beta_2m^{o/o}$ mice. Consistent with these findings, the transient appearance of $CD4^-CD8^-TCR\alpha\beta^+$ T cells was reported following the adoptive transfer of polyclonal wild-type $CD8^+$ T cells into $\beta_2m^{o/o}$ mice (56) and in OT-I tap-deficient mice (18). This further supports the view that in the absence of Fas, the accumulating T cells have received low to negligible TCR signals.

In a second independent system, K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice manifested a profound increase in $CD4^-CD8^-TCR\alpha\beta^+$ T cells compared with conventional $Fas^{lpr/lpr}$ mice as a result of the global reduction in the number of MHC contacts for polyclonal $CD8^+$ T cells due to the absence of MHC class I expression in the periphery. The expression of MHC class I on thymic cortical epithelial cells restored positive selection of $CD8^+$ T cells in K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice; however, negative selection was impaired due to the absence of MHC class I on thymic hematopoietic and medullary epithelial cells (50). It is possible that $CD8^+$ T cells that escape negative selection could also contribute to the increased number of $CD4^-CD8^-TCR\alpha\beta^+$ T cells in K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice by increasing the size of the precursor pool. However, the ratio of $CD4^-CD8^-TCR\alpha\beta^+$ T cells to $CD8^+$ T cells was substantially higher in K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice compared with $Fas^{lpr/lpr}$ mice. This suggested that $CD8^+$ T cells in K14-β_2m $Fas^{lpr/lpr}\beta_2m^{o/o}$ mice are either more intrinsically primed to become $CD4^-CD8^-TCR\alpha\beta^+$ T cells or their $\beta_2m^{o/o}$ environment promotes the transition to a $CD4^-CD8^-TCR\alpha\beta^+$ phenotype.

The peripheral T-cell repertoire may be altered through Fas-mediated death of T cells cycling in response to self-peptide/MHC. We observed that $CD4^+$ T cells from OT-I and OT-I $Fas^{lpr/lpr}$ mice expand to a greater extent than OT-I $CD8^+$ T cells following adoptive transfer into $Rag1^{o/o}$ mice, despite the slower cell cycle initiation of the OT-I $CD4^+$ T cells. One consequence of the selective preservation of OT-I $CD4^+$ T cells in OT-I $Fas^{lpr/lpr}$ mice was the increased ratio of $CD4^+$ to $CD8^+$ T cells with age. The decrease in the number of OT-I $Fas^{lpr/lpr}$ $CD8^+$ T cells also contributed to the increased ratio of $CD4^+$ to $CD8^+$ T cells. This may have resulted from a greater conversion of OT-I $CD8^+$ T cells to $CD4^-CD8^-$ T cells in an environment lacking Fas even if the absence of Fas was not sufficient for their survival. In this regard, previous studies have shown that selective deletion of Fas on T cells did not yield either $CD4^-CD8^-TCR\alpha\beta^+$ T cells or lymphadenopathy (56). This suggests that, in addition to Fas-deficient T cells, loss of Fas expression by antigen-presenting cells may be necessary to promote the optimal conversion OT-I $Fas^{lpr/lpr}$ $CD8^+$ T cells to $CD4^-CD8^-$ T cells.

Peripheral T-cell homeostatic expansion and survival require TCR signals and are dependent on self-peptide/MHC availability (1–7). Competition between T cells for existing MHC complexes may limit T-cell survival, thereby modifying the peripheral T-cell repertoire (1,20–22). Although most TCR-$\alpha\beta$ recognizes peptides in the context of either MHC class I or class II, studies have shown that certain TCR can respond to multiple MHC class I and class II molecules. For example, T cells bearing OT-I, H-Y, 2C, HA and AND transgenic TCR have been shown to interact with both MHC class I and class II complexes (15–18). Since T-cell survival depends on the availability of self-peptide/MHC complexes, the capacity of a given TCR to interact with a spectrum of MHC complexes increases the likelihood of a T cell receiving the needed TCR survival signals. However, the same TCR structural limitations that result in decreased affinity for a given peptide/MHC complex may also result in ability to interact with a broader subset of self-peptides/MHC through degeneracy in recognition of conserved MHC motifs. Thus, the Fas-dependent removal of T cells that proliferate in response to low-avidity interactions with a given self-peptide/MHC may be critical for limiting the degree of TCR promiscuity. A potential undesirable effect of such TCR promiscuity is the increased risk of autoimmune sequelae. This may explain the need for a death receptor such as Fas to remove T cells interacting chronically but with low avidity to self-peptide/MHC complexes.

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