Prevention of Age-related T Cell Apoptosis Defect in CD2-*fas*-transgenic Mice

By T. Zhou,* C. K. Edwards III,‡ and J. D. Mountz*

*From the *Veterans Administration Medical Center and The University of Alabama at Birmingham, Birmingham, Alabama 35294; and the ‡Marion Merrell Dow Research Institute, Cincinnati, Ohio 45215*

Summary

T cell dysfunction and thymic involution are major immunologic abnormalities associated with aging. Fas (CD95) is a bifunctional molecule that is critical for apoptosis and stimulation during T cell development, but the role of Fas during aging has not been determined. Fas expression and function on T cells from old (22–26-mo-old) mice was compared with young (2-mo-old) mice and old CD2-*fas*-transgenic mice. Fas expression and ligand-induced apoptosis were decreased on T cells from old mice compared with young mice. This correlated with an age-related increase in $CD44^+Fas^-$ T cells. There was a marked decrease in the proliferation of T cells from old mice after anti-CD3 stimulation compared with young mice. Anti-CD3–stimulated T cells from young mice exhibited increased production of interleukin (IL)-2 and decreased production of interferon-γ and IL-10 compared with old mice. There was an age-related decrease in the total thymocyte count from 127 ± 10 cells in young mice compared with 26 ± 8 × 10^6 in old mice. In 26-mo-old CD2-*fas*-transgenic mice, Fas and CD44 expression, Fas-induced apoptosis, T cell proliferation, and cytokine production were comparable to that of the young mice. These results suggest that T cell senescence with age is associated with defective apoptosis, and that the CD2-*fas* transgene allows maintenance of Fas apoptosis function and T cell function in aged mice comparable to that of young mice.

Age-related immune dysfunctions of T cells include thymic involution (1), decreased T cell response to mitogens or antigens (2–6), altered cytokine expression (7–10), and altered phenotype (11–13). The decrease in spleen or LN T cell responsiveness has been related to an increase in senescent memory T cells (11, 13), which exhibit defective phosphorylation after stimulation (14–18). Thymic involution has been proposed to be due either to defects in thymocyte precursors derived from stem cells or a defect in expression of thymic factors or growth factors required for normal T cell development (19–23). Recent evidence indicates that depletion of thymocyte stem cells cannot completely account for thymic involution with age (24). Evidence to support an age-related deficiency of growth factors has been provided by the observation that replacement of growth factors or hormones can inhibit thymic involution or T cell dysfunction with aging (19–23).

Fas/Apo-1 (CD95) is a cell surface–signaling molecule that mediates apoptosis (25, 26). The apoptosis defect that results from defective Fas expression in *lpr* mice results in survival of self-reactive T cells, loss of self-tolerance, autoimmunity, and lymphoproliferation (27). There is a close association between apoptosis and proliferation mediated by Fas and other apoptosis molecules, including p53 and c-*myc* (28, 29). Apoptosis that follows stimulation and proliferation has been proposed to be critical for the regulation of the T cell response to stimuli by removing cells with metabolic defects or autoimmune potential (30, 31). We propose that this process of selective depletion of T cells is necessary throughout the life span of the animal to prevent accumulation of dysfunctional senescent T cells.

This article demonstrates that Fas expression and function are decreased in T cells of old mice. Age-related Fas dysfunction can be circumvented in CD2-*fas*-transgenic mice. The presence of the CD2-*fas* transgene allows the maintenance of thymocyte numbers in aged mice comparable to that observed in young mice. The syngeneic mixed lymphocyte reaction (SMLR)[1] and the response of peripheral T cells to stimulation by CD3 cross-linking is comparable to that of young animals. Cytokine expression after stimulation was also comparable to levels observed in young mice.

Materials and Methods

Mice. CD1 mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and kept in specific pathogen-free conditions.

[1] *Abbreviation used in this paper:* SMLR, syngeneic mixed lymphocyte reaction.

Production of CD2-fas Transgenic CD1 Mice. CD1 mice were used for superovulation and production of the CD2-*fas*–transgenic mice as previously described (32). The CD2-*fas* construct was made by ligation of a full-length murine *fas* cDNA into an EcoRI site in front of exon 1 of a human CD2 minigene consisting of 5.5 kb of the 5′ flanking sequence, exon 1, the first intron, fused exons 2–5, and 2.1 kb of the 3′ flanking sequence. The 3′ sequence of this CD2 minigene has been shown to be sufficient to allow copy-dependent, integration-independent expression primarily in $CD2^+$ T cells in transgenic mice (33, 34). Transgenic mice were identified by tail DNA analysis and housed with littermate controls for 24 mo while being maintained on food and water ad libitum. The young 2-mo-old CD1 mice used in the experiments were derived from a breeding colony of CD1 mice.

Southern Blot Analysis. Tail DNA was prepared and digested with the indicated restriction enzymes. Approximately 10 μg of the digested DNA was separated on a 0.7% agarose gel, blotted to a nylon membrane, and hybridized with a ^{32}P-labeled full-length *fas* cDNA probe extending from 49 to 1,033 bp of the murine sequence as previously described (32).

Expression of Murine Fas Ligand. The murine Fas ligand was cloned and sequenced using PCR primers that were prepared according to the sequence of the known mouse Fas ligand (35, 36). The full-length murine Fas ligand cDNA was cloned into the pcDNA1 expression vector (Invitrogen, San Diego, CA). 20 μg of purified plasmid DNA was precipitated with 500 μg/ml of DEAE-Dextran (Sigma Chemical Co., St. Louis, MO), added to 5×10^6 COS7 cells in a 10-cm cell culture dish containing 10 ml of serum-free RPMI 1640 and 10 mM chloroquine (Sigma Chemical Co., St. Louis, MO), and incubated at 37°C for 4 h. The transfected cells were then treated with 10% DMSO + RPMI 1640 for 2 min, and grown in 12% FCS + RPMI 1640 for 72 h. The supernatant was collected and then stored at −20°C until use.

Preparation of Murine Fas–Human IgG1 Fusion Protein. A murine Fas–human IgG1 (*fas*-hIg) fusion protein was prepared as previously described (37). Briefly, the extracellular domain of the mouse *fas* cDNA was fused with the Fc portion of human IgG1 gene to form an open reading frame. The fusion gene was cloned into the pcDNA1 eukaryotic expression vector (Invitrogen) and transfected into COS7 cells as described above. The fusion protein was purified on an anti–human IgG agarose column (Sigma Chemical Co.) and concentrated to 1 mg/ml in PBS.

T Cell Enrichment. Single-cell suspensions of lymphocytes or spleen cells were enriched using a T cell enrichment column (R&D Systems, Inc., Minneapolis, MN) according to the directions of the manufacturer. Purified T cell populations were >98% T cells as determined by flow cytometry analysis of CD3 expression.

Flow Cytometry Analysis. Anti-CD3 (clone: 145.2C11), anti-CD4 (clone: GK 1.5), anti-CD8 (clone: 53-47) and anti-CD44 (clone: 1M7), and anti–mouse Fas (clone: Jo2) were purchased from PharMingen (San Diego, CA). Single cell suspensions of lymphocytes, spleen cells, or thymocytes (10^6 per sample) were stained in FACS® buffer (PBS with 5% FCS and 0.1% sodium azide; Becton Dickinson & Co., Mountain View, CA) and optimal concentrations of antibodies. For two- and three-color analyses, cells were labeled with optimal concentrations of FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 or biotin-conjugated anti-Fas or anti-CD44. Biotintylated conjugated antibodies were revealed by Tandem–avidin. Viable cells were determined by forward and side light scatter using standard methods and were <6% of the sample size. Viable cells (10,000 per sample) were analyzed by flow cytometry on a FACScan® (Becton Dickinson & Co.) with logarithmic scales.

Stimulation by SMLR or Anti-CD3. Anti-CD3 (clone 145.2C11) mAb was diluted to 1 μg/ml and coated on flat-bottom 96-well plates (Costar Corp., Cambridge, MA) by incubation for 12 h at 4°C. The plates were thoroughly washed using sterile PBS, and enriched T cells were added in duplicate cultures for 72 h. For SMLR proliferative responses, 10^6 enriched T cells were cultured in 200 μl of RPMI supplemented with 10% FCS. For some experiments, the SMLR was carried out in the presence of the Fas fusion protein (0.1 μg/ml) or plate-bound anti–mouse Fas mAb (1 μg/ml). Proliferation was determined at different time points by a 12-h pulse of 1 μCi [^3H]TdR (Amersham Corp., Arlington Heights, IL), and [^3H]TdR uptake was measured by liquid scintillation counting.

Assay of Cytokines. Cytokines were assayed using ELISA kits according to the manufacturer's instructions (Genzyme Corp., Cambridge, MA). The OD_{405} was measured on a microplate reader (Emax; Molecular Devices Corp., Menlo Park, CA). Duplicate samples were assayed in three separate experiments and the mean was determined. The statistical significance was determined using the Wilcoxon sign–rank nonparametric test.

In Situ Nick Translation Apoptosis Staining. Nuclear DNA fragmentation was carried out as previously described (38). For cell suspensions, 10^5 cells were cytospun onto poly-L-lysine (Sigma Chemical Co.) –precoated slides. The slides were fixed for 30 min in 10% formalin–PBS buffer and then washed with H_2O for 2 min, six times. Fresh proteinase K (20 μg/ml; Boehringer Mannheim Corp., Indianapolis, IN) was added onto the slides and incubated at room temperature for 15 min. After washing with H_2O, terminal deoxynucleotide transferase (TdT) (1 U/ml; Boehringer Mannheim Corp.) and digitonigen-modified dUTP (0.15 μl/ml; Boehringer Mannheim Corp.) was added in TdT buffer. A coverglass was applied and the slides incubated for 1 h at 37°C in a humidified chamber. After removing the coverglass, the slide was washed once with PBS, followed by incubation in 5% BSA/FACS® buffer for 15 min at room temperature. The slides were incubated with an antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Corp.) at 1:50 dilution with FACS® buffer for 30 min at room temperature. After washing six times in PBS, the slides were incubated in alkaline phosphatase buffer with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma Chemical Co.) and incubated for 30 min at room temperature.

Statistical Analysis. Statistical analysis of proliferation and apoptosis after SMLR or anti-CD3 stimulation was carried out using the analysis of variance model at each time point.

Results

CD2-fas–transgenic Mice. Tail DNA was prepared from mice at 4 wk of age, digested with EcoRI restriction enzyme, and hybridized with the full-length mouse Fas cDNA probe. One of the four founder CD1 CD2-*fas*–transgenic mice was identified as having a single 1.1-kb transgenic Fas cDNA integration band (Fig. 1, lane *3*) and therefore mated with CD1 breeder mice. Transgenic and nontransgenic offspring of this mating were studied at ages 22–26 mo.

Fas Expression and Function on Lymphocytes. Cell surface Fas expression was decreased on lymphocytes from 26-mo-old nontransgenic CD1 mice compared with 2-mo-old nontransgenic mice (Fig. 2). Fas expression was increased to youthful levels in 26-mo-old CD1 CD2-*fas*–transgenic mice. Fas ligand–induced apoptosis was markedly decreased in LN

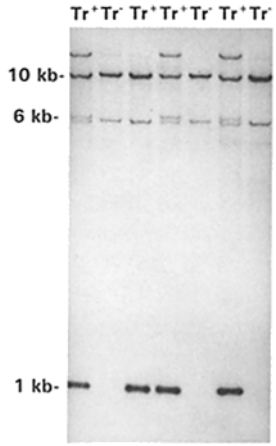


Figure 1. Analysis of CD2-*fas*–transgenic CD1 mice by Southern blot analysis. Tail DNA was digested with the EcoRI restriction enzyme, which releases the 1.1-kb *fas* cDNA insert from CD2-*fas*–transgenic mice.

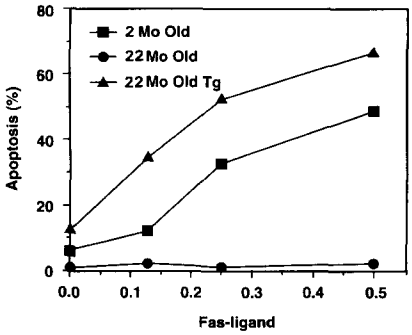


Figure 3. Correction of Fas ligand–induced apoptosis in CD2-*fas*–transgenic aged mice. Lymphocyte suspensions (10^6 per sample) were cultured for 18 h with different dilutions of supernatant of COS7 cells expressing the Fas ligand ranging from 0 to 0.5 (50:50 vol/vol). Lymphocytes were obtained from 2- (■), 22- (●), or 22-mo-old CD2-*fas*–transgenic mice (▲). Apoptosis was determined by the in situ nick translation assay of single-cell suspensions of lymphocytes as previously described (50). The results represent the mean ± SEM for three separate mice assayed individually in triplicate culture wells.

cells from aged mice, and development of this apoptosis defect was prevented in CD2-*fas*–transgenic aged mice (Fig. 3).

Syngeneic Mixed Lymphocyte Response. Spleen cells from 2-mo-old mice exhibited a weak SMLR characterized by a minimal proliferative response (Fig. 4 *A*). This was not caused by an absence of IL-2 since the cells were cultured in the presence of 50 U/ml of IL-2. In contrast, there was a statistically significant increased proliferative response ($p < 0.001$) after SMLR which peaked at 96 h using enriched spleen T cells of 26-mo-old mice. The SMLR response of spleen cells from 26-mo-old CD2-*fas*–transgenic mice was similar to that observed in 2-mo-old mice. On days 1 and 3 of the SMLR, there was minimum apoptosis (20–30%) in all three groups of mice ($p < 0.05$). However, on days 5 and 7, apoptosis increased to 60–70% of spleen cells in both 2- and 26-mo-old CD2-*fas*–transgenic mice (Fig. 4 B). In contrast, the percentage of cells undergoing apoptosis remained significantly lower in aged mice on days 5 and 7 of the SMLR ($p < 0.001$).

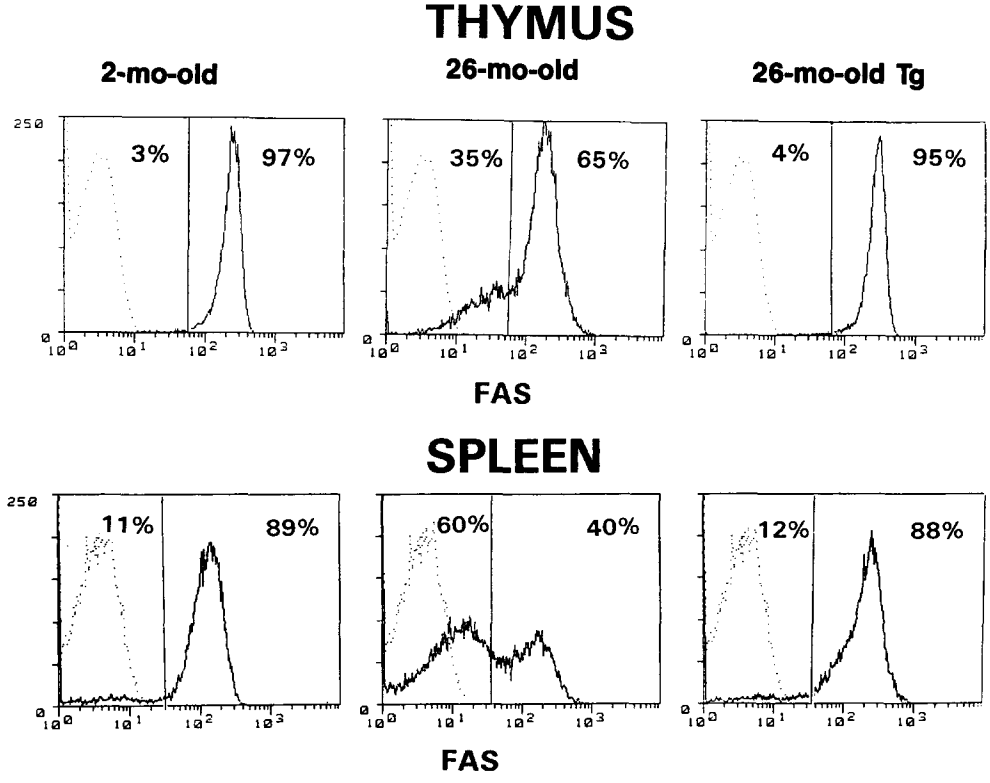


Figure 2. Expression of Fas on thymocytes and spleen cells. Single-cell suspensions of thymocytes and spleen cells from 2-mo-old, 26-mo-old, and 26-mo-old Fas-transgenic mice (10^6 per sample) were stained with anti-Fas. The histograms of Fas expression are representative of at least 10 mice from each group. The gates used to define lymphocyte populations are indicated by the vertical lines. Negative control staining is shown as the dotted curves. There was a statistically significant increase in Fas$^-$ cells in the groups of 26-mo-old mice compared with 2-mo-old and 26-mo-old Fas-transgenic mice ($p < 0.01$). Viable cells (10,000 per sample) were analyzed by flow cytometry on a FACScan® with logarithmic scales.

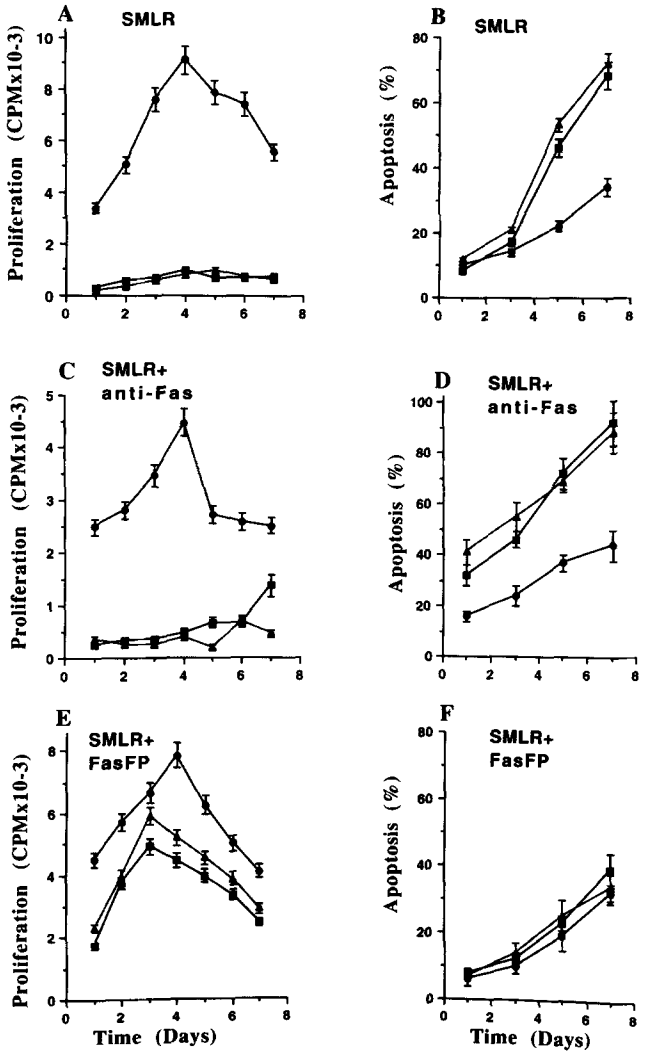


Figure 4. Effect of anti-Fas and Fas fusion protein on SMLR-induced proliferation and apoptosis. Lymphocyte suspensions (10^6 per sample) were cultured in 96-well plates in the presence of IL-2 (50 μm/ml). The proliferation was determined at the indicated times by [^{3}H]thymidine incorporation and apoptosis determined by the in situ nick translation method. (*A*) SMLR proliferative response. (*B*) Apoptosis of SMLR stimulated cells. (*C*) SMLR proliferative response in plates that had been precoated with anti-mouse Fas antibody (1 μg/ml). (*D*) Apoptosis of SMLR-stimulated cells carried out on plates precoated with anti-mouse Fas (1 μg/ml). (*E*) SMLR proliferative response in the presence of Fas fusion protein (*FasFP*; 0.1 μg/ml). (*F*) Apoptosis of SMLR-stimulated cells carried out in the presence of Fas fusion protein (0.1 μg/ml). —■—, 2-mo-old; —●—, 26-mo-old; —▲—, 26-mo-old transgenic.

To determine if the cells undergoing SMLR were sensitive to Fas mediated apoptosis, SMLR was carried out in the presence of 1 μg/ml of purified anti-Fas antibody. Anti-Fas antibody did not prevent the SMLR proliferative responses by spleen cells from 26-mo-old nontransgenic mice and had no effect on the percentage of cells undergoing apoptosis (compare Fig. 4, *A* and *B* with Fig. 4, *C* and *D*). Blocking of the Fas–Fas ligand interaction during SMLR with a Fas fusion protein resulted in a statistically increased proliferative response and decreased apoptosis in young 2-mo-old mice and in 26-mo-old CD2-*fas*-transgenic mice (Fig. 4, *E* and *F*) ($p < 0.001$). In contrast, the SMLR and apoptosis responses from 26-mo-old mice was not significantly affected by the presence of Fas fusion protein ($p > 0.05$). These results indicate that the increased SMLR exhibited by spleen cells from 26-mo-old mice is associated with defective Fas signaling and apoptosis, which is present in 2- and in 26-mo-old CD2-*fas*-transgenic mice, and that the increased SMLR in aged mice is caused by a defect in apoptosis. This failure could not be corrected by stimulation with anti-Fas antibody, consistent with the finding that Fas expression is defective on T cells from aged mice.

Prevention of Age-related Decrease in Proliferation and Apoptosis in CD2-fas-transgenic Mice. T cell proliferation after anti-CD3 cross-linking peaked at 72 h in 2-mo-old CD1 mice (Fig. 5 *A*). In contrast, T cells from 26-mo-old mice exhibited a statistically significant decreased proliferative response ($p < 0.01$) on days 2–4 after anti-CD3 cross-linking and a statistically significant increase ($p < 0.01$) on days 5–7 after anti-CD3 cross-linking. The anti-CD3 proliferative responses in 26-mo-old CD2-*fas*-transgenic mice (and 2-mo-old CD2-*fas*-transgenic mice, data not shown) were significantly decreased ($p < 0.01$) but similar to those observed in 2-mo-old mice with peak proliferation on day 3 followed by a decrease in proliferation. Apoptosis after anti-CD3 stimulation was significantly increased ($p < 0.01$) in 2-mo-old CD1 mice and in 26-mo-old CD2-*fas*-transgenic mice compared with 26-mo-old nontransgenic mice (Fig. 5 *B*).

Correction of Cytokine Response in CD2-fas-transgenic Aged Mice. Decreased IL-2 production after anti-CD3 cross-linking has been noted in old mice and humans (3–5). The 50% decrease in IL-2 production after anti-CD3 cross-linking in 22-mo-old mice was partially corrected in 22-mo-old CD2-*fas*-transgenic mice (Fig. 6 *A*). There was an increase in IL-4 production on days 2 and 4 after anti-CD3 cross-linking in aged mice compared with 2-mo-old mice, and this increase was partially prevented in 22-mo-old CD2-*fas*-transgenic mice (Fig. 6 *B*). There was also an increased production of IL-10, which peaked on day 6 in 22- and 2-mo-old mice in response to anti-CD3 cross-linking (Fig. 6 *C*). This increased IL-10 production was prevented in CD2-*fas*-transgenic mice. These results indicate that the aged mice exhibit a shift toward a Th2-type cytokine response (IL-4, IL-10) rather than a Th1 cytokine response (IL-2), and that this shift can be prevented in CD2-*fas*-transgenic aged mice.

Correction of CD44 Expression on Thymocytes and Spleen Cells. Thymocyte subpopulations exhibiting defective Fas expression were primarily $CD4^+CD8^+$ (double-positive) and $CD4^-CD8^-$ (double-negative) thymocytes in 26-mo-old nontransgenic CD1 mice (Fig. 7 *A*). Most of the thymocytes with low Fas expression exhibited increased expression of CD44. Thymocytes from 26-mo-old CD2-*fas*-transgenic mice were phenotypically similar to those from 2-mo-old mice. Less than 20% of enriched T cells from the spleen of 2-mo-old nontransgenic mice express CD44, and ~90% were Fas^+. In 26-mo-old nontransgenic CD1 mice, 60% of

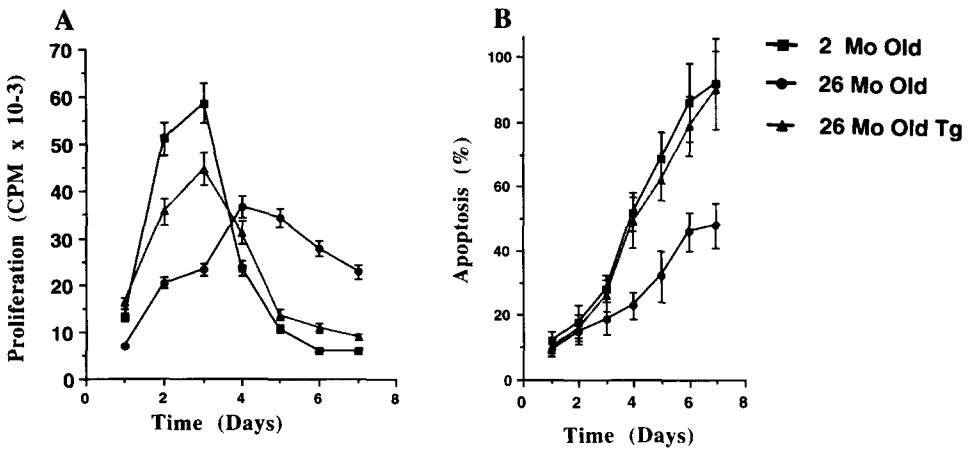


Figure 5. Correction of proliferation and apoptosis after CD3 stimulation in aged CD2-*fas*-transgenic mice. Lymphocyte suspensions (10^6 per sample) were cultured in 96-well plates that had been precoated with anti-CD3 (10 μg/ml). (*A*) The proliferation was determined at the indicated times by the [^{3}H]thymidine incorporation assay. (*B*) Apoptosis was determined by the in situ nick translation assay of single-cell suspensions of lymphocytes as previously described (50). The results represent the mean ± SEM for three separate mice assayed individually in triplicate culture wells.

enriched spleen T cells were Fas$^-$, and ~80% were CD44$^+$. In 26-mo-old Fas-transgenic mice, expression of CD44, CD4, and Fas was similar to that observed in 2-mo-old mice.

Prevention of Thymic Involution in Aged CD2-fas-transgenic Mice. There was a markedly decreased number of thymocytes in 22-mo-old CD1 mice compared with 2-mo-old CD1 mice, which was prevented in the presence of the 22-mo-old CD2-*fas*-transgenic mice (Fig. 8 *A*). Consistent with the finding of increased thymocyte numbers in young and aged CD2 Fas-transgenic mice, thymocyte apoptosis was decreased in young and aged CD2-*fas*-transgenic mice on days 2–6 after CD3 cross-linking (Fig. 8 *B*). In contrast, in 22-mo-old mice, there was increased apoptosis on days 2–6 after CD3 cross-linking, suggesting high utilization of programmed cell death mechanisms within the aged thymus. These results indicate that the CD2-*fas* transgene might provide an intrathymic signal for thymic development, thereby preventing apoptosis after CD3 cross-linking in vitro.

Discussion

The immunologic mechanism underlying defective Fas function with age is not known. One possibility is that there is an intrinsic age-related defect in transcriptional or the post-transcriptional factors that regulate Fas expression. This view is supported by the fact that Fas is a member of a family of receptors that contain cysteine repeat domains and this family of receptors includes TNF-R and CD40-L, which also exhibit decreased expression with age (39–41). Another possibility is that the Fas signaling-function might be impaired with aging, which could further accentuate an expression defect. Fas signaling involves a phosphatase dependent pathway, which is decreased with aging (42). Further investigation into the mechanisms of defective Fas signaling with aging could lead to novel intervention therapies to restore normal Fas signaling.

Our data indicate that Fas function is decreased more than Fas expression in old mice. One possibility is that high Fas expression is required for Fas-induced apoptosis. Enforced upregulation of Fas in the CD2-*fas*-transgenic mice allows the maintenance of Fas function and production of Fas-sensitive T cells in aged mice. This dissociation of Fas expression and function has also been observed in the thymus, where all cells express intermediate to high levels of Fas, but not all thymocytes are sensitive to Fas-induced apoptosis (Zhou, T., P. Yang, Z. Wang, and J. D. Mountz, manuscript submitted for publication). Another possibility may be that alternatively spliced or nonfunctional forms of the Fas molecule might be present

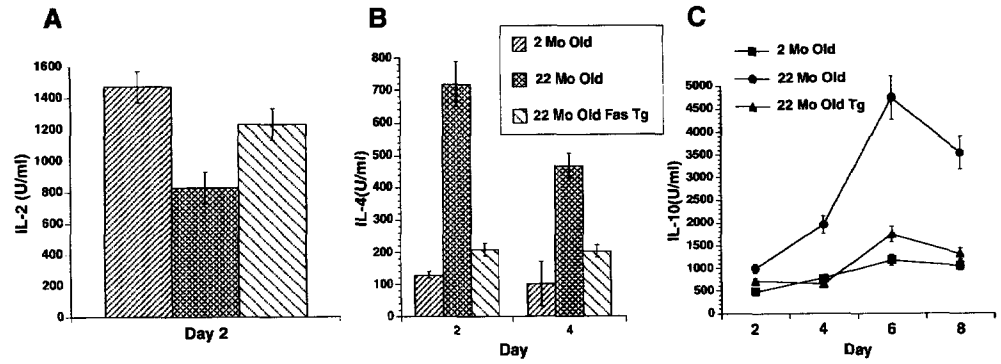
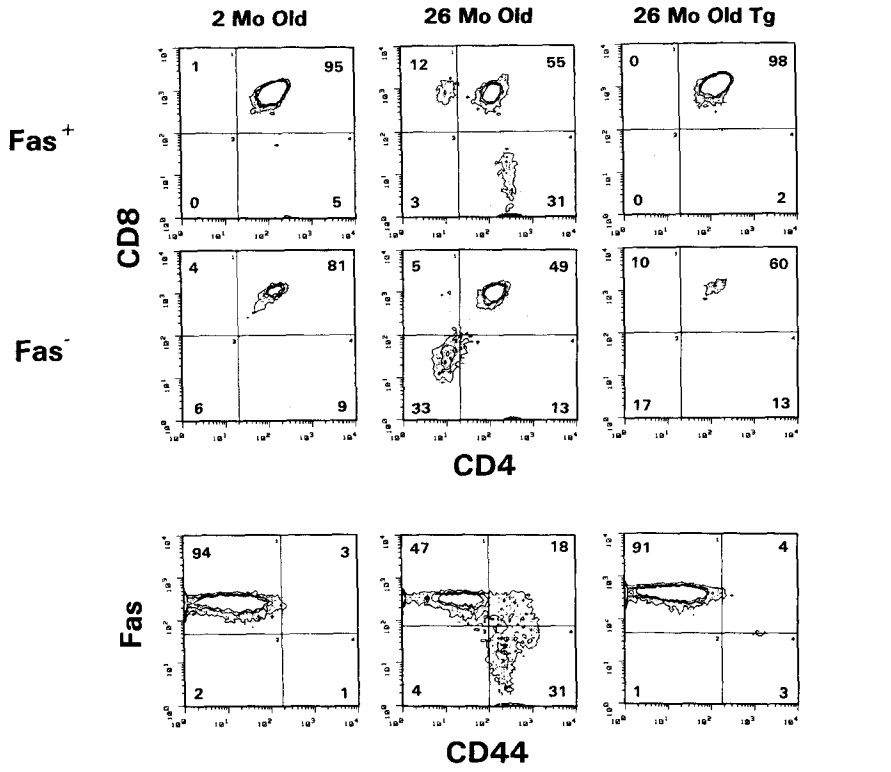


Figure 6. Correction of cytokine production after anti-CD3 cross-linking in aged CD2-*fas*-transgenic mice. Lymphocyte suspensions (2 × 10^6 cells per ml) from 22-mo-old mice were cultured in 24-well plates that had been precoated with anti-CD3 (10 μg/ml). Cytokine concentrations were determined at the indicated times by ELISA. (*A*) IL-2 after 2-d culture. (*B*) IL-4 after 2- and 4-d culture. (*C*) IL-10 at 2–8-d culture. Each data point represents the average of five mice per group, and the error bars represent the SEM.



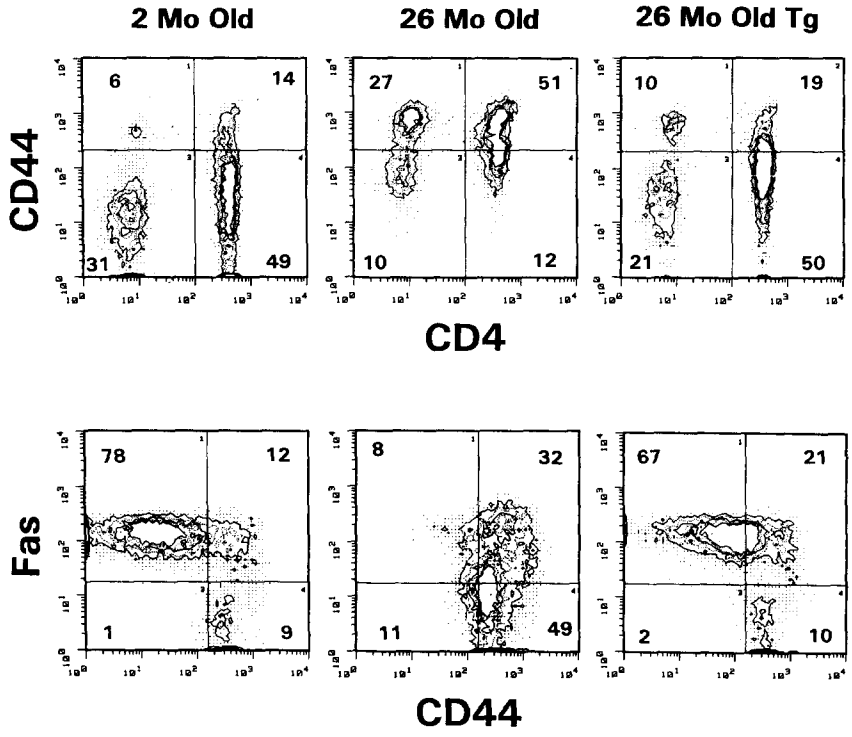


Figure 7. Correction of CD44 expression on thymocytes and spleen cells in aged CD2-*fas*-transgenic mice. Single-cell suspensions of thymocytes and spleen cells from 2-, 26-, and 26-mo-old Fas-transgenic mice (10^6 per sample) were analyzed for expression of CD4 and/or CD8, Fas, and CD44. (*A*) Thymocytes were labeled with anti-Fas, anti-CD4, and anti-CD8, or anti-Fas and anti-CD44. Fas^+ and Fas^- thymocyte populations are defined as in Fig. 2. (*B*) Enriched spleen T cells were labeled with anti-CD44, anti-CD4, and anti-Fas. The gates used to define lymphocyte populations are indicated by the vertical and horizontal lines. Viable cells (10,000 per sample) were analyzed by flow cytometry on a FACScan® with logarithmic scales.

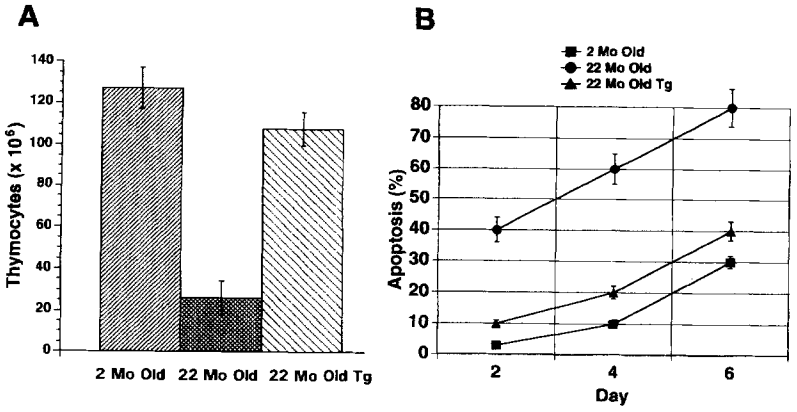


Figure 8. Thymocyte cell count and apoptosis after anti-CD3 cross-linking. Single-cell suspensions of thymocytes were prepared from 2-, 22-, or 22-mo-old CD2-*fas*-transgenic mice. Thymocyte suspensions (10^6 per sample) were cultured in 96-well plates that had been precoated with anti-CD3 (10 μg/ml). (*A*) Total number of thymocytes. (*B*) Percentage of thymocytes undergoing apoptosis at different time points after CD3 stimulation as determined by the in situ nick translation method. Each data point represents the average of five mice per group, and the error bars represent the SEM.

on T cells from aged mice, and that these forms can interact with the Fas antibody but do not signal apoptosis. We have reported the presence of an alternatively spliced Fas molecule that results in the secretion of soluble Fas and inhibition of apoptosis (37). Additionally, other investigators have reported a mutation of Fas leading to defective apoptosis signaling in CBA/ HeN-*lpr/lpr* mice (27).

Previous results indicate that there are at least two pathways for apoptosis of thymocytes (28, 29, 43). Apoptosis of thymocytes that have undergone DNA damage by x-irradiation require p53, whereas steroid-induced apoptosis does not. In the Fas-mutant *lpr* mice, there is an increase in steroid-induced apoptosis of thymocytes, but a decrease in Fas-induced apoptosis caused by the Fas mutation (44). The present results also indicate that there is more than one pathway for apoptosis of T cells in the thymus and LN. One is active in young mice, whereas a second Fas-independent pathway is active for deletion of T cells from aged mice after stimulation. The shift from Fas-dependent to Fas-independent apoptosis with aging may account for altered phenotype and function of T cells with aging.

Loss of Fas function with age is associated with a decrease of the Th1 cytokine IL-2 and an increase of the Th2 cytokines IL-4 and IL-10. The overuse of the Th2 pathway with aging has also been previously reported by other investigators (45, 46). We have shown recently that T cells that have been stimulated to undergo preferential development since Th1 T cells express Fas and Fas ligand, whereas T cells stimulated to undergo development as Th2 T cells do not express Fas or Fas ligand (Zheng, R., J. Pan, J. Mountz, T. Zhou, and C. T. Weaver, manuscript submitted for publication). The shift of Th1 to Th2 T cells with aging may be caused by defective Fas–Fas ligand signaling. The restoration of preferential development of Th1 cells by the *fas* transgene in aged mice suggests that Fas interaction with its ligand has been corrected, and that Fas interaction may play a critical role in T cell development as well as T cell apoptosis with aging.

Immune system aging is associated with an increase in percentage of memory T cells characterized by expression of CD44 and CD45RO (11–13, 47–50). In 26-mo-old nontransgenic mice, approximately one third of thymocytes and 50–60% of purified spleen T cells, increased CD44 expression was associated with low expression of cell surface Fas. $CD44^+$ memory T cells would therefore be insensitive to Fas-induced apoptosis. The percentage of $CD44^+$ T cells was greatly reduced in CD2-*fas*-transgenic aged mice. These results indicate that development of $CD44^+$ memory T cells in aged mice is related to several aspects of cell development, including Fas expression. In both the spleen and thymus, there was also a small population of cells that express high Fas and also high CD44, indicating that other factors in addition to Fas expression influence CD44 expression.

Rescue of thymocytes from apoptosis by the provision of necessary signaling molecules or growth factors may be an important mechanism in the restoration of T cell function with aging. This is consistent with a number of reports that certain growth factors, including growth hormone from transplanted pituitary glands (23), can prevent age-related thymic atrophy. Fas has been proposed to serve as a costimulatory molecule during thymic development of T cells (51), and our data show that decreased Fas expression and function with age leads to increased apoptosis of thymocytes. These results were unexpected because Fas was first identified as a molecule capable of inducing apoptosis in certain cell lines (25, 26). However, it is likely that Fas signaling leads to different responses, depending on the cell type and stage of differentiation. In support of this, in Fas-defective *lpr/lpr* mice, and Fas ligand–defective *gld/gld* mice, defective apoptosis of thymocytes has been difficult to detect (52), and the primary defect in these mice is abnormal T cell differentiation and tolerance loss (53, 54). Therefore, we propose that defective intrathymic Fas signaling with age leads to a developmental defect and increases thymocyte apoptosis by a Fas-independent pathway, and that defective Fas signaling plays a major role in development of thymic atrophy with aging.

We thank Yi Chun Jian for assistance in production of the Fas fusion protein, Fiona Hunter for review of the manuscript, and Brenda Bunn for expert secretarial assistance.

This work was supported by National Institute on Aging grant R01-AG11653, VA Merit Review and Career Development Award. T. Zhou is a recipient of an Arthritis Foundation Investigator Award.

Address correspondence to J. D. Mountz, MD, PhD, The University of Alabama at Birmingham, Division of Clinical Immunology and Rheumatology, 701 South 19th Street, UAB Station, LHRB 473, Birmingham, AL 35294-0007. The present address for C. K. Edwards III, PhD, is Amgen Boulder, Inc., Department of Inflammation, 3200 Walnut Street, Boulder, CO 80301-2546.

Received for publication 7 October 1994 and in revised form 24 February 1995.

References

1. Hadden, J.W, P.H. Malec, J. Coto, and E.M. Hadden. 1992. Thymic involution in aging. Prospects for correction. *Ann. NY Acad. Sci.* 673:231–239.
2. Powers, D.C., and R.B. Belshe. 1993. Effect of age on cytotoxic T lymphocyte memory as well as serum and local antibody responses elicited by inactivated influenza virus vaccine. *J. Infect. Dis.* 167:584–592.
3. Flurkey, K., R.A. Miller, and D.E. Harrison. 1992. Cellular determinants of age-related decrements in the T-cell mitogen response of B6CBAF1 mice. *J. Gerontol.* 47:B115–B120.
4. Kirschmann, D.A., and D.M. Murasko. 1992. Splenic and inguinal lymph node T cells of aged mice respond differently to polyclonal and antigen-specific stimuli. *Cell. Immunol.* 139: 426–437.
5. Song, L.J., J.E. Nagel, F.J. Chrest, G.D. Collins, and W.H. Adler. 1993. Comparison of CD3 and CD2 activation pathways in T cells from young and elderly adults. *Aging (Milano).* 4: 307–315.
6. McElhaney, J.E., G.S. Meneilly, B.L. Beattie, C.D. Helgason, S.F. Lee, R.D. Devine, and R.C. Bleackley. 1992. The effect of influenza vaccination on IL2 production in healthy elderly: implications for current vaccination practices. *J. Gerontol.* 47:M3–M8.
7. Ernst, D.N., W.O. Weigle, D.J. Noonan, D.N. McQuitty, and M.V. Hobbs. 1993. The age-associated increase in IFN-gamma synthesis by mouse $CD8^+$ T cells correlates with shifts in the frequencies of cell subsets defined by membrane CD44, CD45RB, 3G11, and MEL-14 expression. *J. Immunol.* 151: 575–587.
8. Ershler, W.B. 1993. Interleukin-6: a cytokine for gerontologists. *J. Am. Geriatr. Soc.* 41:176–181.
9. al-Rayes, H., W. Pachas, N. Mirza, D.J. Ahern, R.S. Geha, and D. Vercelli. 1992. IgE regulation and lymphokine patterns in aging humans. *J. Allergy Clin. Immunol.* 90:630–636.
10. Daynes, R.A., and B.A. Araneo. 1992. Natural regulators of T-cell lymphokine production *in vivo*. *J. Immunother.* 12:174–179.
11. Okumura, M., Y. Fujii, Y. Takeuchi, K. Inada, K. Nakahara, and H. Matsuda. 1993. Age-related accumulation of $LFA\text{-}1^{high}$ cells in a $CD8^+CD45RA^{high}$ T cell population. *Eur. J. Immunol.* 23:1057–1063.
12. Howard, C.J., P. Sopp, and K.R. Parsons. 1992. L-selectin expression differentiates T cells isolated from different lymphoid tissues in cattle but does not correlate with memory. *Immunology.* 77:228–234.
13. Thoman, M.L., D.N. Ernst, M.V. Hobbs, and W.O. Weigle. 1993. T cell differentiation and functional maturation in aging mice. *Adv. Exp. Med. Biol.* 330:93–106.
14. Shi, J., and R.A. Miller. 1993. Differential tyrosine-specific protein phosphorylation in mouse T lymphocyte subsets. Effect of age. *J. Immunol.* 151:730–739.
15. Heyeck, S.D., and L.J. Berg. 1993. Developmental regulation of a murine T-cell-specific tyrosine kinase gene. *Proc. Natl. Acad. Sci. USA.* 90:669–673.
16. Patel, H.R., and R.A. Miller. 1992. Age-associated changes in mitogen-induced protein phosphorylation in murine T lymphocytes. *Eur. J. Immunol.* 22:253–260.
17. Witkowski, J., and R.A. Miller. 1993. Increased function of P-glycoprotein in T lymphocyte subsets of aging mice. *J. Immunol.* 150:1296–1306.
18. Saini, A., and Y. Sei. 1993. Age-related impairment of early and late events of signal transduction in mouse immune cells. *Life Sci.* 52:1759–1765.
19. Hadden, E.M., P. Malec, M. Sosa, and J.W. Hadden. 1992. Mixed interleukins and thymosin fraction V synergistically induce T lymphocyte development in hydrocortisone-treated aged mice. *Cell. Immunol.* 144:228–236.
20. Candore, G., G. DiLorenzo, C. Caruso, M.A. Modica, A.T. Colucci, G. Crescimanno, A. Ingrassia, G.B. Sangiorgi, and A. Salerno. 1992. The effect of age on mitogen responsive T cell precursors in human beings is completely restored by interleukin-2. *Mech. Ageing Dev.* 63:297–307.
21. Goso, C., D. Frasca, and G. Doria. 1993. Effect of synthetic thymic humoral factor (THF-gamma 2) on T cell activities in immunodeficient ageing mice. *Clin. Exp. Immunol.* 87:346–351.
22. Fridkis-Hareli, M., L. Abel, and A. Globerson. 1992. Patterns of dual lymphocyte development in co-cultures of foetal thymus and lymphohaemopoietic cells from young and old mice. *Immunology.* 77:185–188.
23. Li, Y.M., D.L. Brunke, R. Dantzer, and K.W. Kelley. 1992. Pituitary epithelial cell implants reverse the accumulation of $CD4^-CD8^-$ lymphocytes in thymus glands of aged rats. *Endocrinology.* 130:2703–2709.
24. Mehr, R., L. Abel, P. Ubezio, A. Globerson, and Z. Agur. 1993. A mathematical model of the effect of aging on bone marrow cells colonizing the thymus. *Mech. Ageing Dev.* 67: 159–172.
25. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:233–241.
26. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor.

J. Exp. Med. 169:1747–1756.
27. Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (Lond.).* 356:314–317.
28. Clarke, A.R., C.A. Purdie, D.J. Harrison, R.G. Morris, C.C. Bird, M.L. Hooper, and A.H. Wyllie. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature (Lond.).* 362:849–852.
29. Lowe, S.W., E.M. Schmitt, S.W. Smith, B.A. Osborne, and T. Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature (Lond.).* 362:847–849.
30. Goya, R.G. 1992. Hormones, genetic program and immunosenescence. *Clin. Exp. Immunol.* 9:188–194.
31. Collins, M.K., G.R. Perkins, G. Rodriguez-Tarduchy, M.A. Nieto, and A. Lopez-Rivas. 1994. Growth factors as survival factors: regulation of apoptosis. *Bioessays.* 16:133–138.
32. Wu, J., T. Zhou, J. He, and J.D. Mountz. 1993. Autoimmune disease in mice due to integration of an endogenous retrovirus in an apoptosis gene. *J. Exp. Med.* 178:461–468.
33. Greaves, D.R., F.D. Wilson, G. Lang, and D. Kioussis. 1989. Human CD2 3'-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell.* 56:979–986.
34. Lang, G., D. Wotton, M.J. Owen, W.A. Sewell, M.H. Brown, D.Y. Mason, M.J. Crumpton, and D. Kioussis. 1988. The structure of the human CD2 gene and its expression in transgenic mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1675–1682.
35. Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 75:1169–1178.
36. Takahashi, T., M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell.* 76:969–976.
37. Cheng, J., T. Zhou, C. Liu, J.P. Shapiro, M.J. Brauer, M.C. Kiefer, P.J. Barr, and J.D. Mountz. 1994. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science (Wash. DC).* 263:1759–1762.
38. Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* 119:492–503.
39. Song, L., Y.H. Kim, R.K. Chopra, J.J. Proust, J.E. Nagel, A.A. Nordin, and W.H. Adler. 1993. Age-related effects in T cell activation and proliferation. *Exp. Gerontol.* 28:313–321.
40. Whisler, R.L., J.W. Williams, Jr., and Y.G. Newhouse. 1991. Human B cell proliferative responses during aging. Reduced RNA synthesis and DNA replication after signal transduction by surface immunoglobulins compared to B cell antigenic determinants CD20 and CD40. *Mech. Ageing Dev.* 61:209–222.
41. Whisler, R.L., B.Q. Liu, Y.G. Newhouse, J.D. Walters, M.B. Breckenridge, and I.S. Grants. 1991. Signal transduction in human B cells during aging: alterations in stimulus-induced phosphorylations of tyrosine and serine/threonine substrates and in cytosolic calcium responsiveness. *Lymphokine Cytokine Res.* 10:463–473.
42. Su, X., T. Zhou, Z. Wang, P. Yang, R.S. Jope, and J.D. Mountz. 1995. Defective expression of hematopoietic cell protein tyrosine phosphatase (HCP) in lymphoid cells blocks Fas-mediated apoptosis. *Immunity.* 2:353–362.
43. Zacharchuk, C.M., M. Mercep, C.H. June, A.M. Weissman, and J.D. Ashwell. 1991. Variations in thymocyte susceptibility to clonal deletion during ontogeny. Implications for neonatal tolerance. *J. Immunol.* 147:460–465.
44. Van Houten, N., and R.C. Budd. 1992. Accelerated programmed cell death of MRL-*lpr/lpr* T lymphocytes. *J. Immunol.* 149:2513–2517.
45. Kariv, I., F.G. Ferguson, and F.L. Confer. 1992. Age- and strain-related differences in murine spleen cell responses to different activation signals. *Cell. Immunol.* 140:67–80.
46. Cillari, E., S. Milano, M. Dieli, F. Arcoleo, R. Perego, F. Leoni, G. Gromo, A. Severn, and F.Y. Liew. 1992. Thymopoietin reduces the susceptibility of aged mice to cutaneous leishmaniasis by modulating CD4 T-cell subsets. *Immunology.* 76: 362–366.
47. Lerner, A., T. Yamada, and R.A. Miller. 1989. Pgp-1^{hi} T lymphocytes accumulate with age in mice and respond poorly to concanavalin A. *Eur. J. Immunol.* 19:977–984.
48. Ernst, D.N., M.V. Hobbs, G.E. Torbett, A.L. Glasebrook, M.A. Rehse, K. Bottomly, K. Hayakawa, R.R. Hardy, and W.O. Weigle. 1990. Differences in the expression profiles of CD45RB, Pgp-1, and Cg11 splenic CD4$^+$ T cells from young and aged mice. *J. Immunol.* 145:1295–1302.
49. Nagelkerken, L., A. Hertogh-Huijbregts, R. Dobber, and A. Drager. 1991. Age-related changes in lymphokine production related to a decreased number of CD45RB^{hi} CD4$^+$ T cells. *Eur. J. Immunol.* 21:273–281.
50. Vitetta, E.S., M.T. Berton, C. Burger, M. Kepron, W.T. Lee, and X-M, Yin. 1991. Memory B and T cells. *Annu. Rev. Immunol.* 9:193–217.
51. Alderson, M.R., R.J. Armitage, E. Maraskovsky, T.W. Tough, E. Roux, K. Schooley, F. Ramsdell, and D.H. Lynch. 1993. Fas transduces activation signals in normal human T lymphocytes. *J. Exp. Med.* 178:2231–2235.
52. Zhou, T., H. Bluethmann, J. Eldridge, K. Berry, and J.D. Mountz. 1993. Origin of CD4$^-$CD8$^-$B220$^+$ T cells in MRL-*lpr/lpr* mice. Clues from a T cell receptor beta transgenic mouse. *J. Immunol.* 150:3651–3667.
53. Zhou, T., H. Bluethmann, J. Zhang, C.K. Edwards, and J.D. Mountz. 1992. Defective maintenance of T cell tolerance to a superantigen in MRL-*lpr/lpr* mice. *J. Exp. Med.* 176:1063–1072.
54. Zhou, T., H. Bluethmann, J. Eldridge, M. Brockhaus, K. Berry, and J.D. Mountz. 1991. Abnormal thymocyte development and production of autoreactive T cells in T cell receptor transgenic autoimmune mice. *J. Immunol.* 147:466–474.