Combined deficiency in CD44 and Fas leads to exacerbation of lymphoproliferative and autoimmune disease

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Abstract

Patients with mutations in Fas develop autoimmune lymphoproliferative disease (ALPS), while their family members with similar mutations are often normal, thereby suggesting that additional factors may play a role in the development of ALPS. In the current study, we tested the role of CD44 in the development of lymphoproliferative disease by generating CD44⁻/⁻/Fas⁻/⁻ mice, which failed to express CD44 and Fas, and compared them to CD44⁺/+/Fas⁻/⁻ mice that expressed CD44, but not Fas. The results showed that CD44⁻/⁻/Fas⁻/⁻ mice developed a more severe lymphoproliferative and autoimmune disease when compared to CD44⁺/+/Fas⁻/⁻ mice. This was indicated by increased numbers of cells in their lymph nodes, and a greater proportion of B220⁺CD4⁻CD8⁻ (double-negative) T cells as well as antibodies against single-stranded DNA and chromatin. The heightened severity of lymphoproliferative disease seen in CD44⁻/⁻/Fas⁻/⁻ mice correlated with increased resistance of T cells, but not B cells, to undergo activation-induced cell death (AICD). The current study suggests that deficiency in CD44 in combination with a defect in one of the molecules involved in the death receptor family such as Fas can further down-regulate AICD, and exacerbate the lymphoproliferative and autoimmune disease.

Introduction

Human autoimmune lymphoproliferative syndrome (ALPS) results from mutations in Fas (1,2). Interestingly, however, family members of ALPS patients are devoid of clinical symptoms despite similar Fas mutations (2). Such studies suggest that additional unknown factors play a role in the expression of ALPS phenotype.

Mice bearing the Fas deletion mutant gene, lpr, also develop severe lymphoproliferative and autoimmune disease, and are more susceptible to malignancies (3–5). They develop profound lymphadenopathy, splenomegaly, high titers of circulating autoantibodies and glomerulonephritis leading to premature death (3,4). The clinical disease in lpr mice is similar to that found in human autoimmune diseases including, human systemic lupus erythematosus and ALPS (3–5). Therefore, these mice serve as excellent experimental models for elucidating the pathogenesis of lymphoproliferative and autoimmune disease.

It has been proposed that dysregulation in apoptosis can trigger autoimmunity by failure to eliminate auto-reactive T cells during maturation or in the periphery following exposure to self-antigens (2,6–8). T cells upon activation in the periphery are known to undergo apoptosis, a process referred to as activation-induced cell death (AICD) (9–11). Fas–Fas ligand-based interactions have been proposed to play an important role in AICD. Thus, lpr mice and human patients that have defects in the Fas gene fail to eliminate peripheral T cells that are chronically activated via TCR, and develop severe lymphoproliferative disease (2,6–8). Such T cells accumulate in peripheral lymphoid organs causing massive lymphadenopathy and bear the B220⁺CD4⁻CD8⁻ [double-negative (DN)] phenotype in lpr mice, including high levels of expression of CD44 (12,13).

CD44 is a transmembrane glycoprotein that participates in cell–cell and cell–extracellular matrix interactions, lymphocyte homing, cell migration, metastasis, lymphopoiesis, and T cell activation (14–18). Recent studies from our laboratory demonstrated that CD44 may also serve as an apoptosis signaling molecule (19,20). Thus, T cells from CD44 knockout (KO) mice...
were found to be more resistant to AICD (20). Furthermore, CD44 KO mice exhibited heightened concanavalin A (Con A)-induced hepatitis due to inability of Con A-activated T cells to undergo AICD (19). Although the DN T cells that accumulated in lpr mice have been shown to express high levels of CD44 (15), their role in apoptosis and the progression of lymphoproliferative disease is unknown. In the current study, we developed mice that were deficient in both Fas and CD44, and compared them to those deficient in Fas alone, and observed that such mice developed an early and more severe form of lymphoproliferative and autoimmune disease. The current study demonstrates that CD44, in addition to Fas, may play an important regulatory role in preventing lymphoproliferative and autoimmune disease in the normal host.

Methods

Mice

Adult female C57BL/6/lpr/lpr mice (CD44+/lpr/Fas+/+) were purchased from the Jackson Laboratory (Bar Harbor, ME) and CD44 KO mice with a C57BL/6 background were kindly provided by Dr Tak Mak (Amgen Institute, Toronto, Canada). The phenotype of CD44 KO mice has been described elsewhere (18). To generate CD44+/+/Fas−/− mice, we crossbred CD44 KO mice with C57BL/6/lpr/lpr mice and the F1 mice generated were crossed again to develop F2. The F2 mice were screened for the combined deficiency of CD44 and Fas, and were designated as CD44+/+/Fas−/−. In some experiments, age- and sex-matched wild-type mice (CD44+/+/Fas+/+) and heterozygous littermates (CD44+/−/Fas+/+) were also used. Such mice were bred under specific pathogen-free conditions in the animal facility of Virginia Commonwealth University and the use of mice was approved by the Institutional Animal Care and Use Committee.

Preparation of cells and purification of T lymphocytes and DN T cells

Mice were sacrificed, and cervical, axillary and inguinal lymph nodes (LN) as well as spleens and thymi were removed. A single-cell suspension was prepared using a laboratory homogenizer (Stomacher, Tekmar, Cincinnati, OH) in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 1 mM glutamine and 50 μg/ml gentamycin. The red blood cells were lysed using ammonium chloride and the cells were resuspended in complete medium after three washes. The viable cells were counted using Trypan blue dye exclusion. To purify T cells, LN and spleens cells were incubated at 37°C for 1 h on plastic plates to remove adherent macrophages, and the non-adherent cells were passed over nylon wool columns (21).

In order to purify DN T cells, first, T lymphocytes from LN were purified as described above, and then CD4+ and CD8+ T cells were depleted by using 100 μl of anti-CD4, 100 μl of anti-CD8 mAb and complement as described (22). Briefly, the purified T cells were incubated with anti-CD4 and anti-CD8 antibodies on ice for 1 h, and then washed with medium. Diluted (1:10) rabbit complement (Low-Tox-M; Cederlane, Hornby, Canada) was added to the cell pellet and the cells were incubated at 37°C for 1 h, and then washed twice with medium. The cells thus purified were >98% CD3+ and CD4+ CD8− as determined by flow cytometric analysis (see Fig. 7A).

Flow cytometry

Cells were analyzed phenotypically using FITC-conjugated mAb or phycoerythrin (PE)-conjugated mAb as described (22,23). Briefly, cells were first incubated on ice for 30 min with normal rat serum for blocking Fc receptor. After washing with PBS, cells were incubated on ice for another 30 min with FITC- or PE-labeled mAb and then washed twice before fixing with 4% paraformaldehyde for 30 min at room temperature. For double-staining, cells were incubated with FITC- and PE-labeled mAb at the same time. The mAb used were as follows: PE-anti-CD3, PE- or FITC-anti-CD4, PE-anti-CD8, FITC-anti-B220, PE-anti-CD44, FITC-anti-Fas, PE-anti-Vβ3, PE-anti-Vβ8 and PE-anti-CD19. All antibodies were purchased from PharMingen (San Diego, CA). The cells (10⁴) were analyzed by flow cytometry (Epics V, model 752; Coulter, Miami, FL).

ELISA to detect total serum IgM and IgG

The serum samples were collected and analyzed for total IgM and IgG using ELISA as described (24,25). To detect total serum IgM and IgG antibodies, 96-well microtiter plates were coated with anti-mouse IgG or anti-mouse IgM antibodies (4 μg/ml; Sigma, St Louis, MO) at 4°C overnight. The plates were washed with PBS, blocked with 1% BSA for 2 h at room temperature, washed and then incubated with diluted serum samples to be tested (10⁻⁵ initial dilution). After 2–3 h at room temperature, the bound antibodies were detected with alkaline phosphatase-conjugated anti-IgG or anti-IgM antibodies (Sigma). For the standard, known concentrations of mouse IgG or mouse IgM antibodies (Southern Biotechnology Associates, Birmingham, AL) were used. The absorbance (A₄⁰⁵) was used to calculate the concentration of protein using the standard curve.

ELISA for autoantibodies to single-stranded DNA and chromatin

To detect anti-single-stranded DNA antibody, 96-well microtiter plates were coated with heat-denatured calf thymus DNA (10 μg/ml; Sigma) at 4°C overnight. The plates were washed with PBS/0.05% Tween 20, blocked with 1% BSA for 2 h at room temperature, washed and then incubated with diluted serum samples to be tested (1/500 initial dilution). After 2–3 h at room temperature, the bound antibodies were detected with alkaline phosphatase-conjugated anti-IgG antibody (Sigma). The absorbance (A₄⁰⁵) was measured.

To detect anti-chromatin antibody, 96-well microtiter plates were coated with 200 μl of 2.5 μg/ml of HI-stripped calf thymus chromatin in 50% glycerol and 50% PBS (a generous gift from Dr Robert L. Rubin, University of New Mexico School of Medicine) at 4°C overnight. The plates were washed with PBS/0.05% Tween 20, blocked with 1% BSA for 2 h at room temperature, washed and then incubated with diluted serum samples to be tested (1/500 initial dilution). After 2–3 h at room temperature, the bound antibodies were detected with alkaline phosphatase-conjugated anti-IgG antibody (Sigma). The absorbance (A₄⁰⁵) was measured.
Mice were injected in both rear footpads with 10 μg of staphylococcal enterotoxin A (SEA) to activate Vβ3+ T cells in popliteal LN. From day 1 to 5 following SEA treatment, the draining popliteal LN were collected and viable cells were counted using Trypan blue dye exclusion. Such cells were stained with PE-conjugated anti-Vβ3 and Vβ8 mAb, and fluorescence was measured by flow cytometry.

Proliferative responsiveness of LN cells to re-stimulation with SEA

Draining LN cells were harvested from mice, 3 days after immunization with 10 μg of SEA into the footpads. The viable cells were counted using Trypan blue dye exclusion and such cells (5 × 10⁵) were cultured at 37°C with medium alone or with SEA (4 μg/ml) for 72 h in 96-well tissue culture plates. The cultures were pulsed with [3H]thymidine during the last 8 h. DNA synthesis was determined by [3H]thymidine incorporation using a liquid scintillation counter.

In vitro T cell activation and detection of AICD

To detect spontaneous apoptosis of DN T cells in vitro, purified DN T cells from CD44+/+Fas+/+, CD44+/−/Fas+/-, CD44+/+/Fas±/± and CD44±/±/Fas±/± mice were incubated with tissue culture medium for 24 or 48 h in 96-well plates. The cells were harvested and quantified for apoptosis as described below.

To detect anti-CD3 mAb-induced AICD, splenic T cells were incubated with anti-CD3 mAb (5 μg/ml) for 48 h. The cultures were harvested and viable cells were purified by centrifugation on Ficoll-Hypaque. The cells were added to anti-CD3 mAb-coated plates (1 μg/ml) and incubated for an additional 48 h with IL-2 (50 U/ml). Such cells were harvested, and were double-stained with PE-conjugated anti-CD3 mAb and Annexin-V for detecting apoptosis of T cells.

In vitro B cell activation and detection of AICD

To detect B cell apoptosis, the splenocytes from CD44+/+/Fas+/+, CD44+/−/Fas+/−, CD44+/+/Fas±/± and CD44±/±/Fas±/± mice were stimulated with following reagents in 96-well plates: calcium ionophore (A23187, 100 ng/ml), phorbol myristate acetate (PMA, 10 ng/ml), dexamethasone (1 μM) (26) or anti-CD44 mAb (5 μg/ml). After 48 h, the cells were harvested, washed and double-stained with PE-conjugated anti-CD19 mAb and Annexin-V for detecting apoptosis of B cells. To investigate BCR-induced apoptosis, splenocytes were incubated onto goat F(ab′)² anti-mouse IgM + IgG + IgA (H + L) antibody (10 μg/ml; Southern Biotechnology Associates, Birmingham, AL)-coated plates.
for 48 h as described (27). Such cells were harvested, double-
stained with anti-CD19 mAb and Annexin-V, and analyzed
using a flow cytometer.

Apoptosis
Apoptosis was detected by using two different assays. The
first assay was based on labeling of DNA strand breaks using
the TdT-mediated dUTP nick end-labeling (TUNEL) technique
(Boehringer Mannheim, Indianapolis, IN) (28). The cells to be
analyzed for apoptosis were pooled from four or five mice,
washed twice with PBS and fixed with 4% paraformaldehyde
for 30 min at room temperature. The cells were next washed
with PBS and permeabilized on ice for 2 min, and then
incubated with FITC-dUTP for 1 h at 37°C. Fluorescence of the
cell population was measured by flow cytometry. The second
method used included use of Annexin-V and propidium iodide
(PI), and analysis of cells using a flow cytometer as described
(19). Cells positive for Annexin-V were considered as early
apoptotic cells, and those that were positive for Annexin-V and
PI as late apoptotic/necrotic cells (19). These assays were
repeated at least twice with consistent results.

In vitro culture of lymphocytes with rIL-2 and detection of
AICD following rIL-2 withdrawal
LN from mice were pooled and a single-cell suspension was
prepared as described earlier and incubated with anti-CD3
mAb (5 μg/ml) for 48 h. The cultures were harvested and viable
cells were purified by centrifugation on Ficoll-Hypaque. The
viable cells were further incubated with rIL-2 (50 U/ml) for
another 48 h. For the next 48 h, the cells were cultured with
medium alone and then harvested for apoptosis staining using
Annexin-V.

In vitro culture of lymphocytes with rIL-2, and detection of
cell division using carboxyfluorescein diacetate succinimidyl
ester (CFSE) and PI
The cell division/cycle was detected by using two different
assays. First, LN cells (2 × 10^6/ml) were incubated with the
intracellular fluorescent dye CFSE (50 μM; Molecular Probes,
Eugene, OR) at 37°C for 15 min (29). After three washes with
medium, the cells were cultured in 24-well tissue culture plates
with rIL-2 (50 U/ml) for 6 days. On days 1–6, cells were
harvested and the fluorescence was measured by flow
cytometry. The second method included the use of PI for cell
cycle analysis using a flow cytometer as described (30).

Fig. 3. Comparison of LN of 7-month-old CD44+/+Fas+/+ and CD44+/-Fas+/+ mice. From left to right is shown cervical, axillary, brachial and
inguinal LN taken from a single representative mouse. The bottom row shows a cervical LN from an 11-month-old mouse.
Briefly, at each indicated day, the cells were harvested and washed with PBS. The cells were then incubated on ice for 30 min with hypotonic buffer (0.1% sodium citrate/0.1% Triton X-100) containing PI (50 μg/ml; Roche, Indianapolis, IN). The cells were analyzed by flow cytometry and cell cycle distributions were calculated using WinMDI software.

Statistical analysis
The statistical comparisons between different study groups of mice were carried out using Student's t-test and differences with P < 0.05 were considered to be significant. In some experiments (data shown in Figs 1, 4, 5 and 7–12), LN or
spleen cells from four or five mice were pooled and data from a representative experiment is depicted. Such experiments were repeated at least 3 times with consistent results.

**Results**

CD44+/+ /Fas+/+ mice exhibit early onset of lymphoproliferative disease and increased lymphadenopathy when compared to CD44−/− /Fas−/− mice

In order to study the effect of CD44 on lymphoproliferative disease, we generated CD44−/− /Fas−/− mice and compared them to CD44+/+ /Fas+/+ mice. We confirmed the deficiency of CD44 and Fas using antibody staining and fluorescence analysis. As seen from Fig. 1, LN cells from CD44+/+ /Fas+/+ mice expressed CD44, but not Fas. In contrast, cells from CD44−/− /Fas−/− mice failed to express both CD44 and Fas. As a control, we also tested the LN cells from CD44+/+ /Fas+/+ or CD44−/− /Fas−/− mice, which were found to express both Fas and CD44 to a similar extent (Fig. 1). Inasmuch as autoimmune disease is commonly characterized by lymphadenopathy, we first collected cervical, axillary and inguinal LN from CD44+/+ /Fas+/+ and CD44−/− /Fas−/− mice, and counted the total number of viable cells. The cells were pooled from six LN per mouse and the average number of cells per LN was calculated. Groups of five to eight mice were used and the data expressed as mean cellularity of LN ± SEM. The results shown in Fig. 2(A) demonstrated that the LN cellularity increased with age in both groups of mice. However, CD44−/− /Fas−/− mice showed a more dramatic increase in cellularity in their LN, thereby suggesting enhanced lymphoproliferative disease at all time points and particularly at 11 months of age when compared to CD44+/+ /Fas+/+ mice (Figs 2A and 3). At 4 weeks of age, both groups of mice failed to exhibit lymphoproliferative disease. However, by
6 weeks, the CD44+/+/Fas+/+ mice, but not CD44+/+/Fas−/−, mice showed an increase in LN cellularity (data not shown). In addition to the LN, the CD44+/+/Fas−/− mice also showed a significant increase in spleen weight when compared to CD44+/+/Fas+/+ mice with increasing age (Fig. 2B). Thymic atrophy, characteristic of lpr/lpr mice (31), was seen in both groups of mice, although it was more pronounced in CD44+/+/Fas+/+ mice (data not shown). Together, these data suggested that CD44+/+/Fas+/+ mice developed early-onset and more severe lymphoproliferative disease than CD44+/+/Fas−/− mice. It should be noted that during breeding of F2 mice, we obtained CD44+/+/Fas+/+ as well as CD44+/+/Fas−/− mice, but none of them developed early and rapid onset of lymphoproliferative disease like the CD44+/+/Fas−/− mice (data not shown).

**CD44+/+/Fas−/− mice exhibit increased numbers of B220+CD4+CD8− (DN) T cells in LN, spleen and thymus**

We next studied the phenotype of the cells that caused the increased lymphoproliferative disease in CD44+/+/Fas−/− mice. Because expansion of B220+CD4+CD8− (DN) T cells is responsible for the lymphoproliferative disease seen in lpr/lpr mice (12,13), we stained purified T cells from the LN and spleen with anti-CD4, anti-CD8 and anti-B220 antibodies. The data shown in Fig. 4(A) indicated that CD44+/+/Fas−/− mice had a significant increase in the percentage of CD3+B220+ T cells, which represent the abnormal DN T cells found in mice with lpr mutation. This was also evident from the increase in the percentage of CD4+B220+ and CD8+B220+ T cells both in the LN and spleen. The percentage of CD3+B220−, CD4+B220− and CD8+B220− T cells, which represent the normal T cells, decreased significantly in CD44+/+/Fas−/− mice. These data confirmed that in CD44+/+/Fas−/− mice, there was increased accumulation of B220+ DN T cells, characteristic of the lymphoproliferative disease. It was interesting to note that a small proportion of CD4+, but not CD8+, T cells expressed B220. Furthermore, the percentage of these cells increased in CD44+/+/Fas−/− mice. Such cells have been detected recently in mice immunized with SEB as well as in mice with the gld mutation (32). Studies were also conducted to investigate if CD44+/+/Fas+/+ mice would exhibit B220+ DN T cells in the periphery. The data shown in Fig. 4B indicate that both wild-type CD44+/+/Fas+/+ and heterozygous CD44+/+/Fas+/− mice failed to exhibit B220+CD3+ T cells. Also, the proportions of CD4+ and CD8+ T cells in these groups of mice were similar.

When thymus was screened for CD4+ and CD8+ T cells, CD44+/+/Fas+/+ mice were found to exhibit a decreased percentage of CD4+B220− T cells and an increased percentage of CD4+B220+ T cells, when compared to CD44+/+/Fas+/− mice (Fig. 5A) The percentage of CD4+ and CD8+ T cells remained similar in the two groups of mice. Interestingly, the thymus from CD44+/+/Fas+/+ mice exhibited significant proportions of B220+ T cells that were lacking in CD44+/+/Fas+/− mice (data not shown). Both CD44+/+/Fas+/+ and CD44+/+/Fas+/− mice showed similar percentages of T cell subsets characteristic of normal mice (Fig. 5B).

**Serum IgG and IgM levels in CD44+/+/Fas−/− mice**

Mice with the lpr mutation are known to exhibit increased levels of total serum IgM and IgG (3). We collected sera from CD44+/+/Fas+/+ and CD44+/+/Fas−/− mice, and measured the levels of serum IgM and IgG by ELISA. The data shown in Table 1 suggested that both CD44+/+/Fas+/+ and CD44+/+/Fas−/− mice demonstrated increased levels of IgG and IgM production with increasing age. However, there were no statistically significant differences between the two groups of mice.

**CD44+/+/Fas−/− mice produce increased autoantibodies against single-stranded DNA and chromatin than CD44+/+/Fas+/+ mice**

The lpr mutation on C57BL/6 background triggers a less severe form of autoimmune disease. Such mice produce anti-chromatin/DNA, but not anti-nRNP/Sm, anti-Su or anti-ribosomal P antibodies, nephritis, or arthritis (33). In the current study, therefore, we collected sera from CD44+/+/Fas+/+ and CD44+/+/Fas−/− mice, and measured the levels of serum anti-single-stranded DNA and anti-chromatin antibody by ELISA. The data shown in Fig. 6 indicated that CD44+/+/Fas−/− mice produced more autoantibodies when compared to CD44+/+/Fas+/+ mice. It should be noted that the wild-type CD44+/+/Fas+/+ and CD44+/+/Fas−/− mice did not exhibit significant levels of autoantibodies in the serum.
and data expressed as mean ± SEM.

**Table 1.** Comparison of total IgG/IgM production between CD44+/+/Fas−/− and CD44−/−/Fas−/− mice

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Strain</th>
<th>Sex</th>
<th>Number</th>
<th>Total IgG/IgMµg/ml</th>
<th>IgG</th>
<th>IgM</th>
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</thead>
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<tr>
<td>11</td>
<td>CD44+/+/Fas−/−</td>
<td>F</td>
<td>5</td>
<td>7.5 ± 3.3</td>
<td>0.5 ± 0.07</td>
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<tr>
<td>CD44−/−/Fas−/−</td>
<td>F</td>
<td>5</td>
<td>12.8 ± 4.3</td>
<td>0.4 ± 0.09</td>
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</tr>
<tr>
<td>6</td>
<td>CD44+/+/Fas−/−</td>
<td>F</td>
<td>7</td>
<td>6.9 ± 2.1</td>
<td>0.4 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>CD44−/−/Fas−/−</td>
<td>F</td>
<td>7</td>
<td>5.5 ± 1.4</td>
<td>0.4 ± 0.1</td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>CD44+/+/Fas−/−</td>
<td>F</td>
<td>8</td>
<td>2.9 ± 0.7</td>
<td>0.14 ± 0.06</td>
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</tr>
<tr>
<td>CD44−/−/Fas−/−</td>
<td>F</td>
<td>3</td>
<td>2.9 ± 0.9</td>
<td>0.07 ± 0.03</td>
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*Expressed as µg/ml of serum. Mice were individually screened and data expressed as mean ± SEM.

**Histopathological analysis of organs**

Mice with the *lpr* mutation exhibit lymphoid hyperplasia, splenomegaly, thymic atrophy, vascular disease, arthritis and glomerulonephritis (3,31). However, these clinical findings are dependent on the genetic background of the mice (34). We examined LN, spleen, thymus, liver and kidney tissue from CD44+/+/Fas−/− and CD44−/−/Fas−/− mice at 2, 6 and 11 months of age. Unlike mice with the *lpr* mutation on the MRL background, which suffer severely from glomerulonephritis leading to death within 6 months, the two groups of mice with the C57BL/6 background used in the current study exhibited minimal evidence of kidney dysfunction, similar to a previous study (34). Furthermore, liver tissue remained normal at all ages tested with the exception of one 11-month-old CD44+/+/Fas−/− mouse which exhibited malignancy (data not shown). The thymus from CD44−/−/Fas−/− mice exhibited increased atrophy when compared to the CD44+/+/Fas−/− mice (data not shown).

**T cells from CD44−/−/Fas−/− mice exhibit increased resistance to apoptosis**

In order to investigate why deficiency of CD44 leads to more accumulation of abnormal T cells (DN T cells) in the LN, we tested the hypothesis that CD44-deficient T cells are more resistant to spontaneous cell death or AICD. To this end, first, we purified DN T cells from CD44+/+/Fas−/− and CD44−/−/Fas−/− mice, and incubated them with medium for 24 or 48 h. The purity of DN T cells (CD3−CD4−CD8− B220−) was confirmed by flow cytometry (Fig. 7A). Next, the cells were harvested and analyzed for apoptosis using the TUNEL assay. The data shown in Fig. 7(B) demonstrate that freshly isolated DN T cells from CD44+/+/Fas−/− and CD44−/−/Fas−/− mice failed to exhibit apoptosis. However, following *in vitro* culture, a significant proportion of the cells underwent apoptosis. Interestingly, DN T cells from CD44−/−/Fas−/− mice were more resistant to induction of apoptosis *in vitro* when compared to similar cells from CD44+/+/Fas−/− mice. Moreover, in this assay 16 × 10⁶ cells were cultured from each group of mice. The number of cells recovered in CD44+/+/Fas−/− mice was 8.5 × 10⁶ and 6.3 × 10⁶ after 24 and 48 h culture respectively. In contrast, in CD44−/−/Fas−/− mice the recovery of cells was 13.3 × 10⁶ and 10.9 × 10⁶ for a similar culture period. These data together suggested that CD44 deficiency leads to decreased spontaneous apoptosis of DN T cells and that this may account for the increased accumulation of DN T cells in CD44+/+/Fas−/− mice when compared to CD44−/−/Fas−/− mice.

To study AICD, we investigated the superantigen-induced proliferation and apoptosis in both groups of mice. It is well known that superantigenics such as SEA induce the proliferation of VB3+ T cells, followed by induction of apoptosis. We injected 10 µg of SEA per footpad into CD44+/+/Fas−/− and CD44−/−/Fas−/− mice at 4 weeks of age prior to onset of lymphadenopathy. At 1–5 days later, draining popliteal LN were collected and the VB3+ T cells were analyzed by flow cytometry. In addition, the total LN cellularity and the number of VB3+ T cells per LN were calculated (Fig. 8A). The data indicated that SEA-responsive VB3+ T cells expanded followed by a decline. Moreover, CD44+/+/Fas−/− mice exhibited higher numbers of VB3+ T cells in the LN on all days tested when compared to CD44−/−/Fas−/− mice. However, when we looked at the SEA-unresponsive VB8+ T cell population, we found that CD44+/+/Fas−/− and CD44−/−/Fas−/− mice showed similar cellularity as well as percentage of VB8+ T cells (data not shown). The total number of LN cells prior to immunization with SEA per popliteal LN was similar (~1.5 × 10⁶) in both groups of mice. However, following SEA immunization, the total LN cellularity in CD44+/+/Fas−/− cells was 1.76 × 10⁶ cells on day 2 and 2.75 × 10⁶ cells on day 3. In CD44−/−/Fas−/− mice, the LN cellularity for these days was 6.3 × 10⁶ and 5.4 × 10⁶ cells respectively. The percentage of VB3+ T cells in both groups of mice was ~4%, which increased to 8.1% on day 2 and 7.2% on day 3 in CD44+/+/Fas−/− mice. In CD44−/−/Fas−/− mice, the VB3+ T cells were 9.0 and 7.7% for these respective days (data not shown). When the total number of VB3+ T cells in the LN was calculated, CD44+/+/Fas−/− mice exhibited significantly higher numbers of VB3+ T cells in the LN when compared to the CD44−/−/Fas−/− mice (Fig. 8A). As a control, we also injected CD44−/−/Fas−/− or CD44+/+/Fas−/− mice with SEA and draining popliteal LN were collected at each indicated day, and then the number of VB3+ T cells per LN were calculated (Fig. 8B). The data showed that SEA-responsive VB3+ T cells increased in number on day 3 followed by a decline and, furthermore, there were no significant differences in the responsiveness to SEA between these two groups.

In addition, the draining LN cells from all four groups of mice injected with 10 µg of SEA per footpad 3 days before harvest were investigated for their proliferative responsiveness following *in vitro* SEA re-stimulation. As shown in Fig. 8(C), the cells from CD44+/−/Fas−/− and CD44−/−/Fas−/− mice exhibited increased proliferative response when compared to those from wild-type CD44+/+/Fas−/− and CD44+/+/Fas−/− mice; the latter two groups showing a similar response. Moreover, cells from CD44+/−/Fas−/− mice exhibited the highest levels of cell proliferation.

Next, we studied anti-CD3 mAb-induced apoptosis in splenic T cells in all four groups of mice. To this end, nylon wool-enriched splenic T cells were stimulated with anti-CD3 mAb for 48 h, and viable cells were isolated and re-stimulated by immobilized anti-CD3 mAb or isotype control antibody for 48 h in the presence of exogenous rIL-2 (35). We analyzed the induction of apoptosis in T cells by double-staining the cells with anti-CD3 mAb and Annexin-V (Fig. 9). The data demon-
In vitro responsiveness of lymphocytes to rIL-2

In addition to the stimulation of T cells through the TCR, we investigated whether the T cells from CD44+/+/Fas±/± or CD44+/+/-/Fas⁻/⁻ mice would respond differently upon stimulation with rIL-2. To this end LN from 4-week-old mice were cultured for 48 h with 50 U/ml of rIL-2 and cell proliferation was studied by incorporation of [³H]thymidine. The data shown in Fig. 10(A) demonstrate that LN cells from CD44+/+/-/Fas⁻/⁻ mice cultured with rIL-2 exhibited a significant proliferative response when compared to cells cultured with medium alone. However, LN cells from CD44+/+/-/Fas⁻/⁻ mice exhibited a dramatically increased [³H]thymidine incorporation following rIL-2 stimulation, particularly at 72 h, suggestive of increased cell proliferation and/or survival in culture. Next, we set up bulk cultures with 50 U/ml of rIL-2, and on days 4, 5, 7 and 10 the number of viable cells was enumerated. The data shown in Fig. 10(B) suggest that cultures of LN cells from CD44+/+/-/Fas⁻/⁻ stimulated with rIL-2 showed increased cell numbers until day 4 and declined thereafter. In contrast, LN cells from CD44+/+/-/Fas⁻/⁻ mice showed a significant decrease in viable cell numbers on all days tested.

We also tested the effect of rIL-2 withdrawal on apoptosis induced in T cells (36). To this end, spleen cells were cultured with anti-CD3 mAb for 48 h. The viable cells were purified and cultured with rIL-2 (50 U/ml) for an additional 48 h. The cells were harvested, washed and cultured for 48 h with medium alone, and stained for apoptosis using Annexin-V. The data shown in Fig. 10(C) indicate that the majority of the T cells from CD44+/+/-/Fas⁻/⁻ mice underwent apoptosis upon rIL-2 withdrawal. In contrast, T cells from CD44+/+/-/Fas⁻/⁻ mice were resistant to apoptosis induced by rIL-2 withdrawal.

Cell division/cell cycle analysis following culture with rIL-2

To determine whether the increased proliferative response of LN cells from CD44+/+/-/Fas⁻/⁻ mice seen following culture with rIL-2 (Fig. 10A) was due to enhanced cell division of T cells or increased resistance to apoptosis, we performed cell cycle/division analysis of the lymphocytes using CFSE or PI labeling (29,30). Using CFSE labeling, the fluorescence intensity decreases as the cells divide. As seen from Fig. 11(A), culture of LN cells with rIL-2 induced significant cell division as indicated by a gradual decrease in the intensity of CFSE with increasing days in culture. When examined for 0–6 days, the decrease in CFSE intensity was identical in CD44+/+/-/Fas⁻/⁻ and CD44+/+/-/Fas⁻/⁻ mice, thereby suggesting that the cells from these two groups of mice were dividing at the same rate. This was further corroborated using cell cycle analysis. The same cells cultured with rIL-2 as described above were also analyzed using PI staining. The data depicted in Fig. 11(B) and summarized in Table 2 suggest that the percentage of cells in the G1 or G2/S/M phase of the cell cycle in both groups of mice was somewhat similar. Interestingly, the percentage of cells in the sub-G1 phase, which represent apoptotic cells, was higher in CD44+/+/-/Fas⁻/⁻ mice when compared to CD44+/+/-/Fas⁻/⁻ mice, consistent with the observation that CD44 deficiency confers increased resistance to apoptosis. Together, these data suggest that the rIL-2-induced increase in [³H]thymidine incorporation seen in CD44+/+/-/Fas⁻/⁻ mice may
result from increased survival of cultured cells rather than enhanced proliferation.

**B cells from CD44±/±/Fas±/± mice exhibit similar levels of apoptosis as the CD44+/+/Fas±/± mice**

We next tested the role of CD44 in AICD of B cells. To this end, spleen cells from CD44+/+/Fas±/± or CD44+/+/* ±/± mice were stimulated with various agents previously shown to induce AICD in B cells such as calcium ionophore (100 ng/ml), PMA (10 ng/ml), dexamethasone (1 μM) or immobilized anti-BCR antibodies (10 μg/ml) (26,27). The cells were stained with PE-conjugated anti-CD19 mAb and Annexin-V, and analyzed using a flow cytometer. Figure 12 shows the double-staining histograms gated for CD19+ cells undergoing apoptosis. These data showed that the B cells from CD44+/+/Fas±/± mice exhibited similar levels of apoptosis as the cells from CD44+/+/Fas+/− mice using four different agents to induce apoptosis. Previous studies from our laboratory demonstrated that ligation of CD44 triggers apoptosis in T cells (20). To investigate the role of CD44 on B cell apoptosis, splenocytes from CD44+/+/Fas±/± or CD44+/+/* ±/± mice were stimulated with anti-CD44 mAb (5 μg/ml) for 48 h, and then the cells were stained with PE-conjugated anti-CD19 mAb and Annexin-V. The data show similar levels of apoptosis between the two groups of mice, suggesting that CD44 may not be involved in B cell apoptosis.

**Discussion**

Previous studies from our laboratory and elsewhere demonstrated that CD44 plays an important role as a cytotoxic effector molecule (15–17). Also, we demonstrated that the DN T cells from lpr mice express high levels of CD44 and can mediate cytotoxicity following ligation of CD44 (15). Furthermore, CD44 was shown to participate in rIL-2-induced vascular leak and Con A-induced hepatitis (18,19). These studies together suggested that constitutive expression of high levels of CD44 might trigger an autoimmune reaction. To test this possibility we crossbred CD44 KO mice with lpr/lpr mice, which develop lymphoproliferative and autoimmune disease due to Fas deficiency. We expected the CD44 ±/±/Fas±/± mice to exhibit decreased autoimmune and lymphoproliferative disease. Interestingly, we noted that deficiency of CD44 might increase the lymphoproliferative disease. Using a variety of approaches we found that CD44 deficiency conferred increased resistance to spontaneous cell death and AICD in T cells. These data suggested that CD44 may play a
crucial role in AICD, and that combined deficiency of CD44 and Fas may lead to early onset and increased severity of lymphoproliferative disease.

CD44 deficiency not only led to a more severe form of lymphoproliferative disease in Fas-deficient mice, but it also caused an increase in autoantibody production in CD44+/+ mice, when compared to CD44+/Fas+/+ mice, may not have occurred to an extent that would lead to increased total serum IgG or IgM levels. It is known that genetic factors other than lpr mutation influence the onset and severity of autoimmunity (34). Thus, it is possible that CD44 deficiency in MRL-lpr/lpr mice may trigger enhanced autoantibody production and a more severe form of autoimmune pathology. Alternatively, some studies have suggested that autoantibody production is controlled by an independent and distinct pathway from lymphadenopathy. For example, several studies showed that expression of transgenic Fas or TCR in T cells of lpr mice prevented lymphadenopathy, but had no effect on autoantibody production (24,38). Similarly, treatment of lpr mice with anti-CD8 mAb prevented lymphoproliferative disease, but not the autoantibody production (39). It was interesting to note that one out of six CD44+/Fas+/+ mice examined for ~12 months died of malignancy, whereas none of the CD44+/Fas+/+ mice developed spontaneous tumors during this period. Previous studies have shown that lpr mice, due to a defect in induction of apoptosis, are more susceptible to development of malignancy (5). Thus, a combined defect of CD44 and Fas may lead to early onset and increased susceptibility to malignancy.

Signaling through Fas plays a critical role in the maintenance of immunological tolerance. Thus, mutations in Fas or Fas ligand led to the accumulation of a unique set of T cells that are CD4+CD8- and B220+, commonly called DN T cells (12). Similarly, mice deficient in CTLA-4 also develop massive lymphoproliferative disease and die at an early age (40,41). It is interesting to note that mice deficient only in CD44 molecules have a normal phenotype and do not exhibit any lymphoproliferative disease or accumulation of DN T cells. However, we have observed that such mice (CD44+/+/Fas+/+) exhibit increased T cell response to antigenic challenge and are more resistant to AICD than CD44+/+/Fas+/+ wild-type mice (20). These data along with the data presented in the current study suggest that CD44 on activated T cells plays a critical role in regulating apoptosis. However, such T cells eventually undergo apoptosis due to the presence of Fas. In the absence of Fas, deficiency of CD44 increases the resistance of T cells to apoptosis as seen in the current study.

The exact nature of DN T cells that accumulate in lpr mice is not clear despite extensive research. While most investigators have considered such cells to be anergic and fail to divide, recent studies suggested that DN T cells from normal and DN T cells from lpr mice were shown to rapidly undergo apoptosis in vitro, thereby suggesting that the accumulation of DN T cells may not be due to a defect in apoptosis (46). The DN T cells constitutively express high levels of CD44 (15) and the current study suggests that in addition to Fas, the DN T cells also depend on CD44 to undergo apoptosis.

![Fig. 12. Apoptosis detection in splenic B cells of CD44+/+ and CD44+/+ mice. The splenocytes from 4-week-old mice were stimulated with A23187 (100 ng/ml), PMA (10 ng/ml), dexamethasone (1 μM) or anti-CD44 mAb (5 μg/ml) for 48 h. To detect anti-IgM antibody-induced apoptosis, splenocytes (0.8 × 10⁶) were plated onto anti-mouse IgM antibody-coated plates (10 μg/ml) for 48 h. Following culture, the cells were harvested, and stained with PE-labeled anti-CD19 mAb and Annexin-V for measuring apoptotic B cells. The CD19+ cells were gated and the proportion of cells that was Annexin-V- or Annexin-V+ was gated and is depicted in each of the histograms.](image-url)
CD44 is extensively involved in cell–cell and cell–matrix interactions. In addition, it has been shown to play an important role in signal transduction and activation of T cells, B cells, monocytes and dendritic cells (47–51). CD44 is encoded by 20 exons, seven of which form the invariant extracellular region of the so-called standard form (CD44s). By alternative splicing up to 10 invariant exons (CD44 v1–v10) can be inserted within the extracellular region. Thus, the wide range of functions attributed to CD44 may result from the expression of different CD44 isoforms. For example, B cell activation with PMA has been shown to induce CD44v6 (52). Also, T cell activation leads to the expression of CD44v6, and the delayed-type hypersensitivity reaction can be mitigated by antibodies against CD44v6 and CD44v7 (53,54).

The precise nature of CD44 isoforms involved in apoptosis is not clear. Mice deficient in CD44v7 were shown to be resistant to experimental colitis and, furthermore, such mice exhibited a higher rate of apoptosis at inflamed lesions (55). These data suggested that CD44v7 helped in the survival of effector lymphocytes. These studies are consistent with our observation that CD44 KO mice exhibited decreased vascular leak syndrome and endothelial cell injury following IL-2 therapy (18). Thus IL-2 treatment may up-regulate certain CD44 variant isoforms essential for survival of lymphokine-activated killer cells which in turn cause endothelial cell injury. In contrast, in another model of autoimmune reaction involving Con A-induced hepatitis, we observed that CD44 KO mice exhibited enhanced hepatitis when compared to CD44 wild-type mice (19). In this study, CD44 deficiency led to increased resistance to Con A-induced apoptosis and such activated effector T cells were responsible for causing enhanced hepatitis. Together, these studies stress the need to further investigate the role played by CD44 variant isoforms in T cell activation and apoptosis. The precise mechanism by which CD44 triggers apoptosis is not clear. A recent study suggested that CD44 supports apoptosis by enhancing signal transduction through the TCR (56). However, the CD44-mediated apoptosis in activated T cells was not dependent on Fas because direct ligation of CD44 on Fas-deficient T cells induced apoptosis. Thus CD44 appears to play a critical role in AICD.

Recently, a genetic defect in apoptosis has been shown in humans to lead to lymphoproliferative disease called ALPS (1,2). Patients with mutations in Fas develop ALPS exhibiting lymphoproliferative syndrome characterized by DN T cells and various manifestations of autoimmunity (2,7). Interestingly, family members of ALPS patients are mostly devoid of the clinical symptoms despite exhibiting similar Fas mutations (2,57). These data suggest that environmental factors or other immunoregulatory defects are necessary for full expression of the ALPS phenotype (2). The current study suggests that dysregulation in CD44 expression may influence the severity of Fas-dependent lymphoproliferative disease. Furthermore, targeting CD44 to facilitate apoptosis induction in activated T cells may constitute a novel approach to treat a wide range of clinical diseases including autoimmune reactions. The current study also demonstrates for the first time that CD44, a molecule that does not belong to the death receptor family, may play a crucial role in regulating Fas-mediated apoptosis and development of lymphoproliferative disease.

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Abbreviations
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<tr>
<th>Abbreviation</th>
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<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
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<tr>
<td>ALPS</td>
<td>autoimmune lymphoproliferative syndrome</td>
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<tr>
<td>CFSE</td>
<td>carboxyfluorescein diacetate succinimidyl ester</td>
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<td>Con A</td>
<td>concanavalin A</td>
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<td>DN</td>
<td>double negative</td>
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<td>KO</td>
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<td>lymph node</td>
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<td>PI</td>
<td>propidium iodide</td>
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<td>PMA</td>
<td>phorbol myristate acetate</td>
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<td>SEA</td>
<td>staphylococcal enterotoxin A</td>
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<td>TUNEL</td>
<td>TdT-mediated dUTP nick end-labeling</td>
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