

# Role of CD44 and Its v7 Isoform in Staphylococcal Enterotoxin B-Induced Toxic Shock: CD44 Deficiency on Hepatic Mononuclear Cells Leads to Reduced Activation-Induced Apoptosis That Results in Increased Liver Damage

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**Exposure to bacterial superantigens such as staphylococcal enterotoxin B (SEB) leads to the induction of toxic shock syndrome which results in multiorgan failure, including liver damage. In the present study, we investigated the role of CD44 in SEB-induced liver injury. Injection of SEB into D-galactosamine-sensitized CD44 wild-type (WT) mice led to a significant increase in CD44 expression on liver T cells, NK cells, and NKT cells. Administration of SEB to CD44 knockout (KO) mice caused significantly enhanced liver damage which correlated with elevated numbers of T cells, NK cells, NKT cells, and macrophages in the liver and increased production of tumor necrosis factor alpha and gamma interferon compared to CD44 WT mice. Furthermore, liver mononuclear cells from CD44 KO mice were resistant to SEB-induced apoptosis, and cDNA microarray analysis revealed that SEB activation of such cells led to the induction of several antiapoptotic genes and repression of proapoptotic genes. Examination of CD44 isoforms revealed that SEB exposure altered CD44 variant 7 (v7) isoform expression. Interestingly, mice bearing a specific deletion of the CD44v7 exon exhibited increased susceptibility to SEB-induced hepatitis. Finally, treatment of CD44 WT mice with anti-CD44 monoclonal antibodies reduced expression of CD44 in liver mononuclear cells and caused increased susceptibility to SEB-induced liver injury. Together, these data demonstrate that the expression of CD44 and/or CD44v7 on SEB-activated liver mononuclear cells facilitates their rapid apoptosis, thereby preventing severe liver injury in wild-type mice, and suggest that CD44 plays an important role in the regulation and elimination of immune cells in the liver.**

Toxic shock syndrome (TSS) can result from exposure to bacterial superantigens produced by *Staphylococcus aureus* and is characterized by acute onset of illness which leads to fever, rash formation, multiorgan failure, hepatitis, and possible lethal shock (34). A number of forms of TSS have been associated with staphylococcal enterotoxin B (SEB) exposure. These include postsurgical TSS, influenza-associated TSS, recalcitrant erythematous desquamating syndrome, and TSS with the use of contraceptive diaphragms (34). In addition, SEB has been listed as an agent that could be used in bioterrorism (32). SEB is referred to as a superantigen because of its profound effects on the immune system. Very small concentrations of SEB can activate the immune response due to its high-avidity binding to specific V $\beta$  domains of the T-cell receptor (10).

CD44 is a widely distributed surface glycoprotein expressed by a number of lymphoid and nonlymphoid cells (8, 21). The principal ligand of CD44 is believed to be hyaluronic acid.

CD44 is known to play important roles in a number of immunological processes such as lymphocyte migration, extravasation, activation, and cytolytic activity (2, 6, 13, 14, 20, 23, 42, 45). In addition, recent evidence suggests that CD44 may also be involved in the induction of apoptosis and activation-induced cell death (AICD) (35). The exact role that CD44 plays in apoptosis remains unclear. A number of reports suggested that the signaling through CD44 can protect a cell from apoptosis (12, 55). For example, in mice lacking the CD44v7 exon, treatment with 2,4,6-trinitrobenzene sulfonic acid (TNBS) resulted in a reduced severity of colitis due to increased apoptosis of lamina propria mononuclear cells (55). However, other reports suggested that upregulation and signaling through CD44 can lead to enhanced apoptosis (11, 19, 35). For example, ligation of CD44 leads to apoptosis in fibroblast as well as certain lymphocyte populations (11, 19). Recent evidence from our laboratory demonstrated that CD44 knockout (KO) mice show increased susceptibility to concanavalin A-induced hepatitis, which directly correlated with a decreased incidence of lymphocyte apoptosis (1). It should be noted that in the TNBS model, those authors examined the role of a specific CD44 isoform (CD44v7), while in the later experiments, the investigators used monoclonal antibodies (MAbs) that recognized all

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forms of CD44 including the standard form and all isoforms or they used mice that lacked expression of any CD44 gene products. Although the product of a single gene, CD44 is expressed as multiple isoforms that result from alternatively spliced mRNA species and posttranslational modifications (3, 17, 52). These isoforms are expressed during different stages of activation and by different effector cells (44). Therefore, it is possible that different CD44 isoforms may have unique and sometimes opposite roles in the regulation of apoptosis. In fact, there is evidence that suggests that the individual CD44 isoforms may play specific roles in the regulation of the immune response (9, 16, 27, 48). For example, it has been demonstrated that specific isoforms are expressed only on activated lymphocytes and that antibodies directed against specific CD44 isoforms are able to prevent the development of TNBS-induced colitis (55).

In addition to its clinical importance, SEB stimulation has been used as a model for studying *in vivo* cytokine regulation, T-cell energy, and activation-induced cell death (18, 38, 40). The liver is thought to be an important site for regulation of the immune response. A number of reports have demonstrated that following activation, there is a significant accumulation of apoptotic lymphocytes in the liver (5). Recent studies from our laboratory demonstrated a specific role for CD44 in activation-induced cell death and therefore suggested that CD44 may also regulate activation of T cells by superantigens (35). More specifically, primary as well as secondary responses to staphylococcal enterotoxin A were elevated in CD44-deficient mice (35). These elevated responses were due to a decrease in the susceptibility of CD44-deficient lymphocytes to AICD. However, very little is known about the possible role of CD44 and specific CD44 isoforms in the regulation of the immune response in the liver microenvironment. In the present study, we demonstrate a direct role of CD44 and the CD44v7 isoform in the regulation of the immune response in the liver by using the SEB model of hepatitis.

#### MATERIALS AND METHODS

**Mice.** Adult female C57BL/6 (CD44 wild type [WT]) mice were purchased from the National Institutes of Health (Bethesda, Md.). CD44 KO mice were kindly provided by the AMGEN Institute (Toronto, Ontario, Canada) and bred in our animal facilities and screened for the CD44 mutation. CD44v7 KO mice are those with a targeted deletion of CD44v7 exon products. Such mice, therefore, fail to express CD44 isoforms bearing the variant exon v7 (55). All these strains were on a C57BL/6 background. The CD44 KO and CD44v7 KO mice were bred in the animal facilities at the Virginia Commonwealth University Medical Center and screened for the CD44 and CD44v7 deletions, respectively.

**SEB-induced hepatitis and its evaluation.** To induce hepatitis, female CD44 WT, CD44 KO, and CD44v7 KO mice weighing 20 to 23 g were challenged with SEB (20  $\mu$ g/mouse) admixed with D-galactosamine (20 mg/mouse). Since mice are resistant to the effects of bacterial toxins such as SEB, we increased their sensitivity throughout this study by treatment with D-galactosamine (37). Control mice received 100  $\mu$ l of saline (intravenously). Plasma from individual mice was separated from blood obtained through the orbital plexus of the animal under anesthesia with IsoFlo (Abbott Laboratories, North Chicago, Ill.) at various time intervals after SEB injection. Aspartate aminotransferase activity (AST) levels in plasma were measured by using a commercial kit (Sigma Chemical, St. Louis, Mo.) as described previously (1).

For histopathological studies, the harvested livers were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphological changes as described previously (16). TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay was performed by using an ApopTag peroxidase *in situ* apoptosis detection kit (Serologicals Corp., Norcross, Ga.).

**Isolation of lymphocytes infiltrating the liver.** The isolation of liver-infiltrating mononuclear cells was carried out as described previously by others (18, 19). Briefly, livers obtained after SEB injection from CD44 WT and CD44 KO mice were homogenized with a laboratory homogenizer (Stomacher; Tekmar, Cincinnati, Ohio) and suspended in RPMI medium supplemented with 5% fetal calf serum. After one washing with the medium, the cells were resuspended in 30 ml of medium, and infiltrating lymphocytes were separated from parenchymal hepatocytes and Kupffer cells by Ficoll-Isopaque density (1.09) gradient centrifugation. The cell suspension (35 ml) was overlaid onto 15 ml of Ficoll-Isopaque in a 50-ml conical plastic tube. Centrifugation was performed at  $600 \times g$  for 30 min at room temperature. After centrifugation, 10 ml of the interface was aspirated and mixed with 20 ml of the medium in a 50-ml conical tube, and the cells were washed twice.

**Evaluation of induction of apoptosis.** Apoptosis was measured by using both the annexin-propidium iodide (PI) and TUNEL methods (24, 53). To detect apoptosis with the TUNEL method, the cells were washed twice with phosphate-buffered saline (PBS) and fixed with 4% *p*-formaldehyde for 30 min at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 min, and incubated with fluorescein isothiocyanate (FITC)-dUTP and terminal deoxynucleotidyl transferase (TdT) (Boehringer Mannheim, Indianapolis, Ind.) for 1 h at 37°C and 5% CO<sub>2</sub> (24). To detect apoptosis with the annexin V-PI method, the cells were washed twice with PBS and stained with annexin V and PI for 20 min at room temperature (53). The cells were washed twice with PBS. The levels of apoptosis in both the TUNEL and annexin-PI assays were determined by measuring the fluorescence of the cells by flow cytometric analysis. Five thousand cells were analyzed per sample.

**Analysis of cell surface markers.** Twenty-four hours following exposure to SEB and D-galactosamine, the liver mononuclear cells were screened for various cell surface markers by using flow cytometry. Briefly, 10<sup>6</sup> mononuclear cells were incubated with Fc receptor block (Pharmingen, San Diego, Calif.) followed by culture with phycoerythrin (PE)- or FITC-conjugated anti-mouse CD3, anti-mouse CD8, anti-mouse CD4, anti-mouse Mac-3 (macrophages), anti-NK1.1 (NK cells), or anti-CD19 MAb (B cells) (Pharmingen) on ice for 30 min. The cells were washed with PBS three times and analyzed for fluorescence by using a flow cytometer (11).

**Analysis of protein expression by Western blot analysis.** Protein was isolated from liver mononuclear cells from mice injected with PBS or SEB by freeze-thawing, and the protein concentration was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). The protein (15  $\mu$ g) was run on a 7.5% acrylamide gel. The protein was transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubating the membrane in 5% blotting-grade blocker nonfat dry milk (Bio-Rad, Hercules, Calif.) for 1 h at room temperature. The membrane was rinsed and then washed three times for 10 min with Tris-buffered saline containing 0.1% Tween 20. The membrane was then probed with the specific primary antibody in 5% blocking solution overnight at 4°C. The membrane was then rinsed and washed three times with Tris-buffered saline containing 0.1% Tween 20 and probed with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were then visualized following incubation with enhanced chemiluminescence solution and exposure to X-ray film.

**Cytokine detection.** At various time points following PBS or SEB injection, liver mononuclear cells were isolated as described above. The mononuclear cells were adjusted to  $2.5 \times 10^6$ /ml in RPMI medium containing 10% fetal calf serum and cultured in a 96-well flat-bottomed plate (200  $\mu$ l/well). The supernatants were isolated 24 h later, and the levels of interleukin-2 (IL-2), gamma interferon (IFN- $\gamma$ ), and tumor necrosis factor (TNF) were determined by using the methods described by the manufacturer of the Quantikine M enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minn.).

**CFSE labeling and adoptive transfer.** Splenocytes from CD44 WT or CD44 KO mice were labeled with carboxyl fluorescein succinimidyl ester (CFSE) as described by the manufacturer (Molecular Probes, Eugene, Oreg.). Briefly, 10<sup>7</sup> cells were labeled with 5  $\mu$ M CFSE in PBS for 15 min at 37°C. The cells were washed with PBS, resuspended, and incubated for an additional 30 min at 37°C. The cells were washed twice with PBS and resuspended at  $5 \times 10^8$ /ml. CFSE-labeled splenocytes ( $5 \times 10^7$ ) were adoptively transferred into CD44 WT mice by intravenous injection.

**RT-PCR.** Total RNA was isolated from a single-cell suspension of splenocytes by using an RNeasy Mini kit (QIAGEN, Valencia, Calif.). RNA concentration was determined spectrophotometrically, and the integrity of each preparation was verified by agarose gel electrophoresis. cDNA was synthesized by reverse transcription (RT) of 50 ng of total RNA by using a SensiScript RT kit (QIAGEN). All PCRs were prepared with MasterAmp PCR Premix F (Epicentre Technologies, Madison, Wis.) according to the manufacturer's recommenda-

tions and Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.). The following primers were used:  $\beta$ -actin primers 5'-AAGCCAACCGTGAAAAG ATGACC-3' and 5'-ACCGCTCGTTGCCAATAGTGATGA-3' and Bax primers 5'-GAGCAGCCGCCAGGATG-3' and 5'-GGTGAGCGAGGCGGTG AGGAC-3'. A half-nested RT-PCR approach was used to amplify CD44 isoforms, as endogenous isoform expression is too low to be detected directly by RT-PCR. An initial amplification step with primers referred to as CD44U and CD44L, homologous to the 5' and 3' standard regions of the CD44 gene, was used to amplify the CD44 standard form (CD44s) and all isoforms. The products of this reaction were used as a template in a second reaction using a CD44 isoform-specific primer in conjunction with the appropriate CD44 standard region primer (CD44L and CD44U or CD44U and CD44L). The primers used to detect CD44 isoform expression were CD44U (5'-GCACCCAGAAGGCTAC ATTTT-3'), CD44L (5'-TTCTGCCACACCTTCTCCTACTA-3'), CD44v3L (5'-ATAAAATCTTCATCATCAAT-3'), CD44v5U (5'-GGACCCGGAACC ACAGC-3'), CD44v7U (5'-TTCGCCACAACAACCA-3'), and CD44v7L (5'-GA TGTGAGATTGGGTCGAAGAAAT-3').

**Microarray analysis of gene expression following SEB stimulation.** Total RNA was isolated from mononuclear cells harvested from the livers of CD44 WT and CD44 KO mice 16 h following SEB stimulation by using an RNeasy Mini kit (QIAGEN). Labeled cDNA probes were synthesized from the RNA samples by using an Ampolabeling-LPR kit (SuperArray, Frederick, Md.). The labeled cDNA probes were hybridized to individual GEArray Q series mouse apoptosis array membranes overnight at 60°C with continuous agitation at 5 to 10 rpm. The membranes were washed twice for 10 min at 60°C with a 2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate solution and twice for 10 min at 60°C with 0.1 $\times$  SSC–0.5% sodium dodecyl sulfate. Nonspecific binding was blocked by incubating the membranes with GEA blocking solution (SuperArray, Frederick, Md.) for 40 min. The membranes were labeled with alkaline phosphatase-conjugated streptavidin for 10 min. Excess alkaline phosphatase-conjugated streptavidin was removed by washing the membranes four times with buffer F (SuperArray) for 5 min and rinsing the membranes with buffer G. Gene expression was detected with CDP-Star (Applied Biosystems) chemiluminescent substrate and by exposing the membranes to X-ray film. The data were analyzed by converting the X-ray image into a grayscale TIFF file and using the ScanAlyze software program to convert the data into numerical data. Finally, data analysis was performed by using the GEArray Analyzer data analysis software (SuperArray). Data were normalized by using housekeeping genes including  $\beta$ -actin, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), cyclophilin A, and ribosomal protein L13a.

**Statistical analysis.** Analysis of variance and Student's *t* test were used to determine statistical significance, and a *P* value of <0.05 was considered to be statistically significant.

## RESULTS

**SEB exposure leads to increased CD44 expression on liver mononuclear cells.** We examined the effect of *in vivo* SEB exposure on the levels of CD44 expression in liver mononuclear cells. To this end, CD44 WT and CD44 KO mice were injected with SEB and D-galactosamine. Twenty-four hours posttreatment, mononuclear cells were isolated from the livers of vehicle- or SEB-injected mice. The mononuclear cells were then stained with FITC-labeled anti-CD3 MAbs, PE-labeled anti-NK1.1 MAbs, and Cy-chrome-labeled anti-CD44 MAbs (Fig. 1). The expression levels of the various molecules were determined by using a flow cytometer. The results showed that all populations of mononuclear cells from the CD44 WT mice, including T cells, NK cells, and NKT cells, expressed CD44. The mononuclear cells from naïve mice contained populations with low and high expression levels of CD44. Following exposure to SEB, a majority of the cells expressed high levels of CD44, which led to an overall increase in the mean fluorescence intensity. In contrast, the mononuclear cells from the CD44 KO mice did not express CD44 (data not shown). Together, these results suggest that exposure to SEB *in vivo* leads to an increase in the expression of CD44 in T cells, NK cells, and NKT cells found in the liver.

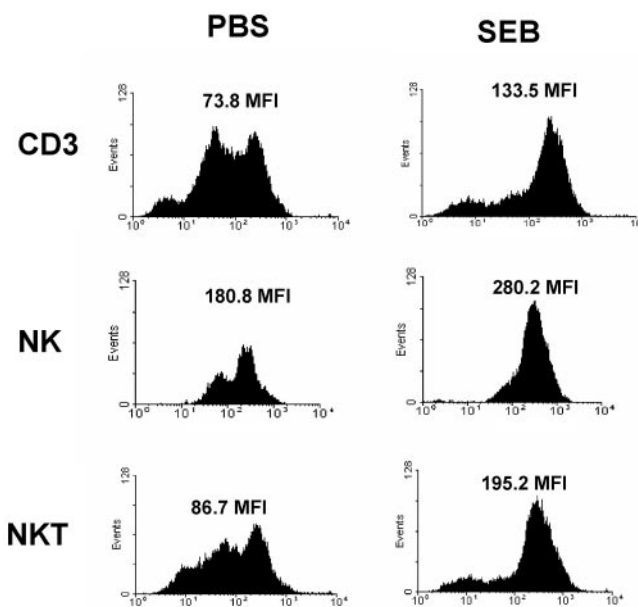


FIG. 1. SEB exposure leads to increased CD44 expression on liver mononuclear cells. CD44 WT mice were injected with SEB (20  $\mu$ g/mouse) and D-galactosamine (20 mg/mouse). Twenty-four hours later, the liver mononuclear cells were stained with FITC-conjugated anti-CD3 MAbs, PE-conjugated anti-NK1.1 MAbs, and Cy-chrome-conjugated anti-CD44 MAbs. Next, CD3<sup>+</sup>, NK1.1<sup>+</sup>, or CD3<sup>+</sup> NK1.1<sup>+</sup> (NKT) cells were gated, and the level of CD44 expression on these populations was determined by FACS analysis. The data are depicted as mean fluorescence intensity (MFI). More than 1,000 events were analyzed per gated population.

**CD44 KO mice are susceptible to SEB-induced hepatocellular damage.** One of the characteristics of SEB-induced toxic shock is liver injury. Therefore, we examined whether the deletion of CD44 had an effect on the susceptibility of C57BL/6 mice to SEB-induced hepatitis. To this end, CD44 WT and CD44 KO mice were injected with SEB and D-galactosamine. Hepatocellular damage was initially quantified by measuring AST levels in plasma at 0, 24, 48, and 72 h following injection of SEB and D-galactosamine (Fig. 2A). The results showed that injection of SEB and D-galactosamine led to a slight increase in the AST levels in the CD44 WT mice, peaking at 24 h following injection. In contrast, injection of SEB and D-galactosamine into CD44 KO mice led to a dramatic increase in AST levels 24 h after injection of SEB. The AST levels in both groups diminished by 48 h postinjection. The AST levels in CD44 WT or CD44 KO mice injected with D-galactosamine and PBS ranged from 30 to 80 U/liter, similar to what was seen in mice injected with PBS alone (data not shown), while AST levels in CD44 KO mice were as high as 2,000 U/liter in the CD44 KO mice 24 h following injection with D-galactosamine and SEB (Fig. 2A), suggesting that D-galactosamine exposure alone was not responsible for the induction of hepatitis in this model.

In addition, we examined SEB-induced liver injury by assessing the level of apoptosis in livers from untreated and SEB-treated mice. To this end, CD44 WT and KO mice were injected with SEB and D-galactosamine. The livers were harvested 24 h following SEB and D-galactosamine injection, sectioned, and stained for apoptosis by using the TUNEL method

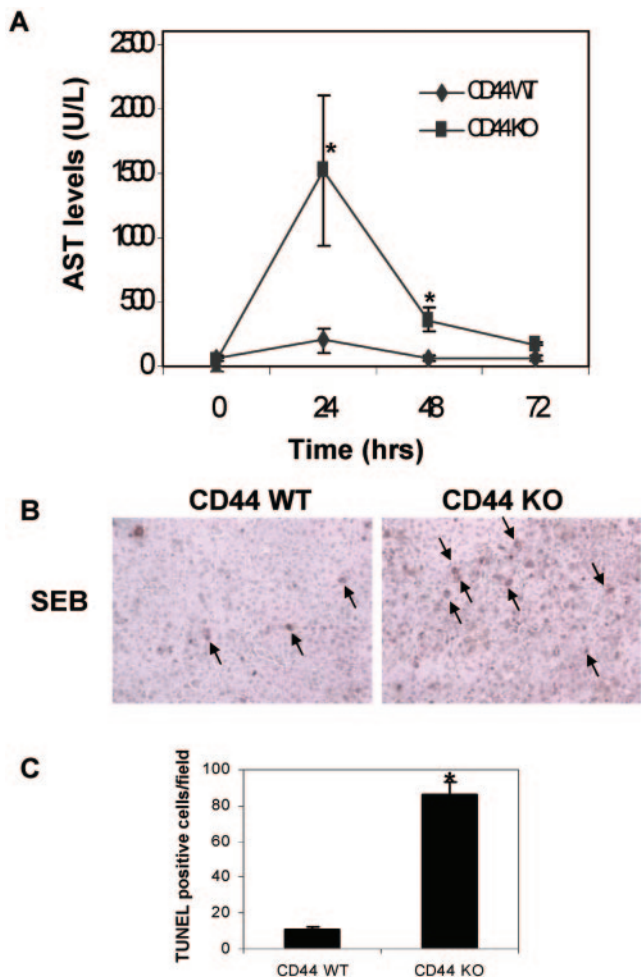


FIG. 2. CD44 KO mice are more susceptible to SEB-induced hepatocellular damage mediated by induction of apoptosis. CD44 WT and CD44 KO mice were injected with SEB (20  $\mu$ g/mouse) and D-galactosamine (20 mg/mouse). Liver damage was assessed 24 to 72 h later by measuring AST levels in plasma (A). The induction of apoptosis in the liver following exposure to SEB was assessed by TUNEL staining of fixed liver sections from SEB-treated CD44 WT and CD44 KO mice (B). Examples of apoptotic cells are indicated with arrows. The level of apoptosis in the liver was quantified by counting the number of TUNEL-positive cells/field from 12 fields (C). The average  $\pm$  the standard error of the mean (SEM) from three mice per treatment group is depicted. Asterisks indicate statistically significant differences compared to the CD44 WT controls ( $P < 0.05$ ).

(Fig. 2B). The level of apoptosis was quantified by determining the number of TUNEL-positive hepatocytes in 12 random fields taken from three individual mice per treatment group (Fig. 2C). The results showed that there were no significant levels of apoptotic cells in the PBS-exposed CD44 WT or CD44 KO mice (data not shown). Following SEB exposure, there was an increase in apoptotic hepatocytes in both the CD44 WT and CD44 KO mice. However, the levels of apoptosis were significantly higher in the SEB-exposed CD44 KO mice than those in the SEB-exposed CD44 WT mice. Together, these results suggested that CD44 KO mice are significantly more susceptible to SEB-induced hepatitis than CD44

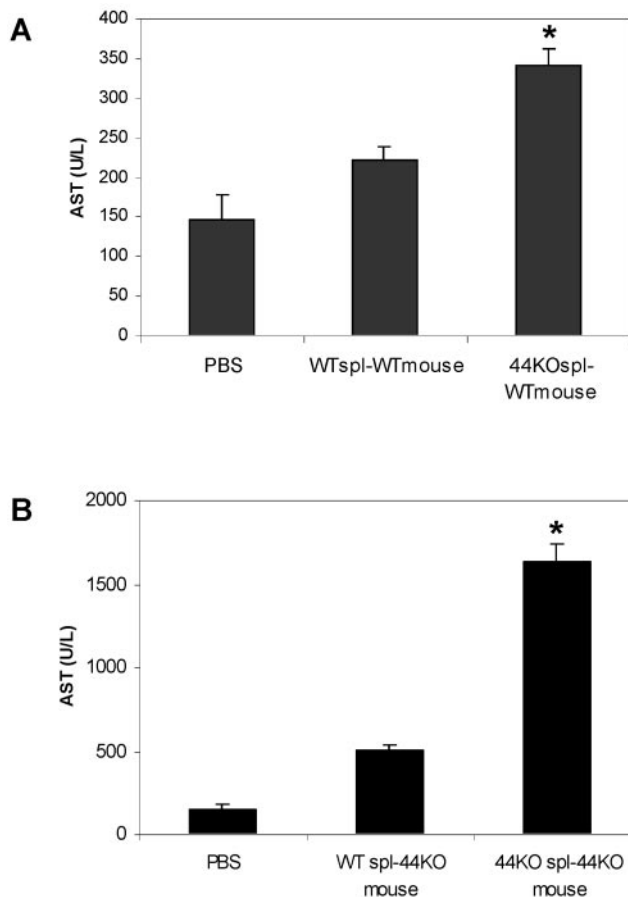


FIG. 3. Adoptive transfer of splenocytes from CD44 KO mice leads to increased SEB-induced hepatitis. Splenocytes from CD44 WT or CD44 KO mice were adoptively transferred into CD44 WT (A) or CD44 KO (B) mice followed by administration of SEB and D-galactosamine, and the induction of hepatitis was determined by measuring AST levels 24 h later. PBS-injected mice were used as controls for normal AST levels. Asterisks indicate statistically significant differences compared to the CD44 WT controls ( $P < 0.05$ ).

WT mice and that this susceptibility correlates with increased induction of apoptosis in the liver.

**Adoptive transfer of splenocytes from CD44 KO mice leads to increased SEB-induced AST levels in CD44 WT mice.** In the above-mentioned experiments, it was not clear whether the increase in SEB-induced hepatitis was the result of a deficiency of CD44 on lymphocytes or on hepatocytes. To address this, splenocytes from CD44 WT or CD44 KO mice were adoptively transferred into CD44 WT (Fig. 3A) or CD44 KO (Fig. 3B) mice followed by administration of SEB and D-galactosamine, and the induction of hepatitis was determined by measuring AST levels in plasma. The results showed that adoptive transfer of CD44 KO splenocytes into CD44 WT or CD44 KO mice followed by exposure to SEB and D-galactosamine led to a significant increase in AST levels compared to the levels of AST from CD44 WT or CD44 KO mice injected with CD44 WT splenocytes. Together, these data suggested that the increased SEB-induced hepatitis seen in CD44 KO mice was

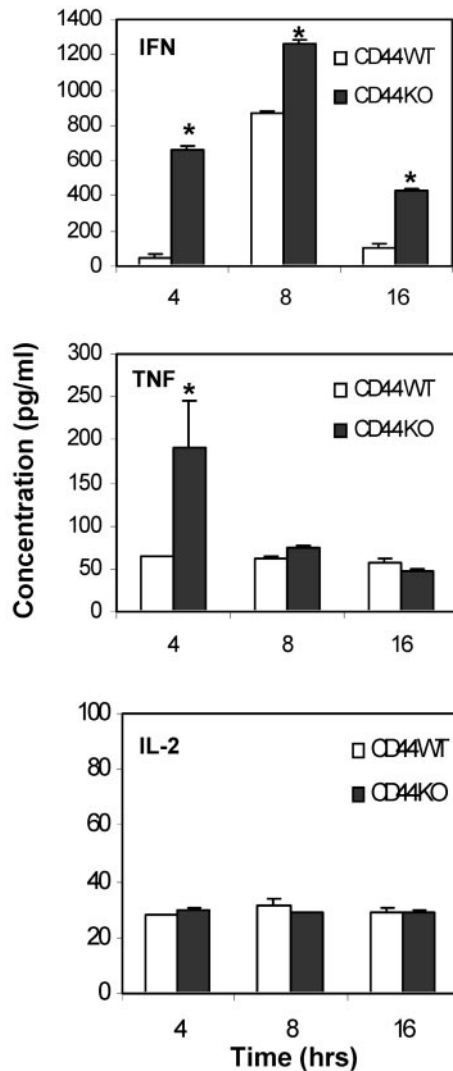


FIG. 4. Increased production of TNF- $\alpha$  and IFN- $\gamma$  from SEB-stimulated CD44 KO liver mononuclear cells. CD44 KO mice and CD44 WT mice were injected with SEB as described in the legend of Fig. 1. Liver mononuclear cells were isolated at various time points (4, 8, and 16 h) following SEB injection and cultured in triplicate for an additional 24 h in vitro. The supernatants were tested for the presence of TNF- $\alpha$ , IFN- $\gamma$ , and IL-2 by ELISA. Vertical bars represent the mean  $\pm$  SEM. Asterisks indicate statistically significant differences compared to CD44 WT liver mononuclear cells ( $P \leq 0.05$ ).

due, at least in part, to a deficiency of CD44 on SEB-activated lymphocytes.

**Role of cytokines in SEB-induced hepatotoxicity in CD44 KO mice.** TNF- $\alpha$ , IFN- $\gamma$ , and IL-2 have been reported to play a significant role in the induction of SEB-induced liver damage (50); therefore, we examined whether the levels of these cytokines were altered in CD44 KO mice following injection with SEB and D-galactosamine compared to similarly treated CD44 WT mice (Fig. 4). Based on previous reports of the kinetics of cytokine production following SEB exposure (15), liver mononuclear cells were isolated at various time points (4, 8, and 16 h) following SEB and D-galactosamine injection. The cells were cultured for an additional 24 h in vitro, after which the

supernatants were collected and tested for the presence of TNF- $\alpha$ , IFN- $\gamma$ , and IL-2 by ELISA. The results showed that mononuclear cells from CD44 KO mice produced significantly elevated levels of TNF- $\alpha$  as early as 4 h following SEB exposure compared to those of mononuclear cells from CD44 WT mice. However, at 8 h, the level of detectable TNF- $\alpha$  was reduced, and no significant differences could be observed between the CD44 WT and CD44 KO mice. The level of IFN- $\gamma$  produced by the CD44 KO liver mononuclear cells was significantly elevated as early as 4 h following SEB exposure. The levels peaked at 8 h and remained significantly higher 16 h following SEB exposure compared to levels of IFN- $\gamma$  produced by CD44 WT liver mononuclear cells. No significant differences were seen in the levels of IL-2 production. Together, these results demonstrate that SEB exposure leads to a rapid increase and subsequent reduction in the production of TNF- $\alpha$  and IFN- $\gamma$ , consistent with the kinetics of cytokine production following SEB exposure (15) and suggest that increased TNF- $\alpha$  and IFN- $\gamma$  production may play a significant role in the increased sensitivity of CD44 KO mice to SEB-induced hepatitis.

**Effect of SEB on liver mononuclear cell populations in CD44 WT and CD44 KO mice.** Next, we examined the effect of SEB exposure on the number and phenotype of liver mononuclear cells in CD44 WT and CD44 KO mice. Injection of SEB and D-galactosamine into CD44 WT mice led to an increase in the total number of mononuclear cells compared to vehicle-treated controls. This increase was first visualized by hematoxylin and eosin staining of liver sections (Fig. 5B) and then further confirmed by direct enumeration of liver mononuclear cells following isolation by Ficoll-Isopaque density gradient centrifugation (Fig. 5A). After exposure to SEB and D-galactosamine, the number of mononuclear cells in the CD44 WT mice rose steadily in a time-dependent manner (Fig. 5A). However, exposure of CD44 KO mice to SEB and D-galactosamine led to a significantly greater increase in mononuclear cells compared to the CD44 WT mice at all time points tested (Fig. 5A). Phenotypic characterization of the liver mononuclear cells revealed a significant increase in CD3<sup>+</sup> cells, V $\beta$ 8<sup>+</sup> cells, NK cells, and NKT cells in the CD44 WT mice (Table 1). In comparison, the CD44 KO mice showed a greater increase in almost all subpopulations of mononuclear cells in both percentage and absolute numbers following SEB injection (Table 1).

**SEB-reactive cells from CD44 KO mice undergo increased numbers of cell divisions.** The elevated number of mononuclear cells found in the livers of CD44 KO mice following SEB injection suggested the possibility that the absence of CD44 leads to increased proliferation and/or decreased susceptibility to SEB-induced apoptosis. To study the number of cell divisions that the SEB-activated CD44<sup>+</sup> or CD44<sup>-</sup> lymphocytes would undergo, we adoptively transferred CFSE-labeled CD44 WT or CD44 KO splenocytes into CD44 WT mice, injected them with SEB and D-galactosamine (as described in Materials and Methods), and enumerated CFSE-positive cells in the liver 24 h later. These data revealed that the proportion of CFSE-positive liver-infiltrating CD44 KO mononuclear cells that underwent six or more cell divisions was significantly higher than that of CFSE-positive cells from CD44 WT mice (Fig. 6). These data suggested that the increased numbers of mononuclear cells in CD44 KO livers as seen earlier (Fig. 5) was likely

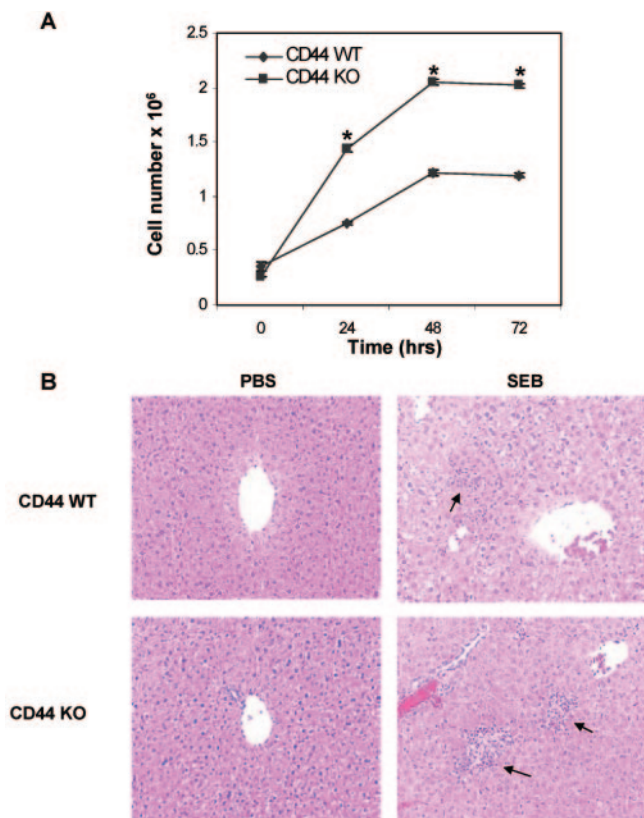


FIG. 5. Quantification of liver mononuclear cell populations following SEB injection. The presence of mononuclear cells was visualized by staining with hematoxylin and eosin (A). CD44 WT and CD44 KO mice were injected with SEB and D-galactosamine as described in the legend of Fig. 1. CD44 WT and CD44 KO mice were injected with PBS or SEB as described in the legend of Fig. 1. Twenty-four hours later, the livers were harvested, fixed, and sectioned. Arrows indicate mononuclear cell populations. To quantify liver mononuclear cells, cells were isolated 0 to 72 h following SEB exposure and quantified by trypan blue dye exclusion and expressed as the absolute number of cells per liver (B). Asterisks indicate statistically significant differences compared to the CD44 WT controls ( $P < 0.05$ ).

due to an increased number of cell divisions in SEB-reactive cells.

**SEB-reactive cells from CD44 KO mice are more resistant to apoptosis.** Upon SEB activation, the T cells rapidly undergo apoptosis. Previous studies from our laboratory showed that CD44 plays a critical role in AICD (35); therefore, we investigated whether this would impact the number of liver mononuclear cells found following SEB injection. To this end, CD44 WT and CD44 KO mice were injected with SEB and D-galactosamine, and 24 h later, the liver mononuclear cells were isolated and cultured in vitro for 24 h. The level of apoptosis was determined by using TUNEL (Fig. 7A). The results showed that following injection of SEB and D-galactosamine, there was significant induction of apoptosis in the mononuclear cells isolated from CD44 WT mice. In contrast, mononuclear cells from CD44 KO mice showed significantly lower levels of apoptosis 24 h following SEB and D-galactosamine injection than mononuclear cells from CD44 WT mice. Similar results were seen when the annexin-PI method for apoptosis detection was used (data not shown). In addition, we examined the levels

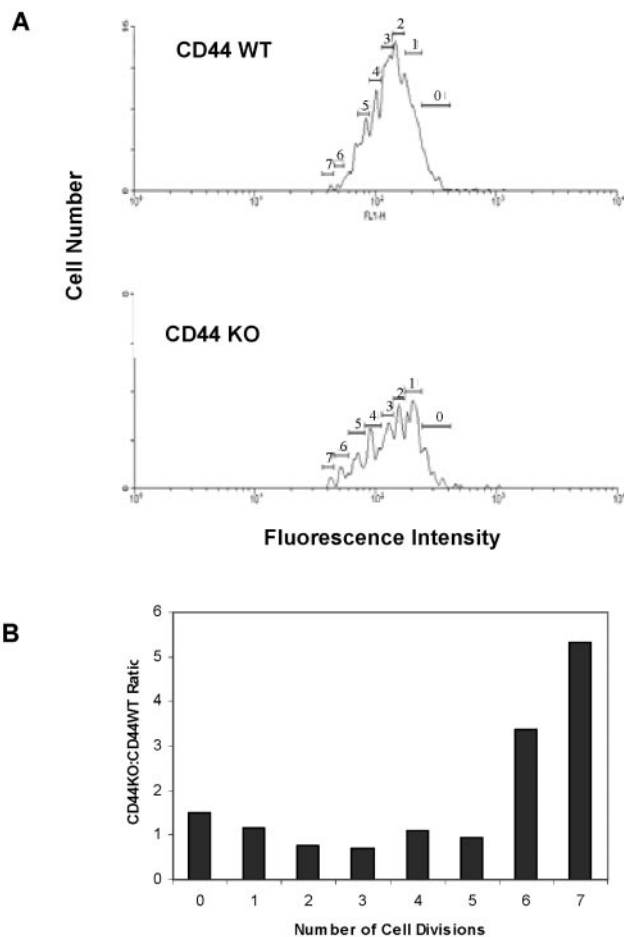


FIG. 6. Adoptive transfer of CFSE-labeled CD44 WT or CD44 KO splenocytes into CD44 WT mice. Splenocytes from CD44 WT or CD44 KO mice were stained with CFSE and then adoptively transferred into CD44 WT mice. The mice were then injected with SEB plus D-galactosamine as described in the text. Twenty-four hours later, the liver mononuclear cells were harvested, and the number of cell divisions was determined by FACS analysis. Each cell division is indicated by brackets. The proportion of CD44 WT cells to CD44 KO cells in each cell division is depicted in panel B. The results are representative data of experimental groups containing three mice. The experiment has been repeated three times with similar results.

of cleaved-active caspase-3 in mononuclear cells isolated from untreated and SEB-treated CD44 WT and CD44 KO mice and found that following exposure to SEB, there was an increase in the levels of cleaved-active caspase-3 in CD44 WT mice (Fig. 7B). In contrast, the levels of cleaved-active caspase-3 were significantly lower in mononuclear cells from SEB-treated CD44 KO mice. Together, these results suggested that liver mononuclear cells from CD44 KO mice are more resistant to SEB-induced apoptosis.

**Microarray analysis of gene expression in liver mononuclear cells following stimulation with SEB in vivo.** Next, we screened for alterations in the expression of genes involved in apoptosis in liver mononuclear cells following exposure to SEB and D-galactosamine with cDNA array analysis. Of the 96 genes screened, the expression of 25 genes was significantly (greater than twofold) altered in the liver mononuclear cells

TABLE 1. Effects of SEB on liver mononuclear cell populations in vivo

Treatment	Cellularity <sup>a</sup> (cells [10 <sup>5</sup> ]/ liver)	Liver mononuclear cell populations <sup>b</sup>			
		Vβ8 <sup>+</sup>	CD3 <sup>+</sup> NK1.1 <sup>-</sup>	CD3 <sup>-</sup> NK1.1 <sup>+</sup>	CD3 <sup>+</sup> NK1.1 <sup>+</sup>
CD44 WT + PBS	3.6 ± 0.4	14.7 (0.53)	25.5 (0.92)	2.1 (0.08)	5.0 (0.18)
CD44 WT + SEB	7.5 ± 0.4	35.3 (2.65)	36.2 (2.72)	11.1 (0.83)	3.3 (0.25)
CD44 KO + PBS	2.5 ± 0.05	21.5 (0.54)	24.5 (0.61)	1.8 (0.05)	4.3 (0.11)
CD44 KO + SEB	14.4 ± 0.1	53.26 (7.67)	37.6 (5.41)	12.7 (1.83)	16.0 (2.3)

<sup>a</sup> D-galactosamine-sensitized mice were treated with SEB or the vehicle. Twenty-four hours later, the liver mononuclear cells were isolated, and the total cell number was determined. Data represent means ± SEM obtained from three mice.

<sup>b</sup> Liver mononuclear cell subsets were determined by using flow cytometry. The data represent the percentage of Vβ8<sup>+</sup> cells, CD3<sup>+</sup> NK1.1<sup>-</sup> cells, CD3<sup>-</sup> NK1.1<sup>+</sup> cells, and CD3<sup>+</sup> NK1.1<sup>+</sup> cells in the livers obtained from three mice. The numbers in parentheses represent the mean total cellularity. (10<sup>5</sup>) of each cell population found in the liver.

from the CD44 KO mice. The expression of 15 genes was reduced while the expression of 10 genes was increased in the CD44 KO liver mononuclear cells (Table 2). Included in the group of genes expressed at lower levels were the proapoptotic *Apaf-1*, *Bak*, *Bax*, *Bid*, *Hrk*, *CRADD*, *caspase-1*, and *caspase-8* genes. In addition, the expression of a number of antiapoptotic genes, including *TANK*, *OX40*, *OX40L*, *TRAF2*, and *TRAF3*, was upregulated. Taken together, the results from the cDNA analysis further supported a role of CD44 in regulating the induction of apoptosis in SEB-activated liver mononuclear cells. More specifically, the data suggested that in the absence of CD44, the expression of a number of proapoptotic genes is decreased while the expression of a number of antiapoptotic molecules is increased.

#### Liver-infiltrating mononuclear cells from CD44 KO mice express lower levels of Bax following SEB stimulation in vivo.

Bcl-2-related proteins play an important role in the induction of apoptosis following SEB stimulation (22). Based on the results from our cDNA array analysis, we further examined the effect of CD44 deficiency on the levels of Bax (Fig. 8). To this end, CD44 WT and CD44 KO mice were treated with SEB and D-galactosamine as described above. Liver-infiltrating mononuclear cells were isolated 24 h following SEB injection, and Bax mRNA levels were determined by RT-PCR (Fig. 8A). The results from several experiments showed that there was a sig-

nificant increase in the level of Bax mRNA in the infiltrating mononuclear cells from SEB-injected CD44 WT mice compared to that of vehicle-treated CD44 WT mice. In contrast, the level of Bax mRNA in the liver mononuclear cells from CD44 KO mice was not significantly altered following SEB stimulation. More specifically, following exposure to SEB, the expression of Bax mRNA was increased by 42% ± 3% in the CD44 WT mice and only by 9% ± 5% in CD44 KO mice. Together, these data validated the results from cDNA array analysis and further suggested that the observed resistance of CD44 KO liver-infiltrating mononuclear cells to apoptosis may be due, at least in part, to a defect in the induction of Bax following activation with SEB in vivo.

**Anti-CD44 MAbs enhance SEB-induced hepatitis in vivo.** In a previous report, Mustafa et al. demonstrated that in vivo treatment with anti-CD44 MAbs leads to reduced cell surface expression of CD44 on splenocytes in vivo (39). Therefore, in the present study, we examined whether treatment with anti-CD44 antibodies and subsequent reduction in CD44 expression would have any effect on SEB-induced hepatitis. To this end, CD44 WT mice were treated with SEB and D-galactosamine plus isotype control antibodies or SEB and D-galactosamine plus anti-CD44 MAbs (IM7). The level of liver damage was determined 24 h later by measuring AST levels in plasma (Fig. 9A). The results showed that treatment with anti-

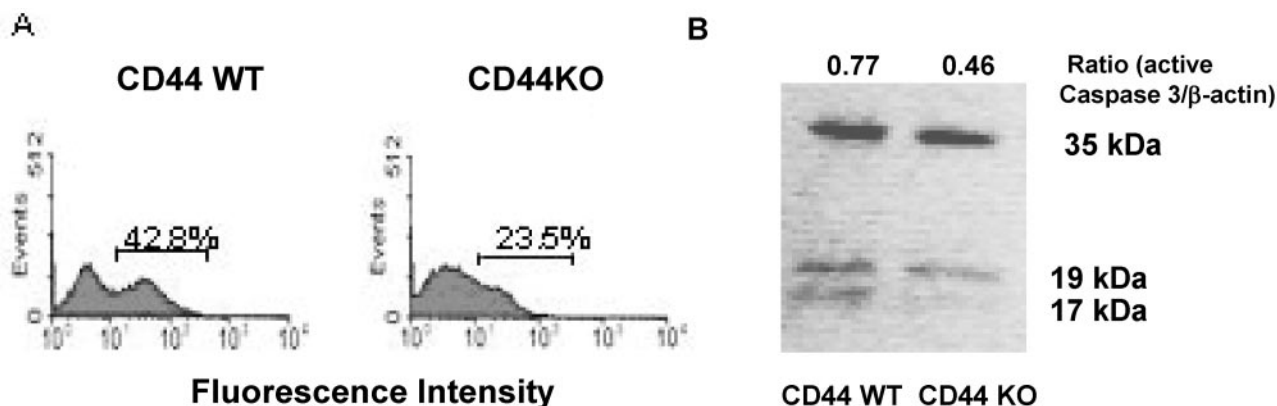


FIG. 7. CD44 KO liver mononuclear cells are resistant to SEB-induced apoptosis. CD44 WT and CD44 KO mice were injected with SEB and D-galactosamine as described in the legend of Fig. 1. Twenty-four hours later, mononuclear cells were isolated from the liver and assayed for the presence of apoptotic cells. Apoptosis in liver mononuclear cells was assayed by using the TUNEL method (A). The level of caspase-3 cleavage was determined by Western blot analysis and depicted as a ratio of caspase-3 levels to β-actin levels (B).

TABLE 2. cDNA array analysis of apoptotic gene expression in liver mononuclear cells from CD44 KO and CD44 WT mice following SEB exposure<sup>a</sup>

Gene name	Description or function	GenBank accession no.	Change (Fold) vs CD44 WT <sup>b</sup>
<i>Apaf-1</i>	Apoptotic protease activating factor-1	NM_009684	-29.1
<i>April</i>	APRIL mRNA	AF294825	-11.3
<i>Bad</i>	Displaces Bax and promotes apoptosis	L37296	-3.0
<i>Bak</i>	Bcl-2 antagonist	NM_007523	-6.0
<i>Bax</i>	Bcl-2-associated X protein	L22472	-33.3
<i>Bcl-w</i>	Bcl-2-like 2	AF030769	-3.7
<i>Bid</i>	BH3-interacting domain, death agonist	NM_007544	-5.6
<i>Hrk</i>	BH3-interacting domain, apoptosis agonist	D83698	-77.5
<i>IAP1</i>	Inhibitor of apoptosis protein 1	U88908	-3.3
<i>IAP2</i>	Inhibitor of apoptosis protein 2	U88909	-4.6
<i>X-linked IAP</i>	X-linked inhibitor of apoptosis	U88990	-22.2
<i>caspase-1</i>	IL-1 $\beta$ convertase	L03799	-5.0
<i>caspase-8</i>	Caspase-8	AF067834	-4.5
<i>CRADD</i>	Death adaptor molecule	AJ224738	-2.8
<i>DAP kinase</i>	Death-associated kinase 2	AF052942	-4.7
<i>caspase-12</i>	Caspase-12	NM_009808	+2.3
<i>caspase-2</i>	Caspase-2	D28492	+2.2
<i>TANK</i>	TRAF family member-associated NF- $\kappa$ B activator	U51907	+3.3
<i>OX40</i>	OX40	Z21674	+2.5
<i>OX40L</i>	OX40 ligand	U12763	+2.3
<i>Fas</i>	Fas antigen	NM_007987	+2.5
<i>FasL</i>	Fas ligand	U06948	+2.2
<i>CD40L</i>	CD40 ligand	X65453	+3.1
<i>TRAF2</i>	TNF receptor-associated factor 2	L35303	+4.7
<i>TRAF3</i>	TRAF-related protein	L08752	+5.7

<sup>a</sup> Summary of gene expression that was found to be increased or decreased in mononuclear cell isolated from the liver of CD44 KO mice following injection with SEB compared to the gene expression in mononuclear cells isolated from the liver of CD44 WT mice following injection with SEB.

<sup>b</sup> Change (fold) represents the change in gene expression following normalization with  $\beta$ -actin gene expression.

CD44 MAbs led to a significant increase in the AST levels compared to those of mice injected with SEB plus isotype control antibodies. In addition, we determined whether treatment with anti-CD44 MAbs had an effect on the level of CD44 expression on liver mononuclear cells (Fig. 9B). To this end, liver mononuclear cells from vehicle-treated and SEB-treated mice which received either anti-CD44 MAbs or isotype control antibodies were stained for the expression of CD44 and analyzed by fluorescence-activated cell sorter (FACS). The results demonstrated that treatment with SEB led to a significant increase in CD44 expression compared to the vehicle-treated mice. Interestingly, treatment of SEB-exposed mice with anti-CD44 MAbs led to a significant reduction in CD44 expression. In addition, the percentage and number of CD3-positive and NK1.1-positive cells were not affected by anti-CD44 MAB treatment (data not shown). Together, the data from this set of experiments suggested that administration of anti-CD44 MAbs into CD44 WT mice leads to increased susceptibility to SEB-

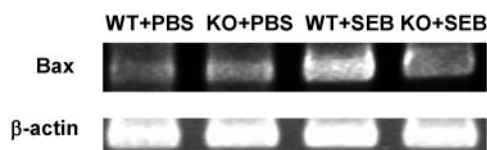


FIG. 8. Expression of Bax by liver mononuclear cells following stimulation with SEB in vivo. CD44 WT and CD44 KO mice were injected with SEB and D-galactosamine as described in the legend of Fig. 1. Twenty-four hours later, the liver mononuclear cells were isolated and whole cell extracts were prepared. Expression of Bax and  $\beta$ -actin was analyzed by RT-PCR analysis.

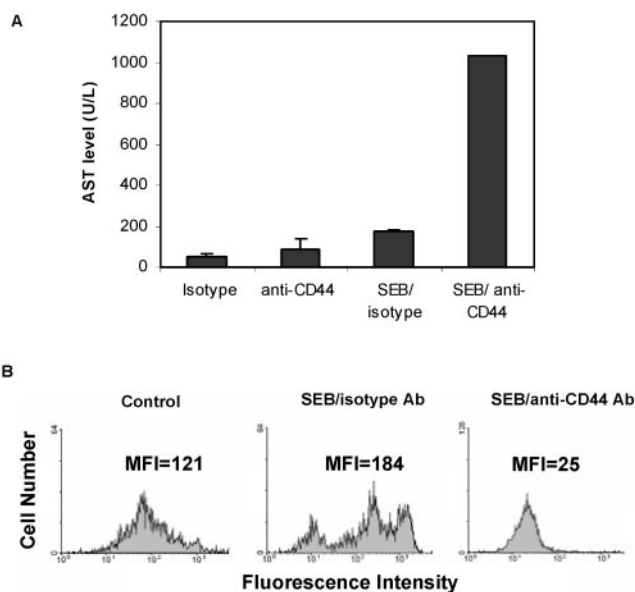


FIG. 9. Administration of anti-CD44 MAbs enhances SEB-induced hepatitis in vivo. Groups of five mice were injected with SEB (20  $\mu$ g/mouse) and D-galactosamine (20 mg/mouse). In addition, groups of mice received 100  $\mu$ g of anti-CD44 MAB (IM7) or isotype control antibody (A). Liver damage was determined 24 h later by measuring levels of AST in plasma. Vertical bars represent the mean level of AST (units/liter [U/L])  $\pm$  SEM. The level of CD44 expression on liver mononuclear cells following anti-CD44 treatment was determined by FACS analysis (B). Asterisks indicate statistically significant differences compared to the isotype controls ( $P < 0.05$ ).



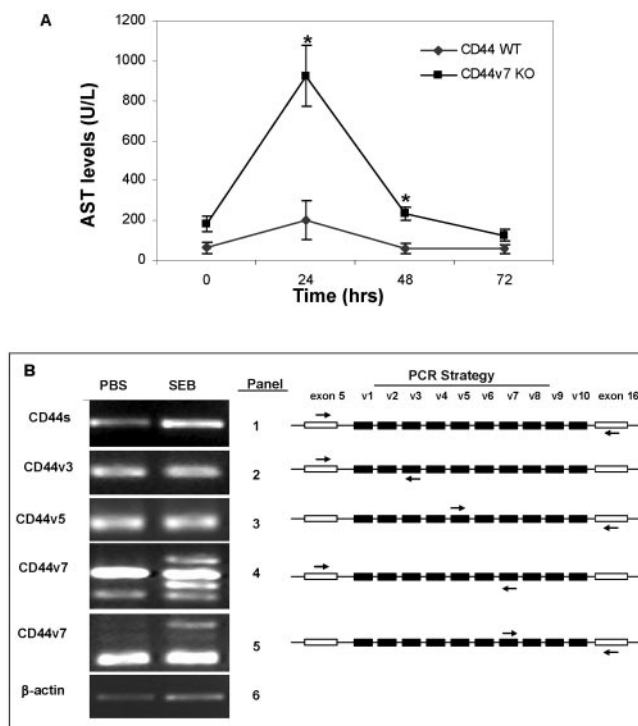


FIG. 10. The role of CD44v7 in SEB-induced activation and induction of hepatitis. (A) Deletion of CD44v7 leads to increased susceptibility to SEB-induced hepatitis. Groups of five CD44 WT and CD44v7 KO mice were injected with SEB (20  $\mu$ g/mouse) and D-galactosamine (20 mg/mouse). Liver damage was determined 0 to 72 h later by measuring levels of AST in plasma. Plots represent the mean level of AST (units/liter [U/L])  $\pm$  SEM. The asterisk indicates a statistically significant difference compared to the CD44 WT controls. (B) RT-PCR-based characterization of CD44 isoform expression in SEB-activated liver mononuclear cells. RNA isolated from splenocytes of PBS-treated and SEB-treated mice was subjected to RT-PCR analysis using the PCR strategy depicted above. PCR products were resolved on 1.2% agarose gels.

induced hepatitis by reduction of CD44 expression on liver mononuclear cells.

**Alteration of CD44v7 variant isoform expression following SEB exposure in vivo.** To determine whether SEB treatment led to the differential regulation of CD44 variant isoform expression, liver mononuclear cells from CD44 WT mice injected with SEB and D-galactosamine or PBS were harvested after 24 h and analyzed for the expression of various CD44 isoforms. The results showed that treatment with SEB in vivo led to the increased expression of the CD44 standard form (CD44s) (Fig. 10B, panel 1) and the variable expression of a number of CD44 variant isoforms (Fig. 10B). Here, the presence of multiple bands is expected due to the combinatorial nature of CD44 isoform expression. Most notable was the dramatic induction of isoforms containing alternatively spliced exon 7 (v7). Amplification using a v7 reverse primer and CD44 conserved region forward primer (Fig. 10B, panel 4) yielded two products in PBS-treated samples, suggesting that two CD44 isoforms containing the v7 gene products were present. In contrast, in SEB-exposed mice, we observed four bands representing four individual CD44 isoforms containing the v7 gene product, suggesting that stimulation with SEB leads to the expression of at

least two additional CD44 isoforms containing the v7 exon gene products. More specifically, since we observed four individual bands, these results suggest that four unique combinations of CD44 (v1 to v7) isoforms are present following SEB stimulation, all of which contain the v7 gene product. Similarly, amplification with a v7 forward primer and CD44 conserved region reverse primer yielded only one band in the PBS-treated sample (Fig. 10B, panel 5). In contrast, two products were generated in the sample from the SEB-treated mice, suggesting that SEB stimulation of liver mononuclear cells results in at least one additional CD44 isoform containing the v7 exon gene product in combination with at least one other CD44v8 to CD44v10 exon gene product. When the expression of CD44 isoforms containing the v3 and v5 exons was analyzed, we noted that exposure to SEB had no significant effect on their expression (Fig. 10B, panels 2 and 3). Together, these data suggested that exposure to SEB leads to differential regulation of CD44 isoforms containing the v7 exon.

**Deletion of CD44v7 leads to increased susceptibility to SEB-induced hepatitis in vivo.** After we determined that exposure to SEB affected the expression of CD44 isoforms containing the v7 exon, we examined whether expression of CD44v7 played a role in the regulation of SEB-induced hepatitis. To this end, CD44 WT and CD44v7 KO mice were injected with SEB and D-galactosamine. Hepatocellular damage was quantified by measuring AST levels in plasma at 0, 24, 48, and 72 h following injection of SEB (Fig. 10A). The results showed that injection of SEB led to a slight increase in the AST levels in the CD44 WT mice, peaking at 24 h following injection. In contrast, injection of SEB and D-galactosamine into CD44v7 KO mice led to a dramatic increase in AST levels 24 h after injection of SEB. The AST levels in both groups diminished by 48 h postinjection.

## DISCUSSION

In the present study, we demonstrated that following exposure to SEB, mice deficient in CD44 have significantly elevated levels of liver damage compared to levels of liver damage in wild-type mice. This increase resulted from enhanced production of cytokines known to be associated with SEB-induced shock. More specifically, TNF- $\alpha$  and IFN- $\gamma$  levels were significantly elevated in CD44 KO mice following SEB exposure. Evaluation of the mechanism of CD44 involvement in SEB-induced hepatotoxicity revealed that the absence of CD44 rendered liver mononuclear cells resistant to the induction of apoptosis, possibly due to an inhibition in the expression of a number of proapoptotic molecules, including Bax. This possible role of CD44 in activated cells is further supported by previous work from our laboratory which demonstrated that CD44 KO mice are more susceptible to concanavalin A-induced hepatitis and that CD44 is important in activation-induced cell death following stimulation with conventional antigens and superantigens (35), and the possible role of Bax is supported by a previous report showing that cross-linking of CD44 on dexamethasone-treated thymic lymphomas leads to upregulation of Bax (19). In the present study, we further demonstrate that CD44v7 may play a critical role in regulating SEB-induced liver injury. Together, our studies suggest that in wild-type mice, SEB triggers CD44 expression on resident lym-

phocytes in the liver, whose activation also leads to production of cytokines, capable of mediating liver injury. However, signaling through CD44 triggers rapid apoptosis in SEB-activated cells, thereby limiting the liver injury. Thus, dysregulation in CD44 expression in liver mononuclear cells may exacerbate SEB-induced hepatic injury.

SEB interacts with major histocompatibility complex-encoded molecules and activates primarily  $V\beta 8^+$  T cells (10). In the present study, we demonstrated that following SEB stimulation *in vivo*, there was a significant increase in the number of  $CD3^+$  and  $V\beta 8^+$  cells in the liver. The liver contains a unique distribution of lymphocytes highly enriched for NK and NKT cells (4). NK cells play an important role in the induction of hepatitis following concanavalin A exposure or infection with adenovirus vectors (30, 51). More specifically, it is believed that NK cells act by producing cytokines such as IFN- $\gamma$ , which leads to the recruitment and accumulation of T cells in the liver (47). Although the liver is known to be rich in NKT cells (4), the exact role of these cells in the immune response in the liver is largely unknown. However, Takeda et al. demonstrated that Fas ligand expression on liver NKT cells played a critical role in the pathogenesis of concanavalin A-induced hepatitis (49). In the present study, we demonstrated that the number and percentage of NK and NKT cells was significantly elevated in the liver of CD44 WT mice following exposure to SEB, suggesting a possible role of these cells in SEB-induced toxic shock. Furthermore, since most NKT cells express  $V\beta 8.2$  (25), it is possible that NKT cells can be directly activated by SEB. Interestingly, the number of NKT cells was significantly higher in CD44 KO mice following SEB exposure than in similarly treated CD44 WT mice. In addition, following stimulation of CD44 WT mice with SEB, we noted a significant increase in the expression of CD44 on NKT cells, suggesting a possible role of CD44 in NKT cell response to SEB.

SEB has been used to examine a number of processes important for the regulation of the immune system, including cytokine production, T-cell anergy, and AICD (18, 38, 40). Following immune activation, there is significant accumulation of apoptotic lymphocytes in the liver. Although there is some controversy as to whether the liver acts as a lymphocyte "graveyard" where dead or dying cells accumulate or as a lymphocyte "killing field" where activated cells receive signals to undergo apoptosis, considerable evidence suggests that activated cells can directly undergo AICD in the liver (5). In the present study, we used SEB to further explore the possible role of CD44 in regulating lymphocyte apoptosis in the liver. Previous reports from our laboratory demonstrated that the expression of CD44 on activated lymphocytes plays an important role in their interactions with endothelial cells (36, 41). Interestingly, the regulation of activated lymphocytes in the liver is postulated to take place through interactions with a number of cells including Kupffer cells and sinusoidal endothelial cells (LSEC) (26). Therefore, we speculate that interactions between activated lymphocytes and activated LSEC or Kupffer cells leads to the induction of apoptosis in the activated lymphocytes and that this signaling is either directly or indirectly regulated by CD44 expression. This possibility is further supported by the fact that LSEC have been shown to bind and express the CD44 ligand hyaluronic acid (28, 57).

The standard form of CD44, designated CD44s, is the most

common form and has a size of 85 to 95 kDa (17). In addition to CD44s, a number of larger CD44 isoforms, ranging in size from 80 to 250 kDa, develop due to alternative splicing of variant exons (52). It has been suggested that expression of specific isoforms may play a role in the regulation of the immune response as well as in the development of autoimmune disorders. In the present study, we demonstrate that exposure to SEB leads to increased expression of CD44 isoforms containing the v7 exon and that deletion of the CD44v7 exon leaves mice more susceptible to SEB-induced hepatitis. Together, these results suggest an important role of CD44v7 in the regulation of the immune response following exposure to the superantigen SEB. In contrast, studies examining the effects of TNBS on the induction of colitis using CD44v7 knockout mice clearly demonstrated that expression of CD44 variant isoforms containing the v7 exon gene product played a role in protecting lamina propria mononuclear cells from the induction of apoptosis (55). These apparent discrepancies can possibly be explained by the fact that these antigenic systems activate distinct components of the immune system, and furthermore, the pathogenesis in these models may involve different mechanisms. For example, TNBS-induced colitis is believed to be mediated by Th1 cells (54). In contrast, SEB is a superantigen that activates a large proportion of T lymphocytes bearing specific  $V\beta$  regions of the T-cell receptor as well as macrophages (10). However, SEB-induced liver injury may result from initial activation of Kupffer cells and subsequent triggering of NK and NKT cells (7, 43, 55). Thus, signaling through CD44 in these different effector cells may play different or opposite roles. To date, little is known about the role of CD44 isoforms in Kupffer, NK, and NKT cells. Also, there could be differences in the intestinal and hepatic microenvironment in terms of the levels of CD44 receptor or the CD44 ligand expression.

To better understand the mechanism of CD44 involvement in the induction of apoptosis in liver mononuclear cells, we used cDNA array analysis. We observed that following stimulation with SEB *in vivo*, there was a significant reduction in the expression of a number of proapoptotic molecules and a significant increase in the expression of a number of antiapoptotic molecules in the mononuclear cells isolated from the livers of CD44 KO mice compared to that of similarly treated CD44 WT mice. Of particular interest was the expression of Bax. We demonstrated that the level of Bax mRNA in CD44 WT mice was significantly elevated following stimulation with SEB. In contrast, stimulation of liver mononuclear cells from CD44 KO mice failed to cause significant upregulation of Bax. In addition, we demonstrated that the levels of caspase-8 and Bid following stimulation with SEB were significantly lower in CD44 KO mice than in SEB-treated CD44 WT mice. Caspase-8 is believed to function primarily in the death receptor pathway, whereas Bax plays an important role in the mitochondrial pathway (33), suggesting that CD44 may play a role in both pathways. Bid serves as an important mediator of cross talk between the death receptor and mitochondrial pathways (29, 31). Since Bid is significantly reduced in SEB-stimulated liver mononuclear cells from CD44 KO mice, it is possible that signaling through CD44 directly leads to the activation of death receptor molecules such as caspase-8 and the eventual participation of the mitochondrial pathway through the in-

volvement of molecules such as Bid, Bax, and HrK. The importance of CD44 in the regulation of Bax is further supported by other reports that demonstrated that the cross-linking of CD44 led to upregulation of Bax expression in dexamethasone-treated thymic lymphomas (19). The role of CD44 signaling in caspase-8 activation was shown in a study that examined the biological activity of a hyaluronan-binding peptide that binds hyaluronan in a fashion similar to that of CD44. In this study, it was shown that treatment with the peptide led to increased expression of caspase-8 and the eventual induction of apoptosis in a human breast cancer cell line (56).

Mice are relatively resistant to the effects of SEB and therefore require prior sensitization with D-galactosamine or endotoxins such as lipopolysaccharide. In contrast, humans are highly sensitive to SEB, and exposure to SEB can lead to the rapid onset of clinical symptoms. SEB is often associated with food poisoning. However, SEB was weaponized by the United States in the 1960s and, when inhaled, leads to rapid incapacitation (32). Symptoms include fever, respiratory distress, headache, and in severe cases multiorgan failure, which are thought to result from the release of large amounts of cytokines such as IFN- $\gamma$  and TNF- $\alpha$  (34). Treatment of SEB-induced toxic shock consists primarily of supportive therapy, and there are currently no available vaccines for preventing SEB-induced toxicities (34). Therefore, developing a better understanding of the mechanism of SEB-induced toxicities may lead to significantly enhanced specific treatments. In the present study, we described a role for CD44 in the regulation of the immune response following SEB exposure and we demonstrated that treatment with anti-CD44 MAbs, which has been shown to reduce cell surface expression of CD44 as shown by others (39, 46), leads to exacerbated toxicity. Together, these results suggest that alterations in CD44 expression or signaling may significantly alter the level of SEB-induced toxicity and that CD44 expression may play an important role in the regulation of the immune response in the liver.

#### ACKNOWLEDGMENTS

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