anti-CD86-PE mAb (BD Bioscience, La Jolla, CA, USA), and anti-LSECs-FITC mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), washed in FACS buffer [PBS containing 1% fetal calf serum (FCS)], and fixed in 2% paraformaldehyde for 30 min at room temperature. Samples were acquired using a FACScalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA), and the data were analyzed using the CELLQuest software (BD Immunocytometry Systems).

Isolation of CD11b-positive cells

CD11b-positive cells were purified by positive selection using antimouse CD11b beads and a magnetic cell separation system column (Miltenyi Biotec) according to the manufacturer's directions. After incubating with isolated $CD11b^+$ cells and FITC-dextran (Sigma), phagocyte activity was analyzed by FACS.

Data analysis

All data are expressed as the mean ± SD. The significance of differences among mean values was evaluated according to the Mann–Whitney *U* test.

Results

To examine the effects of CD44 on liver inflammation, we first generated HBV transgenic (HBVTg) mice backcrossed with CD44KO mice[11] (CD44KO × HBVTg mice). HBV Tg mice named lineage 107-5D (inbred B10.D2, H-2d) in which, constitutively, the HBV envelope coding region is under the control of the mouse albumin promoter.[13] In addition, we confirmed no significant difference with cell number and phenotype in CD44 present or absent intrahepatic leukocytes (IHLs).

We injected 5×10^6 HBV-specific CTL clones (epitope 28–39) into HBVTg or CD44KO × HBVTg mice, and subsequently monitored their sALT activities and intrahepatic leukocyte (IHL) numbers at various time points. As shown in Fig. 1A, the sALT activity in HBVTg mice began to increase at 4 h (287 ± 36 IU/l) and reached a peak at 24 h (8677 ± 1224 IU/l) after CTL injection, as previously reported.[1] On the other hand, the sALT activity in CD44KO × HBVTg mice was significantly reduced at 4 h (168 ± 21 IU/l) compared with HBVTg mice, but exacerbated at 36 h (13650 ± 1490 IU/l) after CTL injection. Consistent with the sALT activity, the total number of IHLs was significantly reduced in CD44KO × HBVTg mice at 4 h, with particular reductions in the neutrophil ($Gr\text{-}1^{++}/CD11b^+$) and macrophage ($Gr\text{-}1^+/CD11b^+$) populations. In contrast, the total number of IHLs, including neutrophils and macrophages, was increased at 24 h in these mice. Furthermore, we analyzed the messenger RNA (mRNA) expression levels for inflammatory cytokines, chemokines, and cell markers in the liver using RNase protection assays (RPAs). The results exhibited similar expression patterns to the elevation of sALT activity, because the mRNA levels of tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and interleukin (IL)-1α/β in the liver of CD44KO × HBVTg mice were reduced at 4 and 8 h but recovered to almost their normal levels at 24 and 36 h (see also Supplementary Fig. 1). In addition, we found that the mRNA levels of various chemokines in the liver of CD44KO × HBVTg mice were reduced at 4 h, in association with the reduced numbers of IHLs.

Regarding the suppressive effects on liver injury at 4 h, we hypothesized that the number of transferred CTLs would be reduced in the liver by the CD44KO conditions. To clarify this hypothesis, we injected 5,6-carboxyfluoroscein succinimidyl ester (CFSE)-labeled CTLs into HBVTg mice with or without CD44 and then counted the numbers of transferred CTLs among the IHLs and PBMCs by fluorescence-activated cell sorting (FACS) and in the liver by immunohistochemistry. The number of CFSE-labeled CTLs was seen to be lower (Fig. 2A) at 4 h, but not at 24 h, in the liver of CD44KO × HBVTg mice compared with the liver of HBVTg mice. In contrast, transferred CTLs were not detected in the PBMCs of both types of mice. Consistent with the FACS data, immunohistochemical analysis revealed that the number of CFSE-labeled CTLs was significantly lower in the liver of CD44KO × HBVTg mice. Collectively, these results suggest that HBV-specific CTLs migrate into the liver in a CD44-dependent manner.

It is well known that CTLs have to pass through liver sinus endothelial cells (LSECs) to enter the hepatocytes.[17–19] Based on this knowledge, we examined the role of LSECs in this model. First, we analyzed CD44 expression on LSECs in the liver of HBVTg mice. LSECs expressed CD44 under steady-state conditions but upregulated its expression at 4 and 24 h after CTL injection (Fig. 3). We also analyzed the levels of a costimulatory molecule (CD86) and an adhesion molecule (ICAM-1) on LSECs in the presence or absence of CD44. We found that these levels were similar in both types of mice, indicating that CD44 on LSECs plays a key role in CTL migration.

As already mentioned, the sALT activity was suppressed in CD44KO × HBVTg mice in association with the reduced number of IHLs and levels of cytokine or chemokine mRNA at 4 h. However, liver inflammation was reinforced at the late phase. To evaluate the mechanism of the latter observation, we analyzed the induction of hepatocyte apoptosis and NF-κB activity in the liver, as we previously demonstrated that low intrahe-

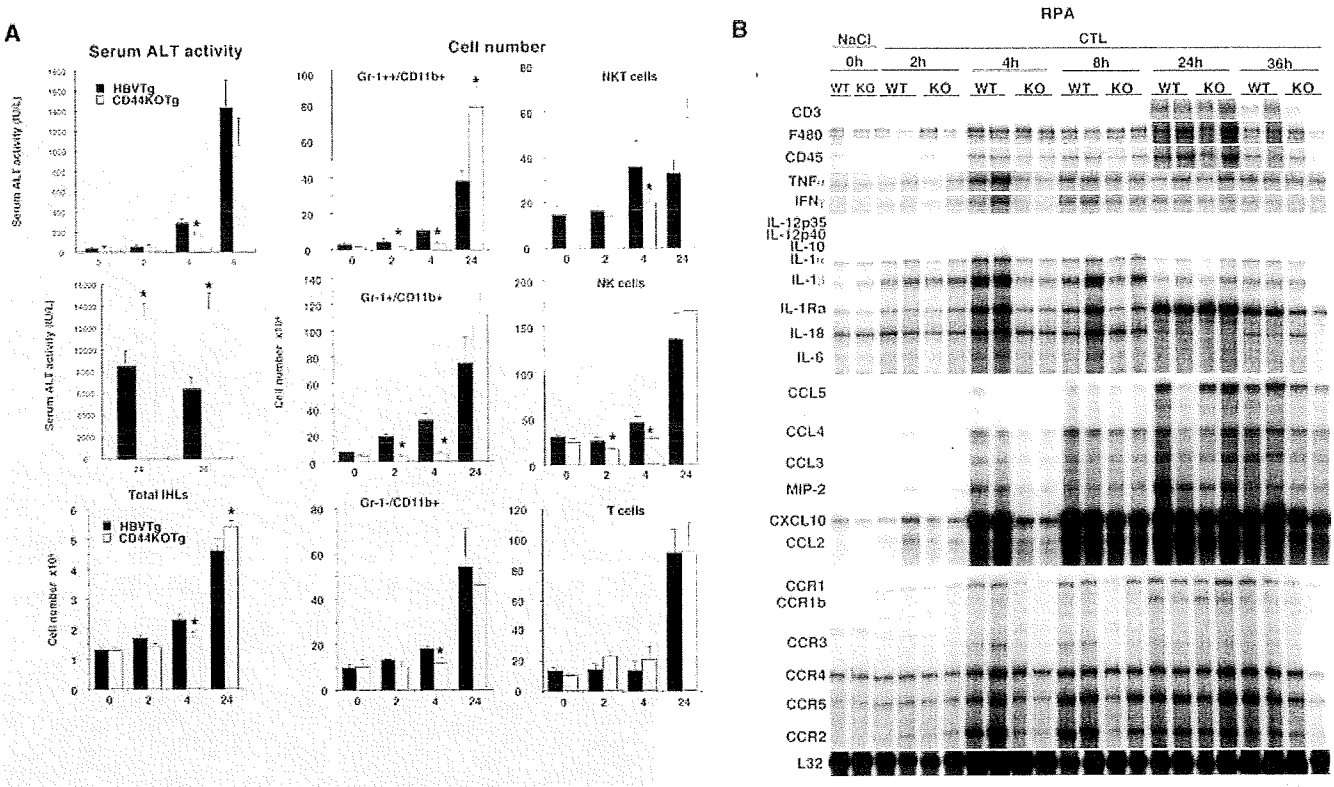


Fig. 1. **A** CD44KO mice exhibit suppressed cytotoxic T lymphocyte (CTL)-induced liver injury and cell recruitment at the early phase but not the late phase. Hepatitis B virus transgenic (HBVTg) or CD44KO × HBVTg mice were injected intravenously with 5×10^6 6C2 cells or NaCl. The mean serum alanine transferase (sALT) activity measured at the time of autopsy is indicated for each group and expressed in IU/l (mean ± SD). $^*P < 0.05$. Intrahepatic leukocytes (IHLs) were isolated from the same animals, and the effects of CD44 blockade were analyzed. The numbers in each subset of cells in the liver were calculated by multiplying the total number of IHLs by the frequency of each subset in the IHL population by fluorescence-activated cell sorting (FACS) analysis. The data represent means ± SD for three mice. $^*P < 0.05$. **B** Inflammatory cytokine and chemokine expression levels in the liver. The mRNA expression levels of the indicated inflammatory cytokines, chemokines, and cell-surface markers in total hepatic RNA samples (20 µg) from the same mice as in **A** were analyzed by RNase protection assays (*RPA*). The mRNA level of the housekeeping gene encoding ribosomal protein L32 was used to normalize the quantity of RNA loaded in each lane

patic NF-κB activity easily induces hepatocyte apoptosis.[15,20] To determine the histological changes in the liver of HBVTg mice with or without CD44 at 24 h after CTL injection, we stained liver tissues with hematoxylin and eosin and in situ TUNEL. The histological analysis revealed widely scattered inflammatory foci in the liver parenchyma and around the portal tract, containing mostly lymph mononuclear cells and a few apoptotic hepatocytes, in HBVTg mice (Fig. 4A). On the other hand, there was a marked increase in apoptotic hepatocytes in the parenchyma (Fig. 4A) in CD44KO × HBVTg mice, and lymph mononuclear cells had infiltrated around the central vein. In addition, the liver of CD44KO × HBVTg mice showed a marked increase in the number of TUNEL-positive cells after CTL injection (Fig. 4A), as quantified in Fig. 4B. These results suggest that the liver of CD44KO × HBVTg mice underwent massive apoptosis of hepatocytes, in association with the marked elevation of sALT activity. Furthermore, we analyzed the difference in CTL-induced NF-kB activation in the liver by an electrophoretic mobility shift assay (EMSA). We found that induction of NF-kB activity revealed the same level in the liver with CD44KO × HBVTg or HBVTg mice (Fig. 4C). We observed induction of NF-kB activity of HBVTg mice at 4 h after the injection, as previously reported.[21] In contrast, and consistent with the cytokine profile, NF-κB activity was reduced with CD44KO × HBVTg mice at 4 h after the injection, suggesting that this reduction represents one of the reasons for the increase in apoptotic hepatocytes. Next, we investigated the phagocytic activity of CD44KO macrophages, because recent reports have suggested that CD44 is a competent phagocyte receptor and CD44KO mice exhibited poor clear-

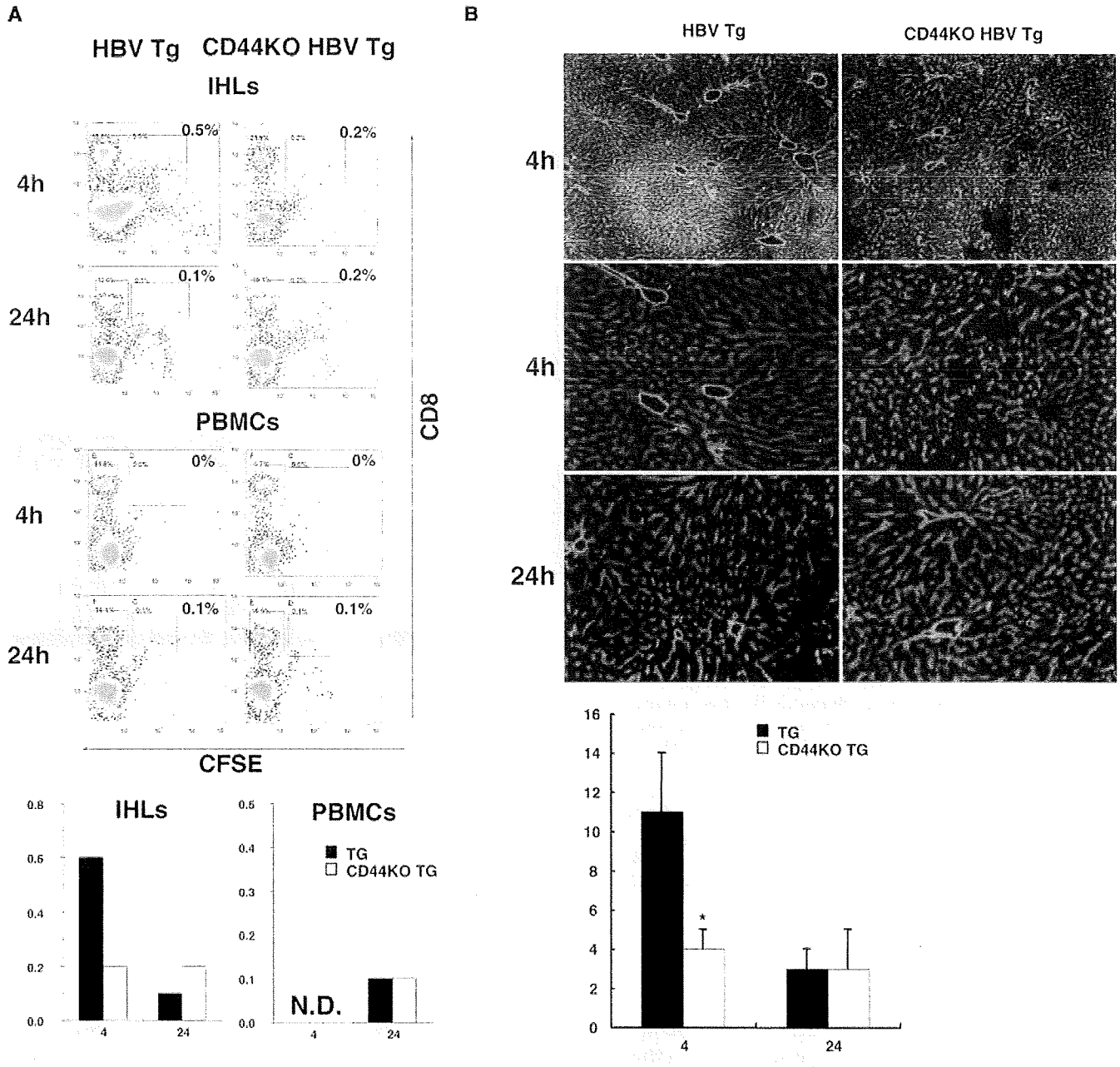


Fig. 2. Numbers of transferred CTLs in the liver labeled by 5,6-carboxyfluoroscein succinimidyl ester (CFSE). **A** To detect CFSE-labeled CTLs in the liver and peripheral blood mononuclear cells (PBMCs), we isolated IHLs and PBMCs from CD44KO × HBVTg and HBVTg mice at 4 and 24 h after injection. The samples were stained with an antimouse APC-CD8 antibody and analyzed by FACS. *N.D.*, not detected. **B** Liver sections were obtained from the same mice after injection of CFSE-labeled CTLs and stained with an antimouse type 4 collagen antibody and Alexa Fluor 594-conjugated goat antirabbit IgG as a secondary antibody to distinguish liver sinus endothelial cells (LSECs). The percentages of CFSE-positive cells in the liver were calculated. Data are expressed as means ± SD for three mice. $^*P < 0.05$

ance of apoptotic cells by macrophages.[22,23] We found that dextran uptake by isolated $CD11b^+$ cells (mostly macrophages) from CD44KOxHBVTg mice was decreased compared with the uptake by $CD11b^+$ cells from HBVTg mice (Fig. 4D), indicating that the increase in apoptotic hepatocytes may be partially the result of dysfunction of clearance by macrophages.

Discussion

In the present study, we have demonstrated that, although CD44 deficiency has no suppressive effect on CTL-induced acute hepatitis, it contributes to CTL migration into the liver. It is already established that adhesion on lymphocytes, including CTLs and endothe-

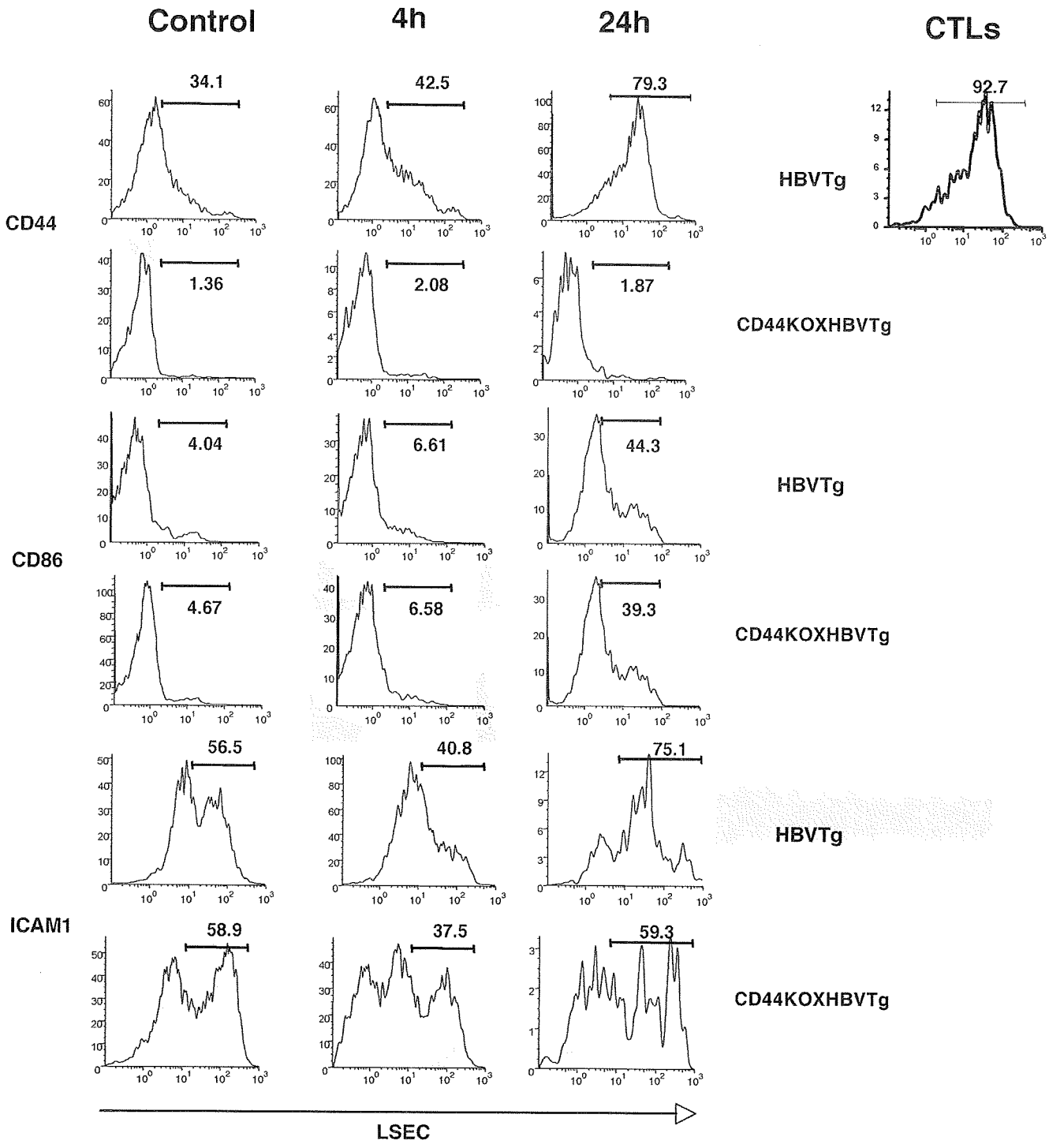


Fig. 3. FACS analysis of LSECs in the liver. The expression levels of CD44, CD86, and ICAM-1 on LSECs were examined at 4 and 24 h after CTL injection in CD44KO × HBVTg or HBVTg mice. IHLs were stained with anti-LSEC fluorescein isothiocyanate (FITC) and anti-CD44-PE, anti-CD86-PE, or anti-ICAM-1-PE antibodies. Representative results of three independent experiments are shown

lial cells, involves multiple steps at inflamed sites.[24–26] Although CD44 on lymphocytes is thought to be essential for adherence to endothelial cells, we have shown that CD44 on LSECs is also important for inflammatory cell migration into the liver. However, the precise mechanism of the interaction between CTLs and CD44 on LSECs remains unknown. As previously reported,[27,28] CD44 is a major receptor for hyaluronan, a nonsulfated

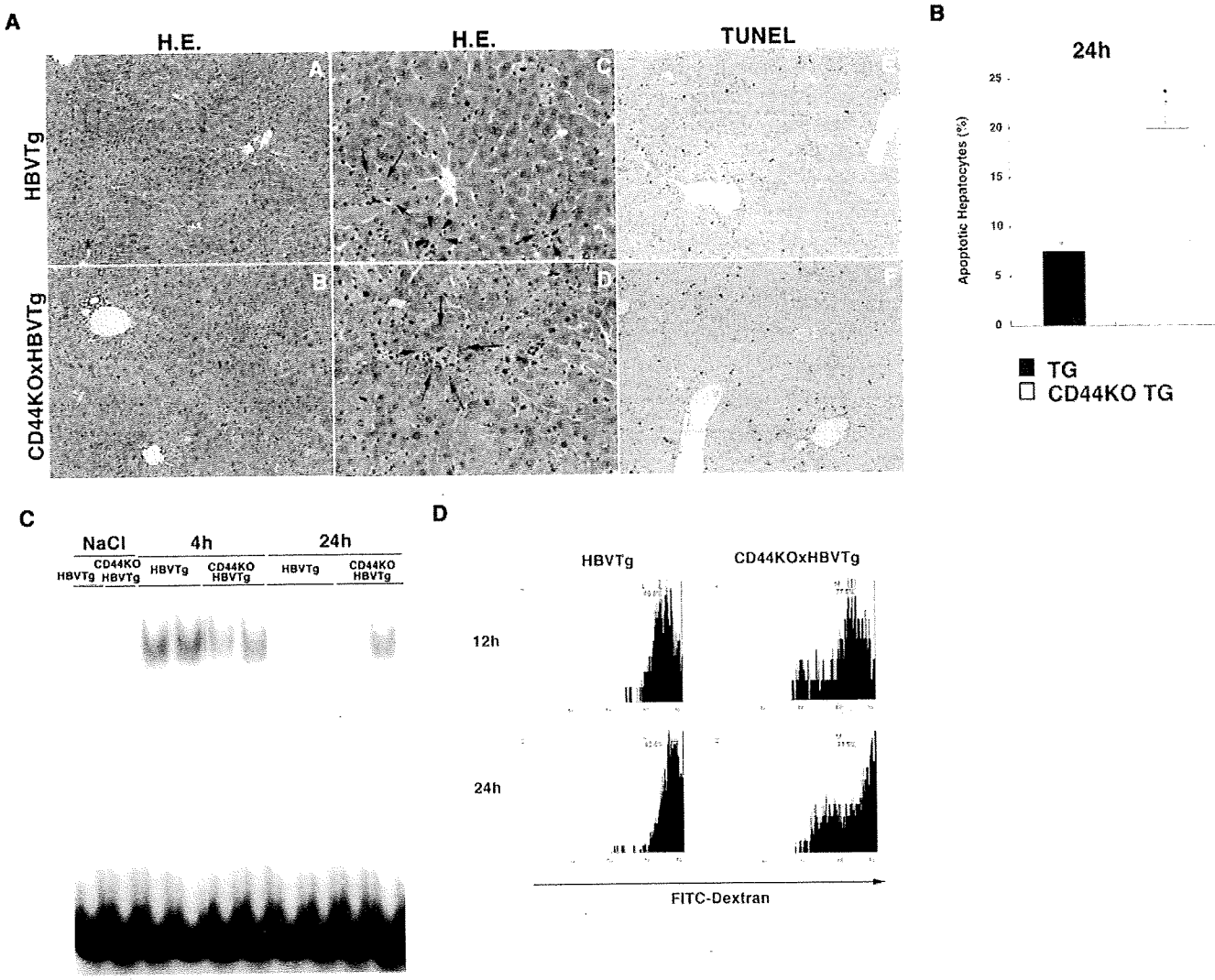
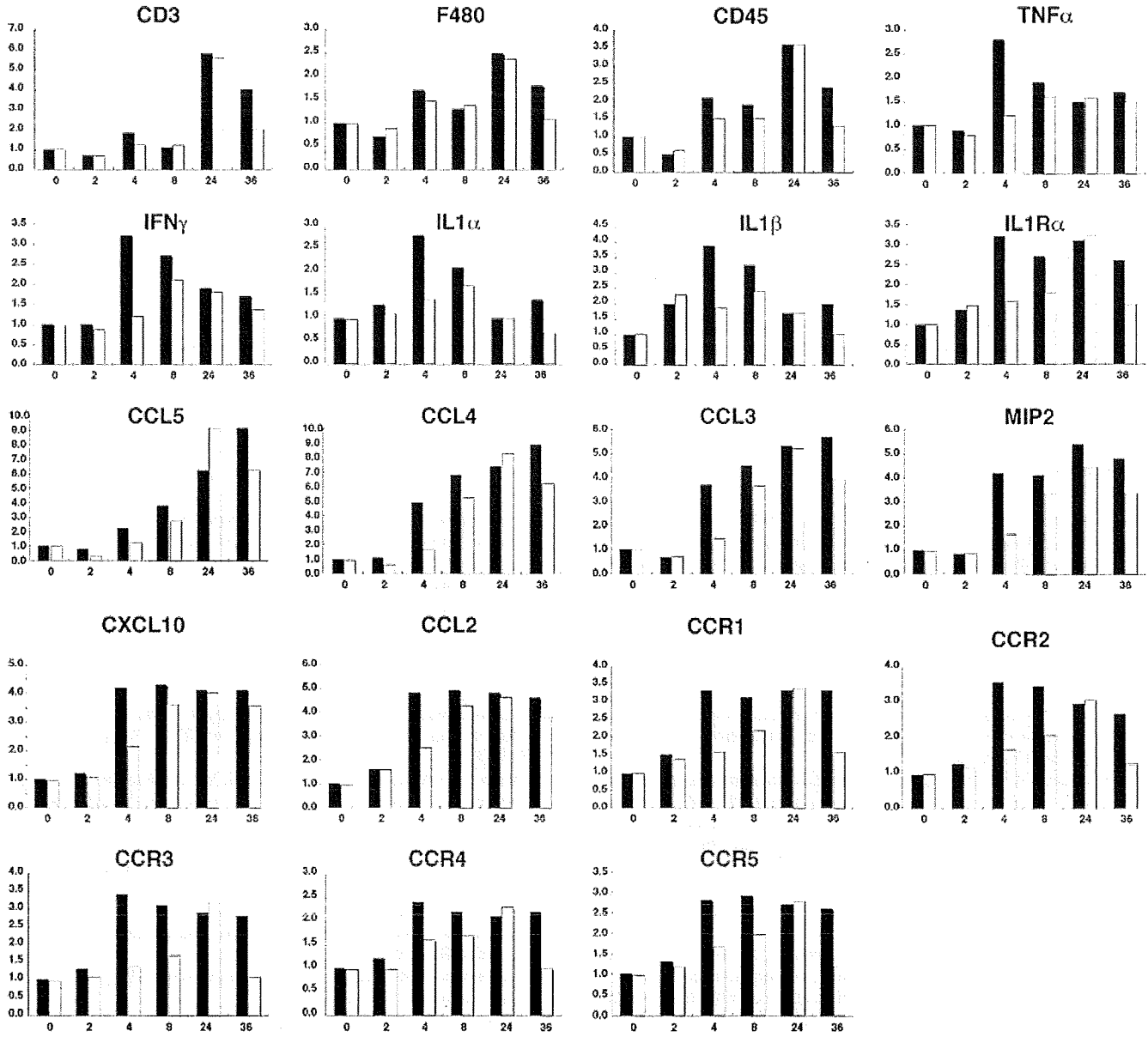


Fig. 4. Histological analysis of the livers of CD44KO × HBVTg or HBVTg mice after CTL injection and NF-κB activity. **A** Liver sections were obtained from mice killed at 24 h after CTL injection and stained with hematoxylin and eosin (*H.E.; left panels*). Note, in HBVTg mice at 24 h, that small inflammatory foci containing mostly lymph mononuclear cells (*arrows*) are observed in the liver. In CD44KO × HBVTg mice, larger foci containing more lymph mononuclear cells and liver necrosis are detected in the parenchyma (*arrows*). To evaluate the induction of apoptosis, liver sections were stained by the in situ TUNEL assay (*right panels*). In CD44KO × HBVTg mice at 24 h, TUNEL-positive hepatocytes are detected in the parenchyma (**B**). The percentages of apoptotic hepatocytes among the total hepatocytes were determined by TUNEL staining. Data are expressed as means ± SD for three mice. $^*P < 0.05$. **C** Electrophoretic mobility shift assays (EMSAs) were carried out on nuclear extracts from the livers of mice at 0, 4, or 24 h after CTL administration. **D** Phagocytic activities. To clarify whether CD44KO macrophages exhibit dysfunction of phagocytes, we isolated $CD11b^+$ cells from IHLs from CD44KO × HBVTg and HBVTg mice using MACS beads. After incubating both types of isolated $CD11b^+$ cells with FITC-dextran for 12 and 24 h, FACS analyses were performed

glycosaminoglycan ubiquitously distributed in extracellular spaces.[6] In addition, proteoglycan forms of CD44 exhibit affinities for other matrix components, including fibronectin, collagens, and osteopontin, as well as growth factors and cytokines.[29] Although we found that osteopontin was present on CTLs (data not shown), we cannot currently confirm the counterpart molecule for CD44 on CTLs. It is also curious why CD44KO inflammatory cells were able to infiltrate the liver at the late phase, when their migration was suppressed in the early phase. As one possible reason, we consider that, once the inflammatory cascade has started and many inflammatory cells migrate to the inflamed tissue, blockade of a single molecule, in this case CD44, is insufficient to protect against inflammation. These facts remind us that antiadhesion molecule treatment requires optimal timing and that its clinical application requires further experiments.

On the other hand, controversial findings have indicated that CD44 ligation has anti- or proapoptotic effects in cell lines.[30,31] To rule out the possibility that the transferred CTLs were susceptible to apoptosis in CD44KO mice, we analyzed annexin-V staining of CTLs after coculture with isolated LSECs from CD44KO × HBVTg or HBVTg mice, as previously reported.[15] However, there was no difference in the numbers of annexin V-positive CTLs in the mice (data not shown).

Thus, it is of note to mention that antiadhesion molecule-targeted therapy should be viewed with caution for the liver immune system. Although our findings require confirmation, blockade of CD44 alone in the present study led to inadequate cytokine production as a result of suppressed inflammation brought about by low NF-κB induction and subsequently led to increased numbers of apoptotic hepatocytes and severe liver injury. These dual effects (suppression and exacerbation) on the regulation of liver inflammation have been reported with regard to NF-κB regulation.[15,16] These results suggested that insufficient antiinflammatory effects rather exacerbate hepatic injury, and it seems that it is necessary to suppress the inflammation sufficiently during acute hepatitis in the environment of the liver.



Supplementary Fig. 1. Supplementary data. Densitometric analysis: mRNA expression levels were calculated as relative percentages to the mRNA expression level of the *L32* housekeeping gene

Furthermore, we showed here that HBV-specific CTLs play an important role as the trigger for severe liver injury. As was shown in Fig. 2, the number of CFSE-labeled CTLs was reduced at 24 h compared with 4 h in HBVTg mice when CD44 was either present or absent; however, sALT activity exhibited a peak at 24 h, suggesting that severe liver injury was mainly caused by antigen-nonspecific inflammatory cells, NK cells, macrophages, and neutrophils, as previously reported.[2,3,13]

Although we showed the number of CFSE-labeled CTLs in the liver and PBMCs (see Fig. 2), we also investigated the number in another tissue, the spleen. However, we could not detect CTLs in the CD44 × HBVTg or HBVTg spleen at 2, 4, and 24 h after injection (data not shown). These results suggested to us the importance of the affinity and avidity of HBV-specific CTLs in HBV-expressing hepatocytes.

In conclusion, these findings mean that although CD44 blockade is useful for the migration of virus-specific CTLs to the liver, it may require further improvement for use as a clinical therapy.

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Original Article

Pathological role of CD44 on NKT cells in carbon tetrachloride-mediated liver injury

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Aim: CD44 has a variety of functions in immune regulation and signal transduction. Although CD44 is involved in the induction of several inflammatory diseases, it remains unknown whether CD44-targeting therapies are useful for liver diseases. Here, we examined whether CD44 blockade is effective in a chemical-induced liver injury model.

Methods: We injected CD44 knock out (KO) or wild type mice with carbon tetrachloride (CCl_4) and examined the difference of liver injury by immunological or histological analysis.

Results: Although CD44KO mice exhibited suppressed liver injury at 6 h after CCl_4 injection with decreased inflammatory cell numbers and cytokine production, these mice showed severe liver injury at 24 h. We found that NKT cells played an important role in liver injury with increased infiltration of the liver after migration, which was independent of the CD44 pathway. In CD44NKT double-KO mice, liver injury was suppressed with reduced cytokine production and macrophage infiltration compared with CD44KO mice. Furthermore, MIP-2 derived from NKT cells or tumor necrosis factor alpha from macrophages contributed to exacerbation of the liver injury, since neutralization of MIP-2 provided significant protection against liver injury in CD44KO mice. Finally, we found that CD44KO mice exhibited excessive liver fibrosis compared with wild-type mice after repeated CCl_4 injections.

Conclusion: We found that CD44 has unique characteristics for inflammatory liver diseases associated with NKT cell infiltration and activation. Furthermore, CD44-targeting therapies may need to be viewed with caution for liver diseases due to the actions of the liver immune system.

Key words: CD44, cytokine, inflammation, liver fibrosis, NKT cell

INTRODUCTION

THE LIVER HAS a variety of characteristic features in its immune responses to foreign pathogens.[1,2] The liver contains a large population of lymphocytes, including $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells and natural killer T (NKT) cells.[3–5] These populations can be rapidly expanded during inflammatory liver disease or in response to viral infection. It has been suggested that most lymphocytes in the normal liver are activated terminally-differentiated T cells that are removed from circulation by the liver, where they are destined to die by apoptosis.[1,6] Furthermore, it is well established that antigen-specific $CD8^+$ cells play an important role in the clearance of hepatitis B and C viruses.[3,7] In addition, recent reports have shown that antigen-nonspecific cells, NKT cells, NK cells and B cells could also act as effector cells in the activated state.[8–10] In view of these findings, blockade of inflammatory cell infiltration into the liver is thought to be an ideal therapy for various diseases of the liver.

For a lymphocyte to be recruited from the circulating blood stream, it must first recognize and then bind to adhesion molecules expressed on endothelial cells.[11] A multistep model of leukocyte adhesion to the vascular

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endothelium has been described elsewhere and is broadly applicable in different tissues.[12,13] It has also been demonstrated that tethering or rolling receptors expressed on endothelial cells capture free-floating leukocytes. Recruitment of leukocytes in the post-sinusoidal venules of the liver follow a similar paradigm. However, distinct from most other organs, many leukocytes can also be seen adhering in the sinusoids, which are specialized hepatic capillaries.[11]

CD44-hyaluronan interactions play an important role in regulating leukocyte extravasation into inflammatory sites and mediate efficient phagocytosis.[14–17] In addition, a growing body of evidence suggests that CD44 serves as a key factor in the resolution of inflammation through removal of matrix breakdown products and clearance of apoptotic neutrophils, and mediates fibroblast migration and invasion in the provisional matrix of the wound.[16]

NKT cells express an invariant T cell receptor chain (V14-J281 in mice) and recognize glycolipid antigens, such as α-galactosylceramide (αGalCer), in association with the major histocompatibility complex (MHC) class I-like molecule CD1d.[18,19] NKT cells are unique for cytokine production, since they are autoreactive and produce both Th1 and Th2 cytokines, including interleukin (IL)-4, IL-5 and IFN-γ, upon stimulation with αGalCer.[20–22] A recent report demonstrates that the adhesion molecule lymphocyte function-associated antigen (LFA)-1 plays a role in NKT cell activation (especially, Th2 responses) in response to αGalCer treatment.[23–25] However, the relationship between NKT cells and adhesion molecules during inflammatory events remains unknown.

In the present study, we examine the suppressive effects of CD44 on inflammation in mouse models of hepatotoxin (CCl_4)-induced acute and chronic liver diseases.

EXPERIMENTAL PROCEDURES

Animals

CD44KO MICE (BACKGROUND, C57BL/6) were generously provided by Dr. Tak W. Mak (University of Toronto, Canada).[26] Vα14 NKT-deficient (NKT-KO) mice were generated as described[20] and C57BL/6 (B6) mice were purchased from SLC (Japan). All animals were housed in pathogen-free rooms under strict barrier conditions, and received humane care according to the guidelines of the Animal Care Committee of the University of Tokyo, School of Medicine.

Administration of CCl_4 and αGalCer

For acute CCl_4-induced liver damage studies, a single dose of CCl_4 (2.0 mL/kg body weight; 1 : 4 v/v in mineral oil) was administered by i.p. injection. For chronic CCl_4-induced liver damage studies, a dose of CCl_4 (2.0 mL/kg body weight) was administered twice per week. For activation of Vα14 NKT cells with αGalCer, mice were injected i.p. with αGalCer (100 μg/kg) or vehicle as previously described.[21] For *in vitro* experiments, we added CD44KO and wild type (WT) mice derived intrahepatic leukocytes (IHLs) with αGalCer (10 ng/mL). αGalCer (KRN7000) was kindly provided by Kirin Brewery (Japan).

Tissue RNA analyses

Frozen liver was mechanically pulverized under liquid nitrogen and total RNA was isolated for ribonuclease protection assay (RPAs), as previously described.[10] All RPA reagents were purchased from BD PharMingen (CA, USA).

Biochemical and histological analyses

The extent of hepatocellular injury was monitored biochemically by measuring the activity of serum alanine aminotransferase (sALT) at multiple time points using a standard automatic clinical analyzer. For histological analysis, liver tissue was fixed in 10% zinc-buffered formalin, embedded in paraffin, sectioned (3 μm) and stained with hematoxylin and eosin or AZAN for light-microscopic evaluation.

Immunohistochemistry

Immunohistochemical staining with anti-mouse α-smooth muscle actin (αSMA) and anti-mouse proliferating cell nuclear antigen (PCNA) monoclonal antibodies (mAbs) was performed using an avidin-biotin-peroxidase complex technique as described previously.[10] The numbers of positive cells were counted in at least 50 high-power (× 400) fields and the results were expressed as percentages (positive cells/total cells). Terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) assays were performed according to the manufacturer's instructions, as described previously.[27]

Preparation of intrahepatic leukocytes (IHLs)

To isolate IHLs, single-cell suspensions were prepared from liver perfused with phosphate-buffered saline (PBS) via the inferior vena cava and digested with 10 mL of RPMI 1640 (Life Technologies, MD, USA) containing 0.02% (w/v) collagenase IV (Sigma–Aldrich, MO, USA)

and 0.002% (w/v) DNase I (Sigma–Aldrich) for 40 min at 37°C. Cells were overlaid on Lymphocyte M (Cedarlane Laboratories, Canada) in PBS.[27]

Fluorescence-activated cell sorter (FACS) analysis

The cells were surface-stained with FITC-conjugated anti-CD3 and APC-conjugated anti-NK1.1 mAbs, along with anti-interferon gamma (IFN-γ)-PE and anti-tumor necrosis factor alpha (TNF-α)-PE mAbs for intracellular cytokine detection (BD PharMingen). Samples were acquired using a FACSCalibur flow cytometer, and the data were analyzed using the CELLQuest software (BD Immunocytometry Systems, CA, USA). Apoptosis of IHLs was determined using annexin V-FITC and Via-Probe (a 7-amino-actinomycin D (7-AAD) viability probe) (PharMingen), according to the manufacturer's instructions.

BrdU incorporation

For *in vivo* BrdU labeling, mice received a 100-μL i.p. injection of a 10 mg/mL solution of BrdU in PBS 2 h before sacrifice. Single-cell suspensions of IHLs were prepared 24 h after CCl_4 injection and stained for CD3 and NK1.1. Following the surface staining, the cells were fixed, stained for intracellular BrdU using a FITC-BrdU Flow kit (BD PharMingen) and analyzed by flow cytometry.

5,6-Carboxyfluoroscein succinimidyl ester (CFSE) staining

For isolation of NKT cells, the spleen was stained with anti-CD3-PE and anti-NK1.1-APC mAbs and sorted into $CD3^+/NK1.1^+$ (NKT) cells using a FACS Vantage instrument (Becton Dickinson, CA, USA). The isolated cells were re-suspended in PBS (1 × 105 cells/mL) containing 5-(and 6-) CFSE (Molecular Probes, OR, USA) at a final concentration of 1 μM, incubated at 37°C for 10 min, washed three times and co-incubated with murine recombinant IL-2 (10 U/mL) for 3 days in 96-well plates. The cells were then harvested and analyzed by FACS. Data analysis was performed using the FlowJo Software (Tree Star, CA, USA).

Cell culture and migration assay

The cell migration assay was performed using Multiwell 12-well plates (BD PharMingen). Immortalized liver endothelial cell line M1 cells from the liver of an H-2Kb-tsA58 transgenic mouse harboring the SV40 TAg gene were placed in the bottom wells.[28] Sorted NKT cells from CD44KO or WT mice were placed in the upper chambers of transwell inserts (pore size, 4 μm) and incubated with recombinant murine IL-2 (10 U/mL) at 37°C for 2 h. After incubation, the migrated cell numbers were quantified by MultiTox-Fluor Multiplex Cytotoxicity Assays (Promega, MI, USA).

Data analysis

All values in the figures and text are expressed as the mean ± SD. The significance of differences between mean values was evaluated by the Mann–Whitney *U*-test.

RESULTS

A single injection of CCl_4 induces NKT cell infiltration in CD44KO mice

TO DETERMINE WHETHER CD44 plays a role in liver injury, CD44KO and C57BL/6 WT mice (3 mice/group) were injected i.p. with CCl_4 (2.0 mL/kg body weight; 1 : 4 v/v in mineral oil) or mineral oil as a control. As shown in Figure 1a, similar sALT activities were detected in both types of treated mice after mineral oil injection (0 h). However, the sALT activity was significantly reduced in CD44KO mice at 6 h after CCl_4 injection. On the other hand, the sALT activities in CD44KO mice were significantly increased by about 1.5-fold at 12 and 24 h compared with WT mice.

Next, to determine the effects of CD44 on the infiltration of inflammatory cells in the same livers, we counted the absolute numbers of IHLs and calculated the number of cells in each IHL subset, as described previously.[10] As shown in Figure 1c, although the total number of IHLs was the same after mineral oil injection in CD44KO and WT mice, the numbers of IHLs were reduced in CD44KO mice at 2 and 6 h after CCl_4 injection compared with WT mice ($P < 0.05$), indicating that the absence of CD44 inhibited inflammatory cell infiltration into the liver within 6 h. However, consistent with the increased sALT activities, the total numbers of IHLs in CD44KO mice were significantly increased at 24 and 72 h. Interestingly, although most of the IHL phenotypes exhibited the same tendency to increase in total number, only the $CD3^+/NK1.1^+$ NKT cell population increased during the course of time recorded after CCl_4 injection (Fig. 1b,c). As shown in Figure 1b, the $CD3_+/NK1.1_+$ NKT cell subpopulation increased quickly within 6 h, compared with WT mice, and remained high until 24 h post-CCl_4 injection. It is worth noting that the $CD3_+/NK1.1_-$ T cells subpopulation in the liver of WT

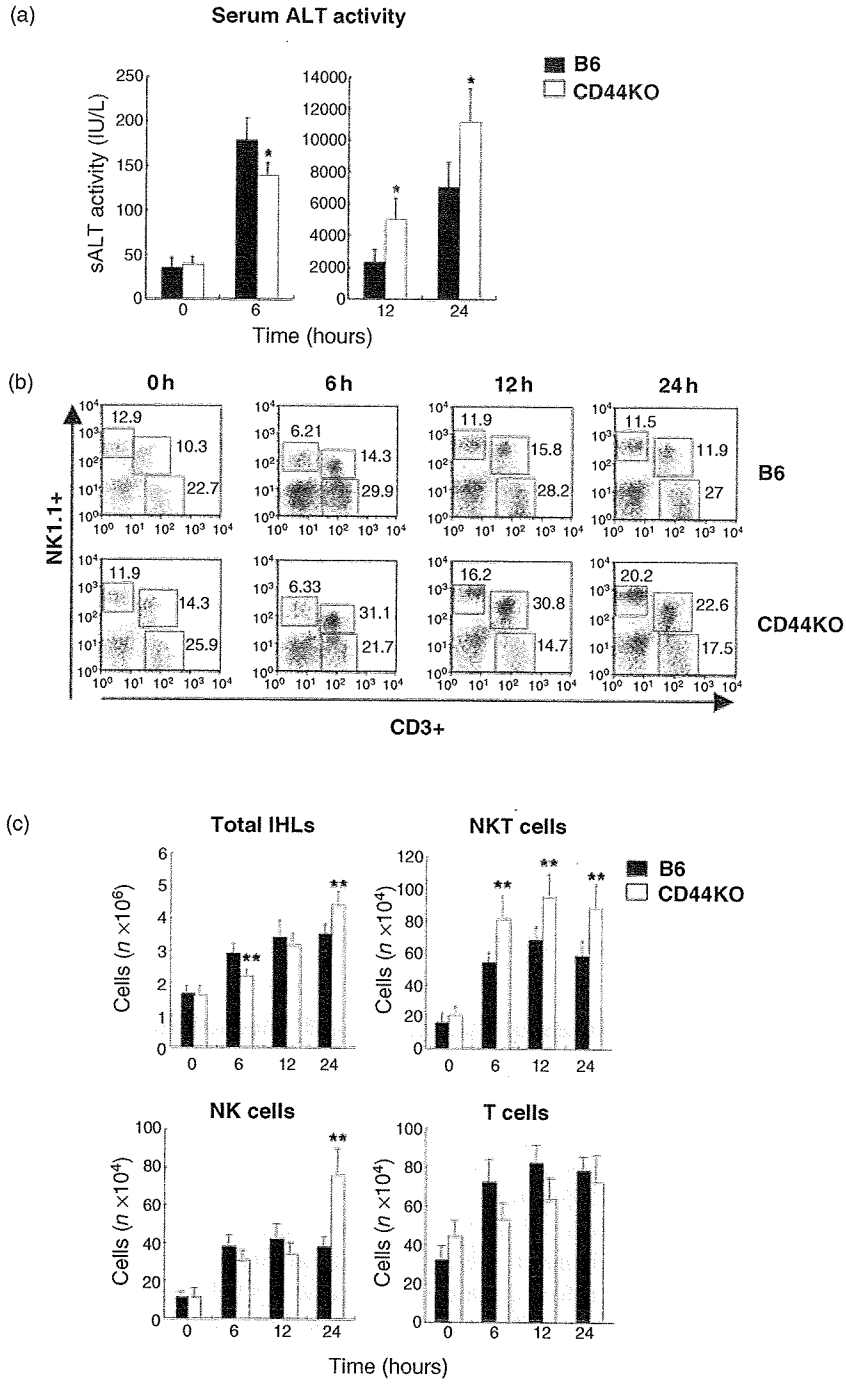


Figure 1 (a) Serum alanine aminotransferase (sALT) activity showing CD44KO mice with exacerbated liver injury after CCl_4 injection. Age-matched male CD44-deficient (CD44KO) and C57BL/6 mice (3 mice/group) were injected i.p. with a single dose of CCl_4 (2.0 mL/kg body weight; 1 : 4 v/v in mineral oil) and killed at the indicated time points. The mean sALT activity measured at the time of autopsy is indicated for each group and expressed in IU/L. (b) FACS analysis of intrahepatic leukocyte (IHL) cell subpopulations examined at 0, 6, 12 and 24 h after injection of CCL_4 or mineral oil as a control into C57BL/6 and CD44KO mice. IHLs were stained with anti-CD3-FITC and anti-NK1.1-PE monoclonal antibodies (mAbs) or anti-Gr-1-FITC and anti-CD11b-PE mAbs. Representative results of three independent experiments are shown. (c) IHLs were isolated from the mice and the effects of CD44 deficiency on cell recruitment were analyzed. The number of each subset of cells in the liver was calculated by multiplying the total number of IHLs by the frequency of each subset in the IHL population by FACS analysis (mean ± SD). $^*P < 0.05$ compared with C57BL/6 mice; $^{**}P < 0.05$.

mice were increased at 6 and 12 h after the injection, unlike the total IHL number.

As previously reported,[29] we found that the mRNA expression levels of inflammatory cytokines and chemokines in the liver showed the same kinetics as the sALT activities, meaning that CD44KO livers exhibited decreased expression at 6 h after CCl_4 injection but increased expression levels at 12 and 24 h compared with WT livers (data not shown). In particular, we found that MIP-2 mRNA expression was distinct, since MIP-2

mRNA expression was increased in CD44KO mouse livers at all the examined time points after CCl_4 injection.

Role of NKT cells

As described above, we found that NKT cells may play a critical role in liver injury in CD44KO mice after CCl_4 injection, as NKT cell migration was apparently increased compared with that of other inflammatory cells (see Fig. 1b,c). To evaluate the role of NKT cells in CD44KO mice, we generated double-KO mice for CD44 and NKT cells (CD44NKT DKO). CD44NKT DKO and CD44KO mice (5 mice/group) were injected i.p. with CCl_4 and killed at 6 and 24 h. As shown in Figure 2a, although there was no significant difference between the sALT activities at 6 h, CD44NKT DKO mice exhibited significantly lower sALT activity than CD44KO mice at 24 h ($P < 0.05$). Interestingly, these results revealed that NKT cells were not required for the early phase of liver injury by CCl_4 (6 h), since the sALT activities were similar with or without NKT cells. Based on the liver injury at 24 h, we found that the absolute numbers of total IHLs, macrophages and neutrophils among IHLs were significantly reduced in CD44NKT DKO mice (Fig. 2b,c). As shown in Figure 2d, consistent with the reduced number of macrophages in CD44NKT DKO mice, TNF-α mRNA expression was suppressed in the liver at 6 and 24 h after CCl_4 injection. Of note, hepatic MIP-2 mRNA expression in CD44NKT DKO mice was decreased at 6 h, indicating that NKT cells play an important role in MIP-2 production during this time. To support the RPA results, we confirmed that TNF-α production was reduced in the macrophages of CD44NKT DKO mice at 24 h relative to CD44KO mice (Fig. 2e).

Analysis of NKT cells

To address the reason why the number of NKT cells increased in CD44KO mice compared with WT mice, we proposed the following two hypotheses: (i) CD44KO NKT cells proliferate highly; and (ii) NKT cells migrate into the liver independent of the CD44 pathway. To examine the first hypothesis, we analyzed the proliferation of NKT cells using BrdU to determine the number of cells that had proliferated or were undergoing proliferation in the following experiments. CD44KO and WT mice were injected i.p. with 2 mg of BrdU 2 h before sacrifice. IHLs were isolated at 24 h after CCl_4 injection, subjected to BrdU labeling and then labeled with anti-mouse BrdU-FITC, CD3-PE and NK1.1-APC mAbs. As shown in Figure 3a, we found that $BrdU^+$ NKT cells were increased by about 10-fold in CD44KO mice compared to only 3-fold in WT mice after CCl_4 injection. To confirm this result, NKT cells were isolated from the liver by electronic sorting, subjected to CFSE staining and incubated with recombinant murine IL-2 (10 U/mL) for 3 days. Next, the co-cultures were harvested, and the percentage of proliferated NKT cells was determined by FACS analysis. As shown in Figure 3b, proliferation of NKT cells in the presence of recombinant IL-2 was greater for CD44KO mice than for WT mice, demonstrating that CD44KO NKT cells have the ability to strongly proliferate. However, there was no difference in the cytokine production levels of NKT cells after αGalCer stimulation in the presence or absence of CD44 (Fig. 3c). Further, we administered αGalcer (100 μg/kg) to the WT and CD 44KO mice, and analyzed sALT activity and the expression of the cytokine mRNA expression in the liver to confirm a difference in the activation of NKT cells by αGalcer in an *in vivo* system. Interestingly, we found sALT activity in CD44KO mice also increased at 24 h compared with WT mice after αGalcer injection (Supporting Information 1).

In vitro migration assays revealed that isolated NKT cells from CD44KO and WT mice showed similar migration toward liver sinus endothelial cell (LSEC) line cells (Fig. 3d). Immunohistochemical analysis suggested that adhesion between NKT cells and LSEC line cells was observed at similar ratios in the two strains of mice, indicating that NKT cell migration was not dependent on the CD44 pathway (Fig. 3e).

Roles of cytokines and chemokines

To evaluate which cytokines or chemokines are responsible for liver injury, we performed antibody neutralization experiments. Groups of 3 CD44KO mice were injected (250 μg/mouse) with anti-IFN-γ mAb, anti-TNF-α mAb, anti-MIP-2 mAb or rat IgG prior to injection of CCl_4, and killed after 24 h. As shown in Figure 4a, treatment with anti-MIP-2 mAb significantly protected against the increase in sALT activity, indicating that MIP-2 plays a key role in liver injury. Consistent with the results for the sALT activity and to a lesser extent than anti-MIP-2 mAb administration, anti-TNF-α mAb administration decreased the number of total IHLs after CCl_4 injection. Further, anti-MIP-2 mAb treatment reduced all phenotypes among the IHLs, indicating that MIP-2 is responsible for cell infiltration as well as liver injury.

Importantly, neutralization of MIP-2 reduced intrahepatic expression of TNF-α mRNA, suggesting that MIP-2

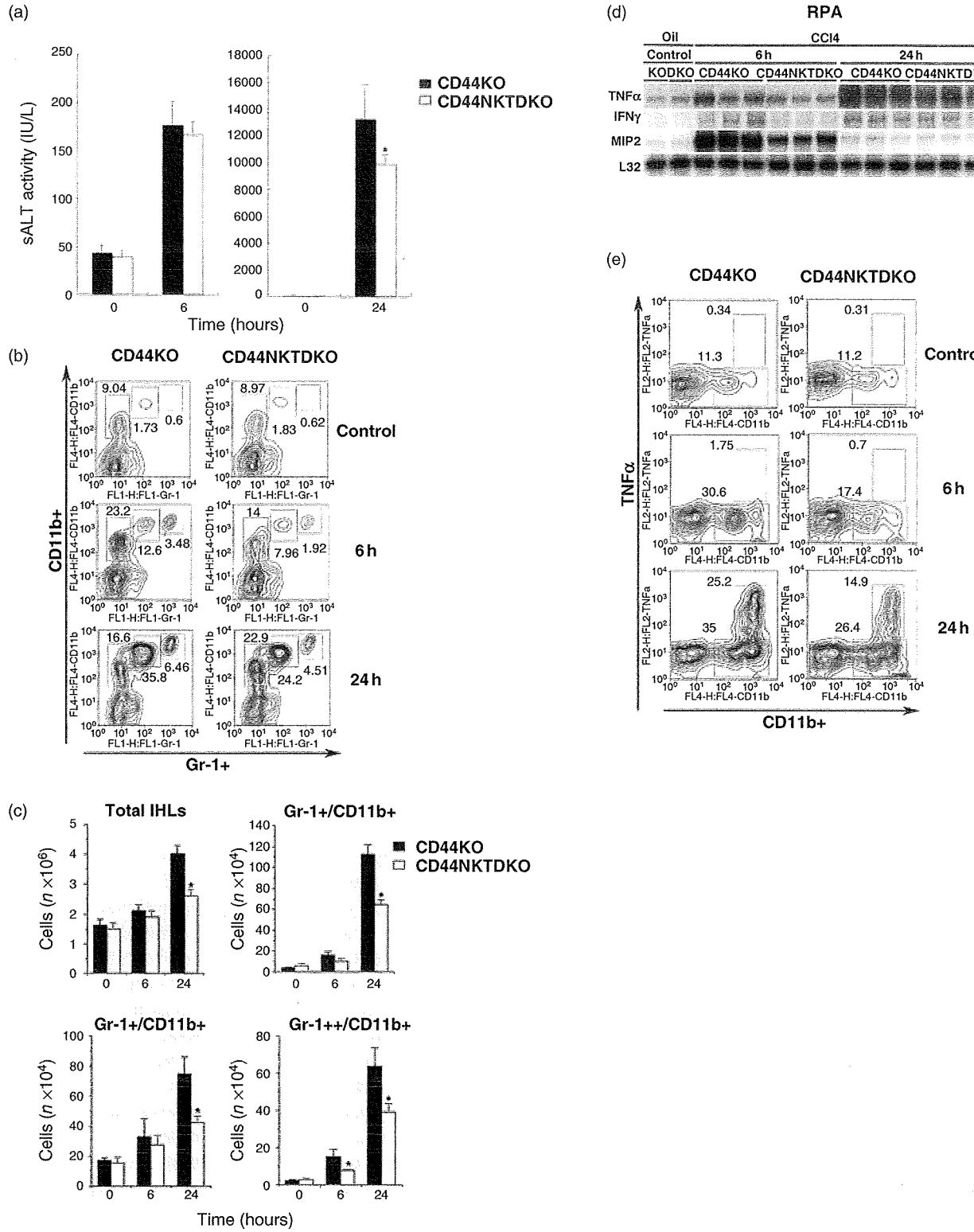


Figure 2 The role of natural killer T (NKT) cells. (a) Serum alanine aminotransferase (sALT) activity in 10 age-matched 8–11-week-old male CD44NKT DKO and CD44KO mice injected with CCl_4 and killed at 6 or 24 h. (b–c) FACS analysis of three animals in both groups. Intrahepatic leukocytes (IHLs) were isolated and stained with anti-Gr-1-FITC and anti-CD11b-APC monoclonal antibodies (mAbs). Representative results of three independent experiments are shown and the absolute cell numbers were calculated. (d) Total hepatic RNA (20 μg) was isolated from livers at the indicated time points and analyzed for cytokine and chemokine expressions by ribonuclease protection assay. (e) Intracellular cytokine expression of macrophages. IHLs were stained with anti-mouse Gr-1-FITC, CD11b-APC and TNF-α-PE mAbs and analyzed using a FACSCalibur system.

production triggers the expression of other inflammatory cytokine cascades (Fig. 4c). Consistent with the cytokine profiles, neutralization of MIP-2 and TNF-α effectively diminished the induction of hepatocyte apoptosis (Fig. 4d) as well as the necrotic parenchymal changes around the periportal vein.

Role of CD44 in the liver fibrosis model

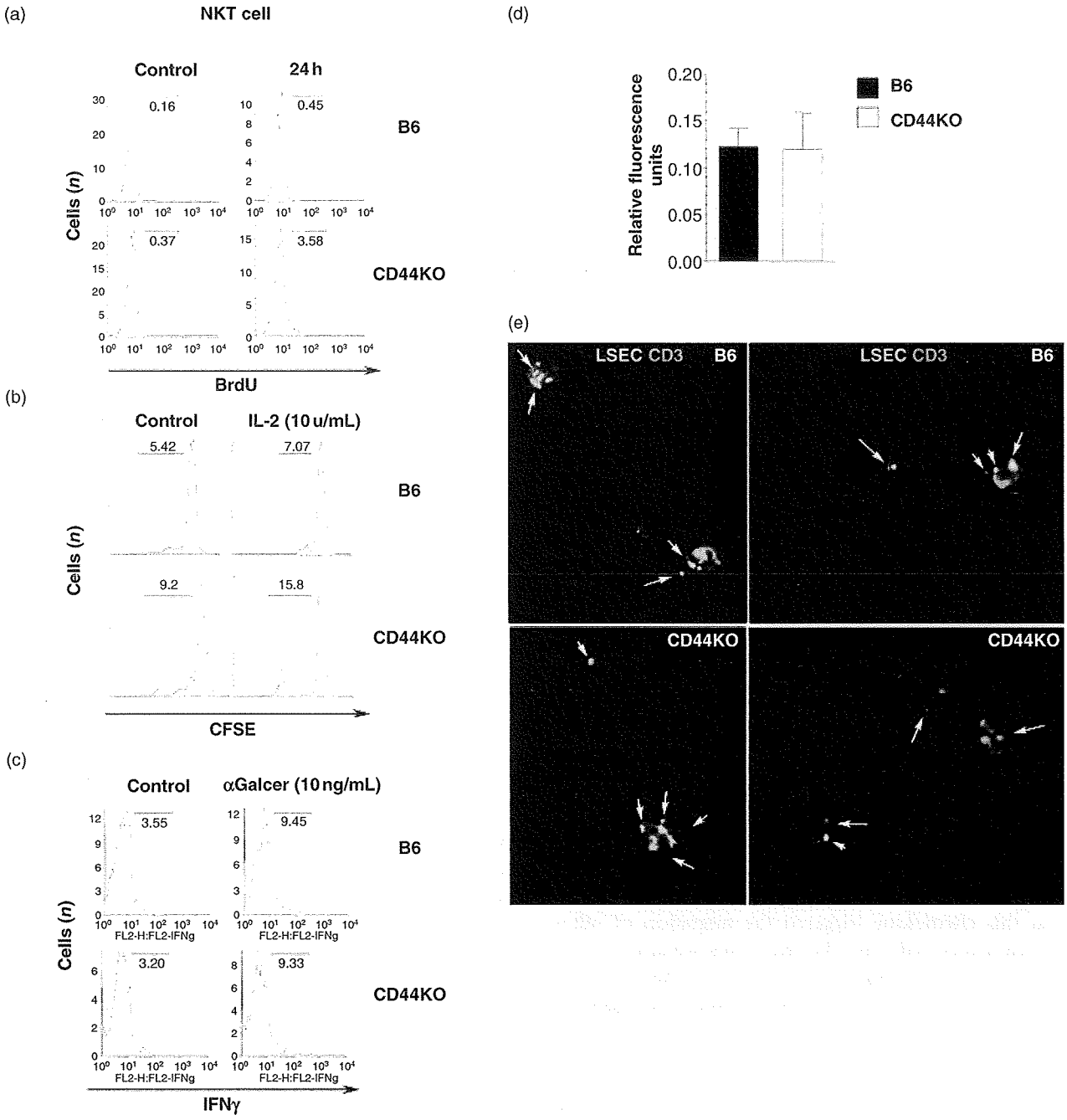
To evaluate whether CD44 deficiency affects chronic liver injury as well as acute liver injury, groups of 3 CD44NKT DKO, CD44KO and WT mice were injected i.p. with CCl_4 twice per week.

Initially, we measured the sALT activity at 24 h after the first injection every week and found that the sALT activity in CD44KO mice was elevated compared with those in CD44NKT DKO and WT mice (Fig. 5a). To address the degree of liver fibrosis in the presence or absence of CD44, we analyzed the liver histology using AZAN staining and immunohistochemistry with an anti-αSMA antibody. As shown in Figure 5c, there was a significant difference between the αSMA-positive areas in CD44KO and WT mice, suggesting that CD44KO mice exhibited enhanced liver fibrosis. On the other hand, immunohistochemical staining for PCNA, a marker for the G_1/S phase of the cell cycle, was used to determine whether the absence of CD44 caused abnormal hepatocyte proliferation after injury. The numbers of PCNA-positive hepatocytes in CD44KO livers were increased, but not significantly, compared to those in WT livers after 2 and 4 weeks of CCl_4 injections. Further, as shown in Figure 5b, intrahepatic expression of TGF-β1 mRNA was apparently increased in the liver of CD44KO mice compared with that of WT mice. However, we could not find a difference between the matrix metallo proteinase (MMPs) and tissue inhibitor of metalloproteinase (TIMPs) family expression in the liver between CD44KO and WT mice. Interestingly, we found that CD44NKT DKO mice exhibited a reduction in the area of liver fibrosis after calculation of the AZAN- and αSMA-positive areas, revealing that NKT cells have an effector function against hepatocytes under CD44-deficient conditions.

DISCUSSION

IN THE PRESENT study, we demonstrated that CD44 has dual effects on liver inflammation using a CCl_4-induced liver injury model. It has already been reported that anti-CD44 antibody treatment for pulmonary eosinophilia,[30] rheumatoid arthritis[31] and experimental encephalomyelitis[32] reduces the inflammatory response by suppressing the migration of inflammatory cells into the inflamed tissue. In contrast, however, CD44KO mice are reported to exhibit exacerbation of *bleomycin-* or *Escherichia coli*-induced lung injury via induction of cell apoptosis in the parenchyma and infiltration of neutrophils in the lung.[15,17] The reason for this contradiction remains unknown. Here, we showed several interesting findings with regards to CD44 and liver inflammatory response using CCl_4.

First, we found that only the population of NKT cells was increased in the liver after CCl_4 treatment, while other cell populations showed reduced numbers during the early phase. Importantly, NKT cells derived from CD44KO mice exhibited higher proliferative activities after CCl_4 treatment compared with those from WT mice, since CFSE- or BrdU-positive NKT cells were increased in our *ex vivo* and *in vivo* systems. It has been demonstrated that liver lymphomononuclear cells from CD44KO mice are highly proliferative in the staphylococcal enterotoxin B-induced liver injury model.[33] Further, we previously showed that IHLs from CD44KO mice were more resistant to apoptosis than those from WT mice at 24 h after CCl_4 treatment.[29] Since IHLs are highly proliferative and resistant to apoptosis in CD44KO mice, theoretically, all cell subpopulations would be expected to increase. However, we found that only NKT cells increased. Based on these results, we conclude that the main mechanism for the increase in CD44KO NKT cells is that NKT cells can adhere to



endothelial cells without utilizing the CD44 pathway. In support of this hypothesis, cell migration assays between isolated NKT cells and LSEC line cells revealed that NKT cells migrated toward to the LSEC line cells at similar levels regardless of the presence or absence of CD44. Recent reports have demonstrated that NKT cells express high levels of LFA-1, which is thought to be required for the development and/or survival of liver NKT cells.[23,24] These results suggest that the major adhesion molecule on NKT cells for infiltration into the liver may be LFA-1 through its interaction with CD54. Although we confirmed the expression of LFA-1 on NKT

Figure 3 Proliferation of intrahepatic leukocytes (IHLs) is higher in CD44KO mice than in wild type (WT) mice. (a) *In vivo*: CD44KO and WT mice were injected i.p. with 2 mg of BrdU at 2 h before sacrifice. IHLs were isolated at 24 h after CCl_4 injection, subjected to BrdU labeling and then labeled with anti-mouse BrdU-FITC, CD3-PE and NK1.1-APC monoclonal antibodies (mAbs). Data shown are representative of triplicate mice. (b) *In vitro*: After isolating IHLs from CD44KO and WT mice, IHLs were isolated using a fluorescence-activated cell sorter (FACS) sorting system, stimulated with recombinant IL-2 (10 U/mL) for 72 h and analyzed for CFSE dye loss by FACS analysis. Data are representative of 3–6 independent experiments. (c) *In vitro*: After isolating IHLs from CD44KO and WT mice, the cells were cultured with αGalCer (10 ng/mL) for 4 h and then labeled with anti-CD3-FITC, anti-NK1.1-APC and anti-IFN-γ-PE mAbs. Representative results of three independent experiments are shown. (d) Migration assay of immortalized liver endothelial cells (M1 cells). Sorted NKT cells (labeled with anti-CD3-PE and anti-NK1.1-APC mAbs) from CD44KO and WT mice were placed in the upper chambers of transwell inserts and incubated with recombinant murine IL-2 (10 U/mL) at 37°C for 2 h. After the incubation, the numbers of migrated cells in the lower chambers were quantified by MultiTox-Fluor Multiplex Cytotoxicity Assay. (e) The migrated cells from CD44KO and WT mice in the lower chambers were stained with FITC-LSEC and observed by light microscopy to determine their immunohistochemistry.

cells from CD44KO and WT mice, there was no difference in the expression levels (data not shown).

Second, we found that expression of MIP-2 mRNA was elevated in the liver at 6 h after CCl_4 treatment. Despite the increased expression of major inflammatory cytokines and chemokines in WT mice compared with CD44KO mice, only the expression of MIP-2 showed different patterns at this time point. Consistent with a recent report,[34] we also found that expression of MIP-2 was reduced in CD44NKT DKO mice compared with CD44KO mice, suggesting that NKT cells may produce MIP-2 in the liver after CCl_4 treatment. It is noteworthy to mention that these results may depend on the reduced numbers of macrophages and neutrophils due to the absence of NKT cells, since we have already shown that depletion of macrophages causes reduced expression of MIP-2 in the liver.[29] Although such a scenario is possible, we suggest that NKT cells from CD44KO mice were the main producers of MIP-2 and that this chemokine triggered the migration of other inflammatory cells into the liver. This scenario is supported by our findings that anti-MIP-2 mAb treatment decreased the sALT activity, inflammatory cell migration and induction of hepatocyte apoptosis in the liver.

MIP-2 has been identified as being functionally analogous to human IL-8 and is produced by macrophages, monocytes, fibroblasts and endothelial cells.[35,36] Some reports have suggested that MIP-2 is involved in the liver injury after hepatic ischemia-reperfusion and in the Con A-induced hepatitis model.[37–39] Thus, we conclude that induction of MIP-2 release from NKT cells and macrophages in CD44KO mice causes migration of other cells into the liver and, as a consequence, reinforces liver inflammation.

We found that CD44KO mice exhibited exacerbated liver fibrosis after repeated CCl_4 injections. This is the first evidence that CD44 is involved in the formation of liver fibrosis. These results suggest that CD44 not only contributes to acute liver injury but also to chronic liver damage. Although we have not shown the results obtained with hepatic stellate cells (HSCs), which are key players in liver fibrosis[40,41] we found that HSCs expressed CD44 and that the number of HSCs was increased in CD44KO mice compared with WT mice at day 7 after CCl_4 injection. It is curious that adhesion molecules, such as CD44, are involved in the formation of liver fibrosis and this represents a novel finding. This might be a candidate for target therapy for liver fibrosis.

In summary, we have shown that inhibition of CD44 has dual effects on liver inflammation. In the liver, CD44 deficiency induced suppression of cell recruitment (except NKT cells) and protected against liver inflammation due to decreased expression of inflammatory cytokines during the early phase. However, despite the suppressed cell recruitment, NKT cells still migrated to the liver in a CD44-independent manner and were highly proliferative. NKT cells induced MIP-2 expression in the liver and, in turn, this chemokine triggered the late phase and severe liver inflammation with increased hepatocyte apoptosis via the NF-κB signaling pathway. Thus, caution is warranted for the protection of the liver immune system when using the adhesion molecule CD44 as a therapeutic target for inflammatory liver diseases.

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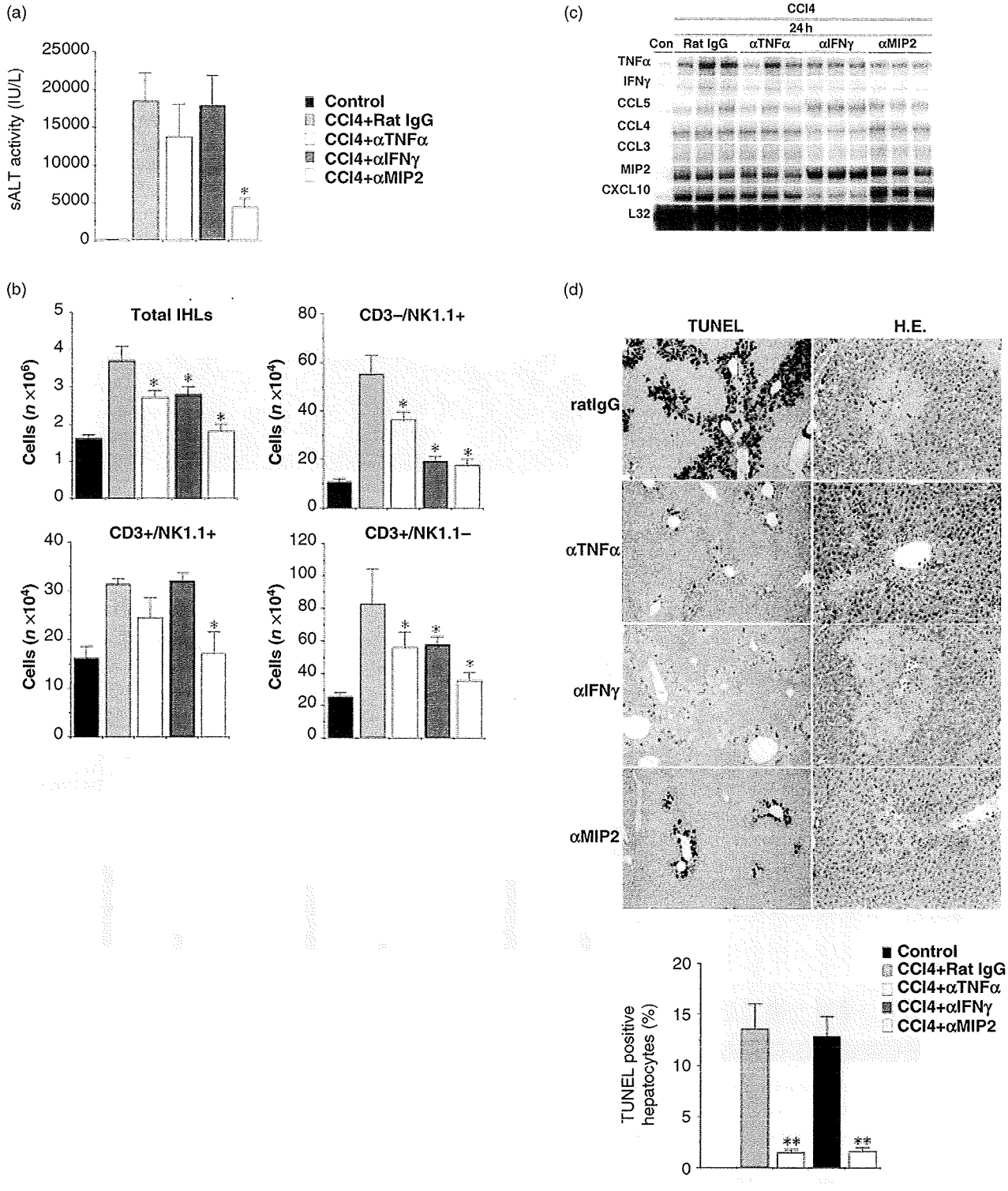

Figure 4 (a) Age-matched male CD44KO mice were injected with anti-IFN-γ monoclonal antibody (mAb) (250 μg), anti-TNF-α mAb (250 μg), anti-MIP-2 mAb (250 μg) or control rat IgG prior to injection with CCl_4 to determine serum alanine aminotransferase (sALT) activity. (b) Intrahepatic leukocytes (IHLs) from five groups of three mice were isolated and stained with anti-CD3-FITC and anti-NK1.1-APC mAbs or anti-Gr-1-FITC and anti-CD11b-APC mAbs. Representative results of three independent experiments are shown and the absolute cell numbers were calculated. (c) Total hepatic RNA (20 μg) was isolated from livers at various time points and analyzed for cytokine and chemokine expression by ribonuclease protection assay (RPA). (d) To evaluate the induction of apoptosis, liver sections were stained using the in situ terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) assay (6). The percentages of apoptotic hepatocytes among the total hepatocytes were determined by TUNEL staining. Data are expressed as the mean ± SD for three mice. $*P < 0.05$; $**P < 0.01$.

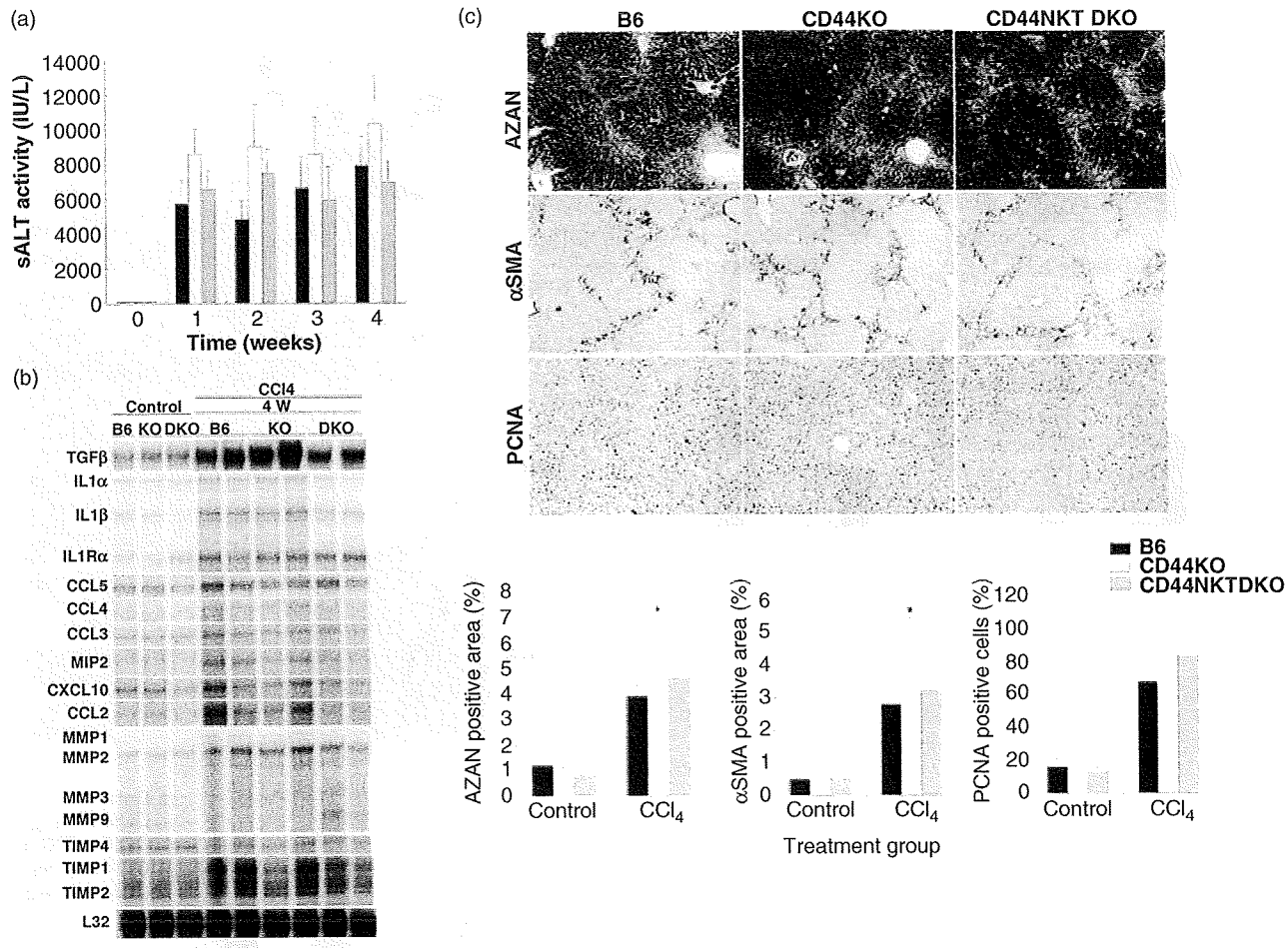

Figure 5 Liver fibrosis model. (a) CD44NKT DKO, CD44KO and C57BL/6 mice were injected i.p. with CCl_4 twice per week for 4 weeks and serum alanine aminotransferase (sALT) activity was measured at 24 h after the second injection. The mice were killed at 4 weeks. (b) Total hepatic RNA (20 μg) was isolated from livers at various time points and analyzed for the expression of cytokines, matrix metallo proteinase (MMPs) and tissue inhibitor of metalloproteinase (TIMPs) by ribonuclease protection assay (RPA). (c) Liver sections were obtained from mice killed after 4 weeks of repeated CCl_4 injections and stained with AZAN and an anti-αSMA monoclonal antibody (mAb) for evaluation of liver fibrosis. Immunohistochemical staining for PCNA, a marker for the G_1/S phase of the cell cycle, was used to determine whether the absence of CD44 caused abnormal hepatocyte proliferation after CCl_4 injections. The percentages of liver fibrosis were determined by the numbers of αSMA-positive cells or the AZAN-stained areas. Data are expressed as the mean ± SD for three mice. $*P < 0.05$.

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