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CD137-Mediated Pathogenesis from Chronic Hepatitis to Hepatocellular Carcinoma in Hepatitis B Virus-Transgenic Mice

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Chronic hepatitis B virus (HBV) infection is characterized by sustained liver inflammation with an influx of lymphocytes, which contributes to the development of cirrhosis and hepatocellular carcinoma. The mechanisms underlying this immune-mediated hepatic pathogenesis remain ill defined. We report in this article that repetitive infusion of anti-CD137 agonist mAb in HBV-transgenic mice closely mimics this process by sequentially inducing hepatitis, fibrosis, cirrhosis, and, ultimately, liver cancer. CD137 mAb initially triggers hepatic inflammatory infiltration due to activation of nonspecific $CD8^+$ T cells with memory phenotype. $CD8^+$ T cell-derived IFN-γ plays a central role in the progression of chronic liver diseases by actively recruiting hepatic macrophages to produce fibrosis-promoting cytokines and chemokines, including TNF-α, IL-6, and MCP-1. Importantly, the natural ligand of CD137 was upregulated significantly in circulating $CD14^+$ monocytes in patients with chronic hepatitis B infection and closely correlated with development of liver cirrhosis. Thus, sustained CD137 stimulation may be a contributing factor for liver immunopathology in chronic HBV infection. Our studies reveal a common molecular pathway that is used to defend against viral infection but also causes chronic hepatic diseases. ***The Journal of Immunology*****, 2010, 185: 7654–7662.**

Persistent infection with hepatitis B virus (HBV) predisposes to the development of chronic inflammatory liver diseases, which often progress to hepatic cirrhosis and hepatocellular carcinoma (HCC) (1). Because HBV is not directly cytolytic for the hepatocyte, liver diseases are thought to be immune mediated. HBV-specific $CD8^+$ CTLs were demonstrated to play a critical role in viral clearance in acute infection or the early stage of liver diseases (2, 3). However, this response is clearly blunted in chronic HBV infection, with scanty responses of low frequency and limited specificity (4, 5). Patients with chronic hepatitis B (CHB) often have large lymphocytic infiltration in the livers with a high ratio of $CD8^+$ T cells that are not specific for HBV and often have memory phenotype (4). However, the characteristics of these $CD8^+$ T cell populations and their potential contribution to liver immunopathology are largely unknown. A recent report indicated that circulating and intrahepatic $CD8^+$ T cells from CHB patients, regardless of their Ag specificity, are impaired in their ability to produce IL-2 and to proliferate upon stimulation by Ag. However, these $CD8^+$ T cells retain the capacity to produce proinflammatory cytokines IFN-γ and TNF-α, which persist even in the patients with high viral load and liver inflammation (6).

CD137 (4-1BB) is an inducible cosignaling receptor of the TNFR superfamily, which is expressed on the surface of activated T cells, NK cells, macrophages, and dendritic cells (7). Its ligand, CD137L, is constitutively expressed on a fraction of dendritic cells and is inducible mainly on activated monocytes, macrophages, B cells, and a small fraction of T cells (8). Engagement of CD137 provides a costimulatory signal to induce T cell expansion, production of IFN-γ, and prevention of activation-induced death of effector T cells (9), leading to enhanced T cell responses against viral infection in animal models (10, 11). We showed recently that CD137 stimulation by an agonist mAb in the absence of Ag induces vigorous growth and cytokine production from $CD8^+$ and $CD4^+$ T cells with memory phenotype in naive mice, whereas the same stimulation does not affect naive T cells (12). Given the possible role of CD137 in Ag-independent stimulation of memory T cells, we speculate that enhanced CD137 stimulation may stimulate HBV-nonspecific memory T cells, leading to chronic inflammation and pathogenesis of liver diseases. We report in this study that expression of CD137L is substantially upregulated in peripheral $CD14^+$ monocytes of CHB patients and is closely associated with liver cirrhosis. Using an agonist CD137 mAb as

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Abbreviations used in this paper: ALT, alanine transaminase; CHB, chronic hepatitis B; CHB/LC, chronic hepatitis B patient with liver cirrhosis; CHB/Non-LC, chronic hepatitis B patient without cirrhosis; CM, cultured media; CMT, mock transfectant; GdCl3, gadolinium chloride; HBsAg, hepatitis B surface Ag; HBV, hepatitis B virus; HC, healthy controls; HCC, hepatocellular carcinoma; IHL, intrahepatic lymphocyte; LTM, wild-type littermates; poly-IC, polyinosinic-polycytidylic acid; Tg, hepatitis B virus-transgenic mice; U.D., undetected; WT, wild-type.

mimicry of CD137L, we examined the consequence of CD137 stimulation on liver inflammation and disease progression in HBV-transgenic mice.

Materials and Methods

Subjects

Ten milliliters of venous blood was drawn from 61 patients with chronic HBV infection (serum positive for hepatitis B surface Ag [HBsAg] for ≥12 mo) and 31 healthy donors (HBsAg negative, anti-HBc negative, and anti-HBe negative). The patients were divided into two groups with respect to the pathological index of liver cirrhosis, the typical morphologic findings on computed tomography or ultrasound, symptoms of portal hypertension, and liver biopsies: 40 patients with liver cirrhosis and 21 patients without cirrhosis. No patient had received anti-HBV agents or immunosuppressive drugs 6 mo before sampling. Patients who had HIV and other types of chronic liver diseases, such as hepatitis C virus, chronic hepatitis E, alcoholic liver disease, or steatohepatitis, were excluded from the current study. The study protocol was approved by the Ethics Committees of the institutions, and informed consent was obtained from all participants before sample collection. The characteristics of the patients and healthy donors are listed in Supplemental Table I.

Experimental animals

HBV-transgenic mice C57BL/6J-TgN (Alb1 HBV)44Bri, which express part of the HBV genome, including S, pre-S, and X genes under the mouse albumin promoter, were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were matched for sex and age (6–8 wk), and the HBV-transgenic mice and their wild-type (WT) littermates were used. The mice were maintained under specific pathogen-free conditions in the animal facility at the Institute of Biophysics, Chinese Academy of Sciences. All studies involving animals were approved by the Institutional Laboratory Animal Care and Use Committee.

Abs

Anti-mouse CD137 agonist mAb (clone 2A, rat IgG2a) was described previously (13). Rat anti-KLH mAb (rat IgG2a) or rat IgG (Sigma-Aldrich, St. Louis, MO) was used as control. All mAbs, including anti-CD8 (TIB210, rat IgG2a), anti-NK1.1 (PK136, rat IgG2a), anti-CD4 (GK1.5, rat IgG2b), and anti–IFN-γ (E4-6A2, rat IgG1) were purified from ascites of nude mice and treated with Triton-X114 for LPS removal.

Cytokine and serum biochemical analysis

TGF-β was detected by a commercial ELISA kit (eBioscience, San Diego, CA). Other cytokines and chemokines were detected using a cytometric bead array kit (BD Biosciences, San Diego, CA). Alanine transaminase (ALT), hyaluronic acid, and total bilirubin levels were measured using commercial kits (Biosino, Beijing, China).

Cell purification, culture, and analysis

Mouse intrahepatic lymphocytes (IHLs) were isolated as described previously (14). Furthermore, the $CD11b^+$ macrophages were isolated by positive selection (Miltenyi Biotec, Auburn, CA), and $CD8^+$ lymphocytes were purified by negative selection with exclusion of $CD4^+$, $NK1.1^+$, $Gr\text{-}1^+$, $CD11b^+$, and $B220^+$ cells. The purity of isolated cells was >95%, as demonstrated by FACS.

Purified $CD8^+$ cells (2×10^5 cells/well) were stimulated with 3 μg/ml immobilized anti-CD3, whereas liver $CD11b^+$ cells (1×10^5 cells/well) were cultured alone without any stimulation or were cultured together with $CD8^+$ cells stimulated with anti-CD3 in 96-well plates for 48 h. Flow-cytometry analysis was performed using a four-laser FACSCalibur instrument equipped with CellQuest Pro software (BD Biosciences, San Jose, CA).

Detection of HBsAg-specific T cells

HBV-transgenic mice were treated with anti-CD137 mAb (clone 2A) or control rat IgG on days 0 and 7 and immunized with 5 μg recombinant HBsAg (Institute of Virology, Academy of Military Medical Sciences, Beijing, China) emulsified in CFA on the day before the first Ab injection. The mice were bled for serum ALT analysis at different time points. On days 10–12, 4×10^6 splenocytes and 2×10^6 IHLs were cultured in 24-well plates with HBsAg or HBsAg peptide s208 (OVA257 peptide served as negative control) or were cocultured with 5×10^4 irradiated HBsAg transfectant (adapted from CMT-93 stably transfected with PIRES2-EGFP-Middle HBsAg plasmid) or mock transfectant in the presence of IL-2 (30 U/ml; eBioscience) for 5 d. IFN-γ levels in the culture media were detected by ELISA (eBioscience).

HBV-transgenic mice on a C57BL/6 background were crossed with BALB/c mice to produce HBV-transgenic F1 strain. HBV-transgenic F1 mice and WT littermates were immunized with 50 μg EN28 peptide and 50 μg polyinosinic-polycytidylic acid (poly-IC) on days 0 and 14 and were treated with 100 μg anti-CD137 mAb (2A) or control rat Ig on days 5 and 12. On day 17, 2×10^6 IHLs and 4×10^6 splenocytes were stimulated in 24-well plates with EN28 or control 2C peptide in the presence of IL-2 (30 U/ml; eBioscience) for 48 h. Intracellular IFN-γ staining of CD8 T cells was performed after restimulation with 50 μg PMA and 500 μg ionomycin for an additional 3 h.

Cell depletion and cytokine blockage

HBV-transgenic mice were treated i.p. with 100 μg 2A or rat Ig weekly. For depletion of CD8 cells, CD4 T cells, or NKT/NK cells, mice were injected i.p. with 100 μg TIB210, GK1.5, or PK136 twice a week 1 d before the first 2A injection, respectively. The depletion effects were confirmed by FACS before each experiment. To investigate whether liver macrophages are involved in anti-CD137–induced liver fibrosis in vivo, the selective Kupffer cell toxicant gadolinium chloride (GdCl3; Sigma-Aldrich) was dissolved in acidic saline (0.9% NaCl solution [pH 3]) and administered to mice (10 mg/kg) twice each week via the tail vein beginning 5 d after the first 2A treatment. For IFN-γ neutralization in vivo, mice were treated i.p. with 200 μg anti–IFN-γ (E4-6A2) or rat IgG twice a week since day 4 after the first 2A injection.

Pathology

Liver sections (5 μM) after paraffin embedding were stained with H&E for inflammation analysis. Collagen was stained with saturated picric acid containing 0.1% Sirius red and 0.1% fast green for 1 h, as described previously (15). Collagen distribution was analyzed by Image Pro Plus software (Media Cybernetics, Bethesda, MD) to quantify the area of tissue occupied by positive staining, and at least four serial images (×40) were taken of the tissue section. To detect the activated hepatic stellate cells, liver sections were stained with anti–α-smooth muscle actin Ab (Neomarker, Fremont, CA). TUNEL staining was done using in situ cell death detection kits (Roche, Indianapolis, IN).

Statistics

The two-sample *t* test was used for comparisons between groups, and the Pearson test was used for correlation assay. Statistical analyses were

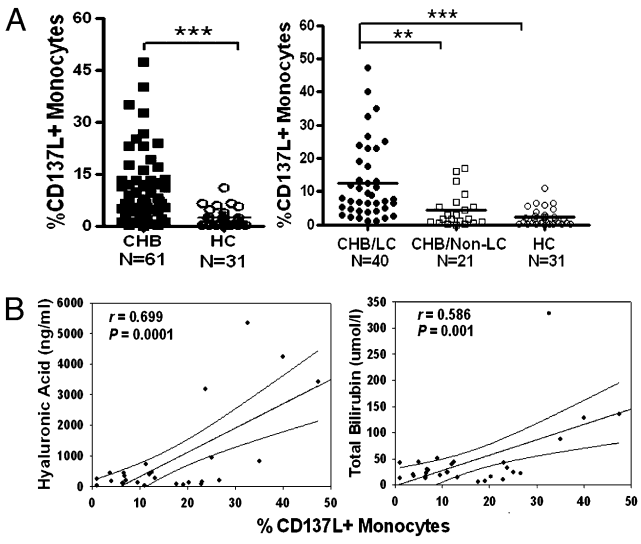

FIGURE 1. Upregulated CD137L in peripheral $CD14^+$ monocytes of CHB patients associated with hepatic cirrhosis. *A*, CD137L expression was analyzed by flow cytometry on peripheral monocytes, gating on $CD14^+$ PBMCs, and compared between CHB patients and healthy controls (HC), (*left panel*) or among CHB patients with liver cirrhosis (CHB/LC), CHB patients without cirrhosis (CHB/Non-LC), and healthy controls (*right panel*). Each dot represents one individual. Horizontal lines represent mean values. *B*, Correlation analysis between the levels of CD137L on monocytes and plasma total bilirubin (*right panel*) and hyaluronic acid (*left panel*) levels in individuals with CHB with liver cirrhosis. Solid line, linear growth trend with 95% CI. $^{**}p < 0.01$; $^{***}p < 0.001$.

performed using GraphPad Prism 4 software (GraphPad, San Diego, CA). Error bars represent SEM. A p value <0.05 was considered significant.

Results

Upregulated CD137L in peripheral CD14$^+$ monocytes of CHB patients associated with hepatic cirrhosis

To determine whether there is increased expression of CD137 and CD137L in CHB, we examined their expression in PBMCs from 61 CHB patients and 31 healthy donors by staining with specific mAbs. CD137L on CD14$^+$ monocytes was significantly higher in CHB patients than in the healthy controls (Fig. 1*A*, *left panel*), whereas it was minimal in other types of cells. CD137 expression was not detected in freshly isolated PBMCs. We further analyzed the relationship of the expression of CD137L with liver cirrhosis. As shown in Fig. 1*A* (*right panel*), the expression of CD137L on CD14$^+$ monocytes was significantly increased in CHB patients with liver cirrhosis compared with patients with no evidence of liver cirrhosis. There was no significant difference between the CHB patients without cirrhosis and the healthy donors ($p = 0.06$). In addition, overexpression of CD137L on CD14$^+$ monocytes positively correlated with the plasma levels of total bilirubin and hyaluronic acid (Fig. 1*B*), which are markers for the progression of liver fibrosis during chronic liver diseases (16), but it did not correlate with plasma ALT level ($p = 0.215$). Our results suggest that upregulated CD137L might trigger aberrant CD137 signaling in CHB patients, which plays a role in the progression of chronic liver diseases.

CD137 stimulation promotes liver disease progression from hepatitis to fibrosis to HCC in HBV-transgenic mice

Although our previous data in CHB patients suggest a role for aberrant CD137 triggering in the progression of liver cirrhosis, a cause–effect relationship could not be established. To test this, we examined the effect of anti-CD137 mAb (clone 2A), which was shown to mimic CD137L to stimulate CD137 (13), in HBV-transgenic mice, which constitutively express HBsAg with features of the HBV chronic carrier status (17). HBV-transgenic mice or WT littermates were injected i.p. weekly with 100 μg of anti-CD137 Ab or control RatIg up to seven times. The sera were collected regularly to monitor the serum ALT, which is released from injured or necrotic hepatocytes. As shown in Fig. 2*A*, the ALT in HBV-transgenic mice rapidly increased from day 8 after

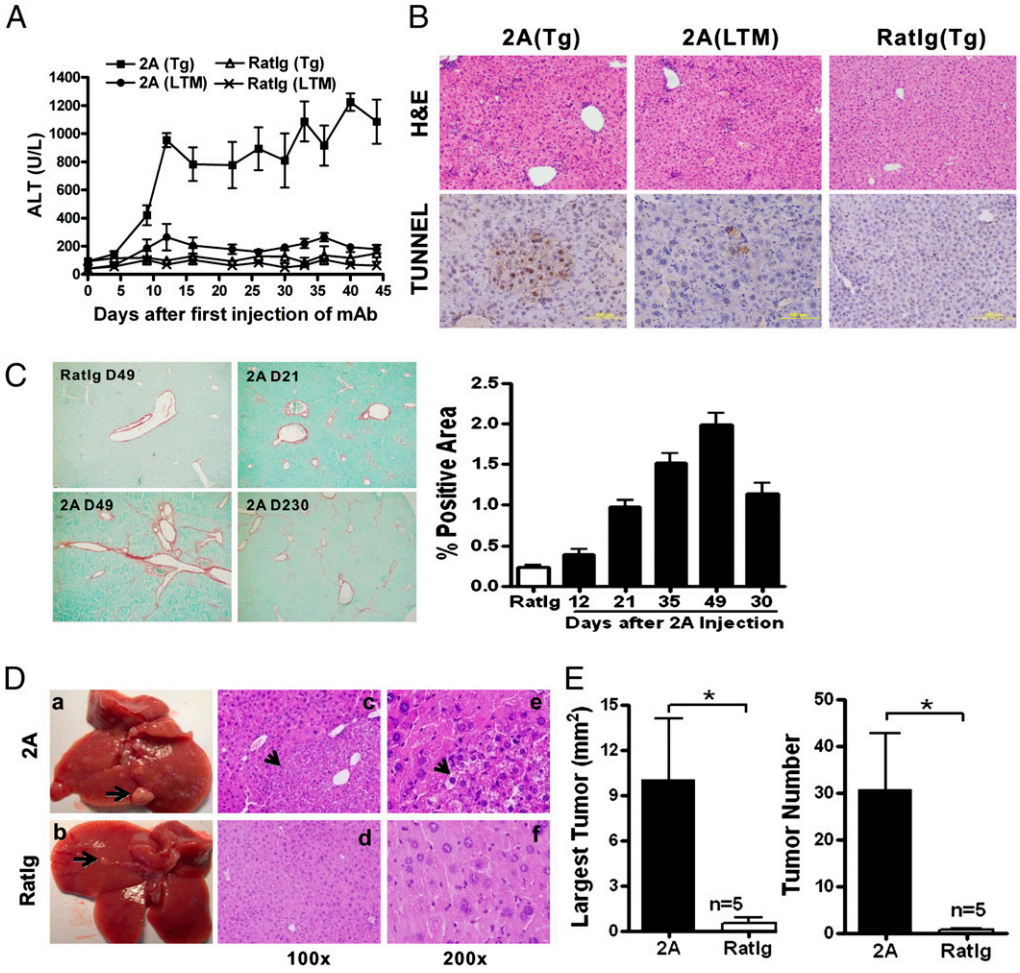

FIGURE 2. CD137 stimulation promotes chronic liver disease progression in HBV-transgenic mice. HBV-transgenic mice (Tg) or WT littermates (LTM) were injected i.p. weekly with 100 μg agonist CD137 mAb or control RatIg since day 0. Serum and liver tissues were collected at the indicated time points. *A*, The kinetics of serum ALT levels in different groups. *B*, H&E (original magnification ×100) or TUNEL (original magnification ×200) staining of liver sections on day 12 after 2A or RatIg injections. *C*, Sirius red staining of liver sections at indicated days for measurement of collagenous bands (*left panels*) in HBV-transgenic mice; the positive areas were quantified by image systems (*right panel*) (original magnification ×40). *D* and *E*, HBV-transgenic mice were given seven weekly mAb injections and were analyzed for liver tumor development 6 mo after the final treatment. *D*, Typical liver morphology (*a*, *b*) and liver sections stained with H&E (*c–f*) (original magnification ×100 [*c*, *d*] and ×200 [*e*, *f*]). Arrows indicate the typical liver tumor nodule. *E*, Comparison of the volume of the largest tumor (*left panel*) or the total number of tumor nodules (*right panel*) on the liver surface. The tumor volume was calculated as length × width. *$p < 0.05$.

the first injection and persisted at high levels during treatment. However, it gradually recovered to normal levels 1 mo after stopping mAb injections (data not shown). Liver sections on day 12 revealed spreading necroinflammatory foci with infiltration of mononuclear cells, by H&E staining, and profound hepatocellular apoptosis, by TUNEL staining. Consistent with serum ALT level, there was minimal inflammation and liver injury in the mice treated with control Ig (Fig. 2*B*). Anti-CD137 mAb treatment of littermates induced an elevation of serum ALT in low levels that were not distinguishable from the mice treated with control Ig (Fig. 2*A*), with similar liver inflammation (Supplemental Fig. 1) and much less severe liver injury compared with anti-CD137 mAb-treated HBV-transgenic mice (Fig. 2*A*, 2*B*).

To analyze the fibrogenesis, hepatic collagen deposition/accumulation was stained with Sirius red and quantified using digital image analysis. Anti-CD137 mAb-treated HBV-transgenic mice began to show overt hepatic fibrosis on day 21 that became more severe with the continuance of mAb injection, whereas the liver of control Ig-treated mice remained normal. Although the extent of the fibrogenesis decreased after stopping the mAb injection, the liver still showed severe fibrogenesis after 6 mo (Fig. 2*C*). After five injections, the liver showed obvious cirrhosis nodes on the surface, and smooth muscle actin staining of live sections revealed marked activation of hepatic stellate cells (Supplemental Fig. 2*A*). In addition, the level of hydroxyproline and hyaluronic acid, two closely related hepatic fibrosis factors, dramatically increased in liver homogenates of anti-CD137 mAb-treated transgenic mice compared with control Ig-treated mice (Supplemental Fig. 2*B*). Anti-CD137 mAb-treated littermates also showed increased fibrogenesis but at much milder levels compared with the HBV-transgenic mice (Supplemental Fig. 3). These results suggest that HBV-transgenic mice are more susceptible to the damage caused by anti-CD137 mAb-induced liver inflammation than healthy littermates.

It was reported that >70% of male HBV-transgenic mice spontaneously develop HCC in ~15 mo (18). Interestingly, only 6 mo after seven injections, we observed the occurrence of large ($\geq$3 mm^2) liver tumor nodules on the liver surface in all five anti-CD137 mAb-treated mice, but only two control Ig-treated mice had one or two smaller ($\leq$2 mm^2) tumor nodules. There were vast differences in tumor nodule numbers and largest tumor sizes between the two groups of mice (Fig. 2*D*, 2*E*). Tumors were not found in the liver of littermates treated with anti-CD137 mAb.

Taken together, our results indicate that sustained CD137 stimulation by repetitive injections of agonist CD137 mAb could induce

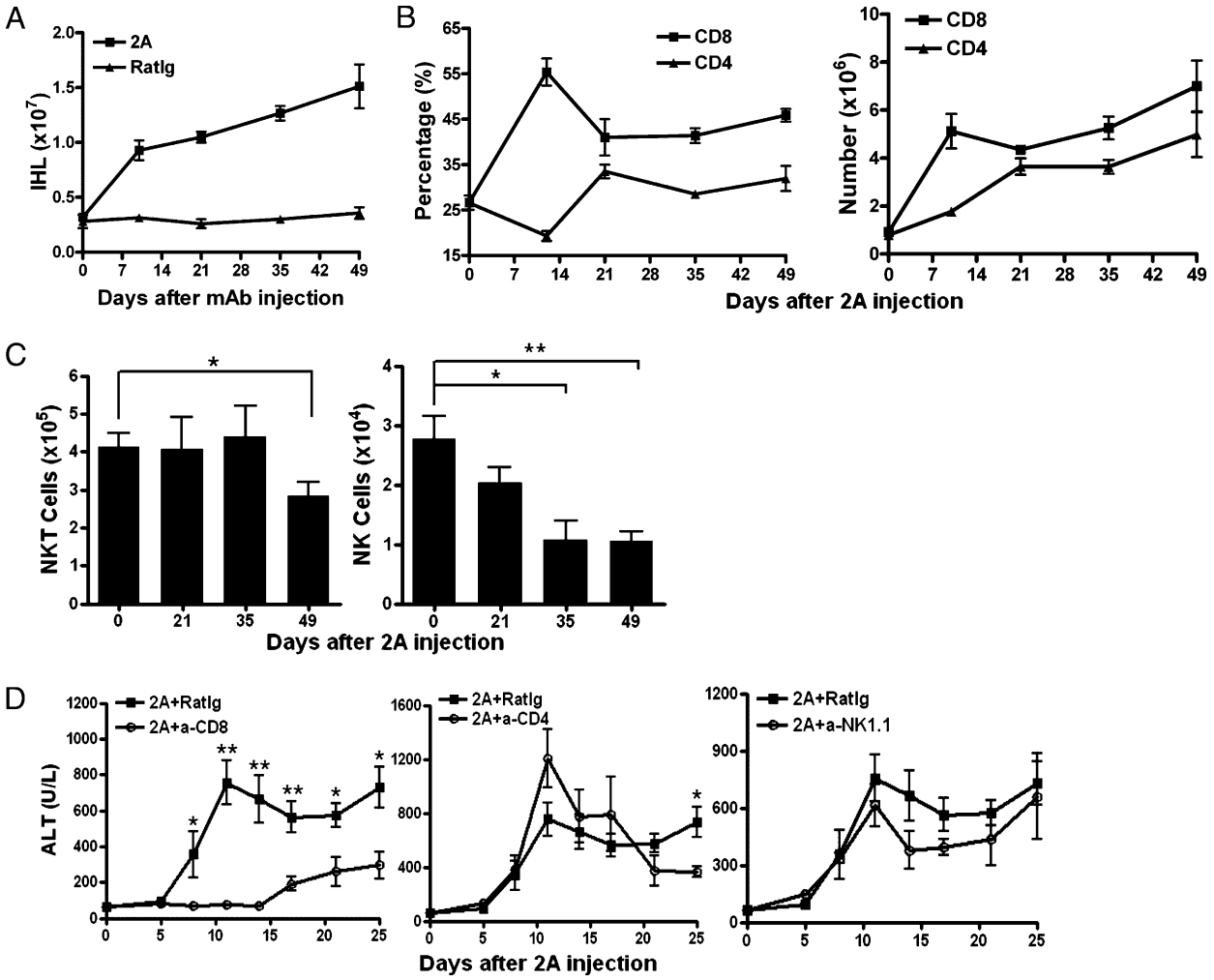

FIGURE 3. CD8$^+$ T cells mediate hepatic pathogenesis induced by CD137 stimulation. HBV-transgenic mice were treated as in Fig. 2, and IHLs were isolated for FACS staining at the indicated time points. *A*, The kinetics of total IHL numbers. *B*, Frequency (*left panel*) and total number (*right panel*) of CD8$^+$ and CD4$^+$ cells. *C*, Total number of NK T and NK cells. *D*, One hundred micrograms of depletion Abs for CD8$^+$ (*left panel*), CD4$^+$ (*middle panel*), or NK1.1$^+$ (*right panel*) cells or control RatIg was injected i.p. into HBV-transgenic mice on the day before anti-CD137 injection and then twice a week to maintain the effect of cell depletion. Serum ALT levels were monitored. One representative result from at least two independent experiments is shown as mean $\pm$ SEM. *$p < 0.05$; **$p < 0.01$.

continued liver inflammation in WT and HBV-transgenic mice, which causes severe liver damage in HBV-transgenic mice that leads to fibrosis, cirrhosis, and HCC. This process is very similar to disease progression in CHB patients.

$CD8^+$ T cells are required for CD137-mediated hepatic pathogenesis

With significant infiltration of leukocytes into the liver upon anti-CD137 mAb treatment, which precedes the development of chronic liver damage, we analyzed IHLs from the HBV-transgenic mice upon mAb treatment. As shown in Fig. 3*A*, the total number of IHLs in anti-CD137 mAb-treated mice had rapidly increased by day 10 and then steadily increased until day 49 by ~5-fold (1.68 ± 0.26 versus 0.36 ± 0.09) more than those observed in control Ig-treated mice. The proportion of $CD8^+$ T cells increased rapidly to 55.4% of IHLs on day 10 and then decreased to 41% on day 21. The total number of intrahepatic $CD8^+$ T cells rapidly increased 5-fold (5.1 ± 0.99 versus 0.92 ± 0.09) on day 10 and then steadily increased slowly. However, the frequency of $CD4^+$ T cells in IHLs decreased significantly on day 10 as a result of the dramatic increase in $CD8^+$ T cells, whereas the number of $CD4^+$ T cells continued to increase steadily (Fig. 3*B*). The numbers of NKT and NK cells in IHLs did not increase, but they decreased in the late stage (Fig. 3*C*). These results suggest that $CD8^+$ T cells are the earliest cells that appear in the liver after mAb injections.

To determine the potential roles of T cell subsets, we depleted $CD8^+$, $CD4^+$, and $NK1.1^+$ cells using specific mAbs in anti-CD137 mAb-treated HBV-transgenic mice and verified the deletion effects by flow cytometry. The depletion of $CD8^+$ T cells delayed ALT secretion and suppressed serum ALT to near basal levels. However, depletion of $CD4^+$ cells, as well as NK1.1 cells, did not affect ALT levels, although it seems that the ALT levels were lower in the mice depleted with $CD4^+$ T cells after day 25 in comparison with the mice treated with control Ig (Fig. 3*D*). Consistent with this observation, the liver fibrosis was greatly reduced in the mice that were depleted of $CD8^+$ and were partially depleted of $CD4^+$ cells, whereas no change was observed in $NK1.1^+$ cell-depleted mice by Sirius red staining of liver tissues 1 wk after five injections of anti-CD137 mAb (Supplemental Fig. 4). Our results indicate that CD137 stimulation-induced liver fibrosis is mainly mediated by $CD8^+$ T cells, whereas $CD4^+$ T cells only partially contribute in the late stage.

CD137 stimulation does not stimulate HBV-specific T cell responses in HBV-transgenic mice

Given that CD137 is known to be a potent costimulator of Ag-specific $CD8^+$ T cell responses (7), it is logical to assume that CD137 mAb treatment stimulates expansion of HBV-specific CTL responses, which may cause liver pathology as the result of expression of the HBV Ag in the liver of HBV-transgenic mice. However, we could not detect $CD8^+$ T cells specific for S208, a peptide encoding HBsAg/K^b-specific epitope (19), after CD137 mAb treatment (Supplemental Fig. 5*A*), suggesting T cell tolerance of HBV Ag in this model. To further address this issue, the transgenic mice were immunized with HBsAg/CFA, followed by weekly anti-CD137 mAb treatment. On day 12 after the mAb injection, the splenocytes and IHLs were restimulated with HBsAg, peptide S208, or HBsAg transfectant in vitro to amplify the

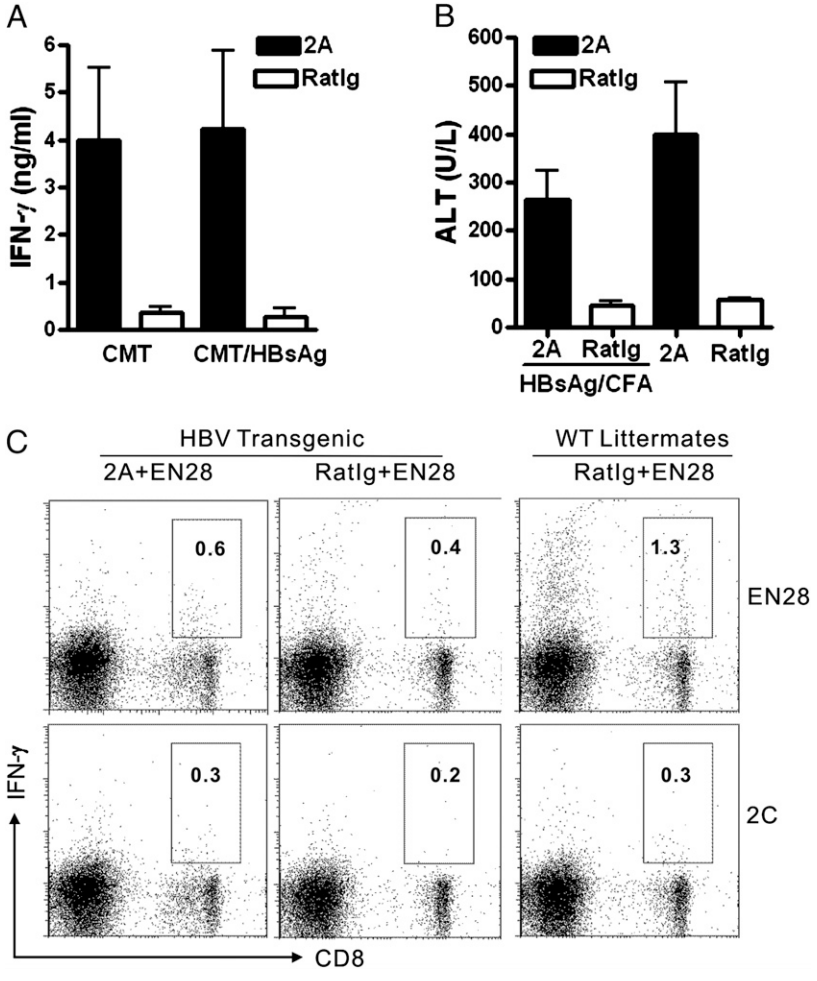

FIGURE 4. Detection of HBsAg-specific T cell responses in HBV-transgenic mice treated with anti-CD137. *A*, HBV-transgenic mice were treated with HBsAg emulsified in CFA on day −1, followed by 2A or RatIg injection on days 0 and 7. On day 12, splenocytes and IHLs were cocultured in 24-well plates with HBsAg transfectant (CMT/HBsAg) or mock transfectant (CMT) for 5 d. IFN-γ in the culture media was detected by ELISA. *B*, HBV-transgenic mice were treated with PBS or HBsAg, as described above, on day 1, followed by 2A/RatIg injections on days 0 and 7. The serum was collected for ALT assay on day 12. *C*, HBV-transgenic F1 ($H\text{-}2^{bxd}$) mice and WT littermates were immunized with EN28 peptide and poly-IC on days −5 and 9 and were further treated with anti-CD137 or RatIg on days 0 and 7. On day 12, splenocytes and IHLs were cocultured in 24-well plates with EN28 or control 2C peptide in vitro for 48 h. IFN-γ–producing cells were detected by intracellular staining after restimulation with PMA and ionomycin for an additional 3 h.

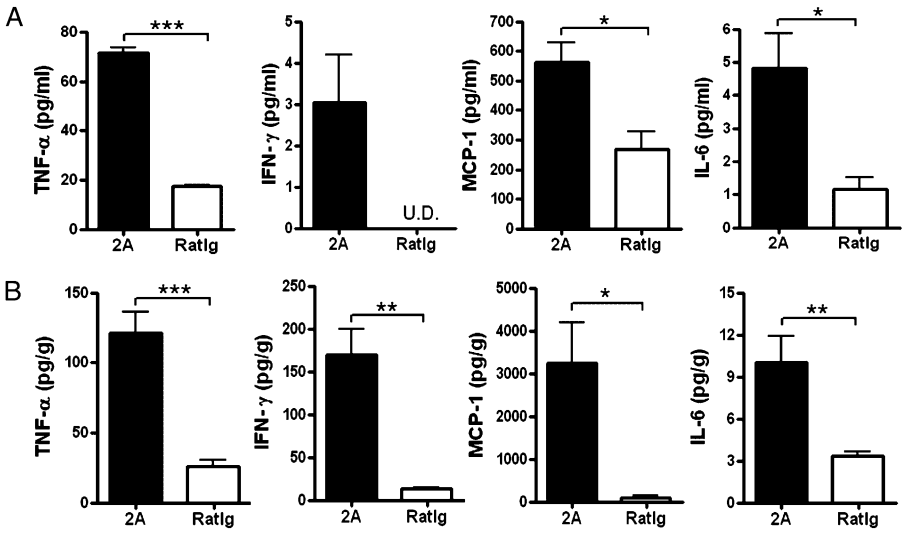

FIGURE 5. Cytokine profiling after anti-CD137 treatment in HBV-transgenic mice. HBV-transgenic mice were treated as in Fig. 2. On day 12, the cytokine in serum (*A*) or liver homogenates (*B*) was determined by cytokine beads array. Representative data from three independent experiments, with at least three mice per group, are shown. U.D., undetected. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

responses. CTL responses against HBsAg epitope still were not detected, as shown by HBsAg peptide S208/H-2K^b dimer staining (data not shown) and IFN-γ production (Fig. 4*A*). Meanwhile, the immunization with HBsAg did not enhance the liver damage induced by anti-CD137 mAb (Fig. 4*B*). We also tested the effect of anti-CD137 mAb in inducing CTL responses to an additional H-2^d–restricted epitope (EN28), which is a dominant epitope of HBsAg (20). To do so, HBV-transgenic F1 (H-2^{bxd}) mice were produced, immunized with EN28 peptide and poly-IC, and treated with anti-CD137 mAb. EN28 peptide immunization induced specific IFN-γ–producing CD8$^+$ T cells detected by IFN-γ production (Fig. 4*C*) and EN28-dimer staining (Supplemental Fig. 5*B*) in WT F1 littermates, but not in HBV-transgenic F1 mice, even after treatment with anti-CD137 mAb. Finally, blockade of MHC class I by a specific neutralizing mAb in HBV-transgenic mice could not alleviate the liver injury induced by anti-CD137 mAb (Supplemental Fig. 6). These data indicate that anti-CD137 mAb does not stimulate HBV-specific T cell responses and that the liver pathogenic effect is not mediated by MHC class I-associated T cell responses.

IFN-γ from CD8$^+$ T cells induces fibrosis-promoting cytokines/chemokines during CD137-mediated liver pathogenesis

Our previous study indicated that anti-CD137 mAb stimulated T cells with memory phenotype independent of MHC class I (12) and induced large numbers of cytokines (21). Proinflammatory

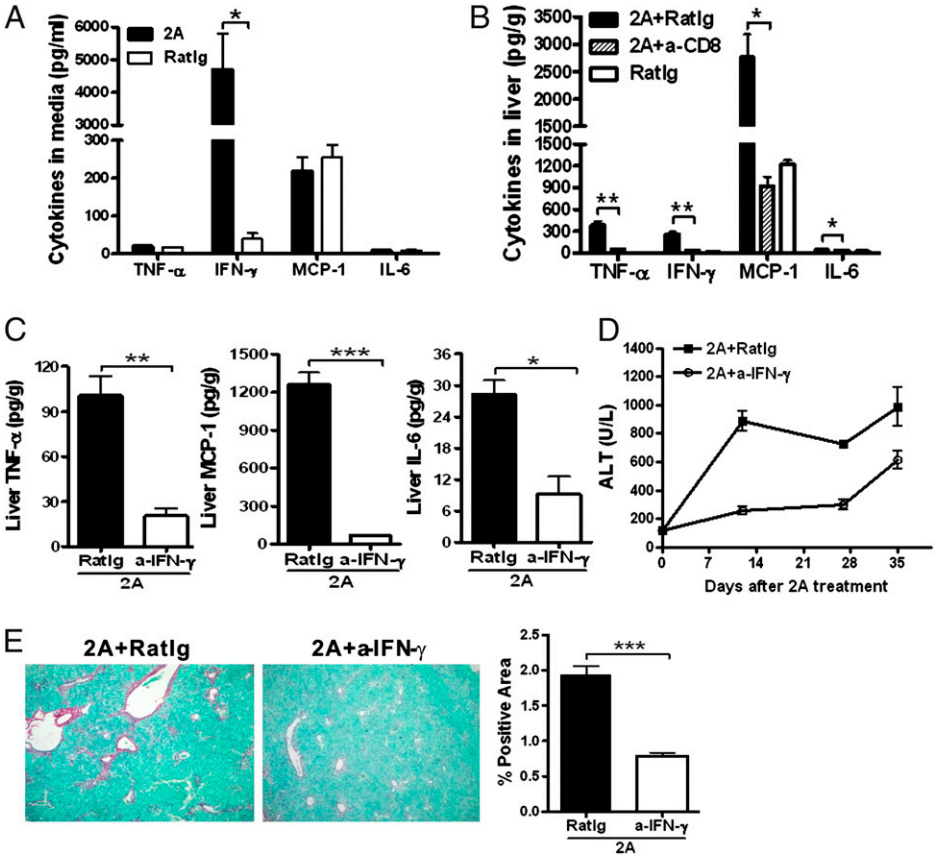

FIGURE 6. IFN-γ from CD8$^+$ T cells induces production of fibrosis-promoting cytokines/chemokines in CD137-mediated liver pathogenesis. HBV-transgenic mice were treated as in Fig. 2. *A*, On day 12, liver CD8$^+$ cells were purified and stimulated with immobilized anti-CD3 for 48 h, and the cytokines in the cultured media were determined. *B*, One hundred micrograms of anti-CD8–depletion Ab or RatIg was injected i.p. twice a week from the day before anti-CD137 injections. On day 12, the cytokines in liver homogenates were determined. *C–E*, Two hundred micrograms of anti–IFN-γ–neutralizing Ab or RatIg was injected i.p. twice a week starting on day 4. *C*, On day 12, the cytokines in liver homogenates were determined. *D*, Serum ALT levels at the indicated time points. *E*, Liver sections from the mice, 1 wk after the fifth 2A injections, were stained with Sirius red (*left panels*), and the collagen deposition was quantified (*right panel*) (original magnification ×40). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

cytokines and chemokines are critical in the progression of liver diseases, including chronic inflammation and fibrosis (1, 22). Treatment with anti-CD137 mAb stimulated significant elevation of IFN-γ, TNF-α, IL-6, and MCP-1 in sera (Fig. 5*A*) and liver homogenates (Fig. 5*B*) of HBV-transgenic mice on day 12 compared with control Ig treatment. There was no significant difference for KC, MIP-2, IL-10, IL-12, IL-4, IL-17, and TGF-β (data not shown). TNF-α, MCP-1, and IL-6 were shown to play a crucial role in liver fibrosis (23–26). Thus, our results establish a potential correlation of these fibrosis-promoting cytokines/chemokines with CD137-mediated liver pathology.

Next, we were interested in the extent of the contribution of $CD8^+$ T cells to the production of these cytokines. $CD8^+$ IHLs were isolated from the livers of HBV-transgenic mice on day 12 after mAb treatment and stimulated with immobilized anti-CD3 mAb in vitro for 48 h. As shown in Fig. 6*A*, $CD8^+$ T cells from anti-CD137 mAb-treated mice produced high levels of IFN-γ, but not TNF-α, MCP-1, or IL-6, compared with control Ig-treated mice.

To test whether the $CD8^+$ T cell-derived IFN-γ is responsible for subsequent induction of other fibrosis-promoting cytokines/chemokines, we eliminated $CD8^+$ T cells by anti-CD8–depleting mAb. Depletion of $CD8^+$ T cells dramatically decreased production of TNF-α, MCP-1, and IL-6, as well as IFN-γ, in the liver homogenates from HBV-transgenic mice (Fig. 6*B*). Furthermore, neutralization of IFN-γ in vivo by specific mAb also dramatically inhibited production of TNF-α, MCP-1, and IL-6 in liver homogenates (Fig. 6*C*) to a level similar to that after deletion of $CD8^+$ T cells (Fig. 6*B*). Importantly, neutralization of IFN-γ in vivo abrogated liver injury, as indicated by decreased serum ALT levels (Fig. 6*D*), and fibrogenesis (Fig. 6*E*). Thus, our results support a central role for IFN-γ in CD137-mediated liver pathogenesis through, at least in part, the induction of fibrosis-promoting cytokines/chemokines.

Increased hepatic infiltration and production of fibrosis-promoting cytokines/chemokines by $CD11b^+$ macrophages in response to T cell-derived IFN-γ

Increased hepatic infiltration of inflammatory cells is a significant phenotype of the HBV-transgenic mice treated with anti-CD137 mAb (Fig. 3*A*). Flow-cytometry analysis showed that anti-CD137 mAb treatment induced a significant increase in $CD11b^+$ cells in the liver of HBV-transgenic mice on day 12 (Fig. 7*A*, 7*B*), and >90% of this population was Gr-1^- and $CD11c^-$ (data not shown), indicating macrophage phenotype. The total number, but not the percentage, of $CD11b^+$ cells in IHLs steadily increased during mAb treatment (Fig. 7*B*), which may indicate that $CD11b^+$ cells are recruited into the liver. However, when the mice were treated with anti–IFN-γ–neutralizing mAb starting the day before anti-CD137 mAb treatment, hepatic infiltration of $CD11b^+$ cells was dramatically decreased (Fig. 7*C*). Therefore, IFN-γ may play a central role in CD137-mediated hepatic infiltration of macrophages during liver-inflammatory responses.

To test whether liver $CD11b^+$ cells are responsible for producing fibrosis-promoting cytokines/chemokines, $CD11b^+$ cells were isolated from the livers on day 12 after mAb treatment and were cultured without stimulation for 48 h. As shown in Fig. 8*A*, anti-CD137 mAb treatment significantly increased TNF-α and IL-6 secretion, whereas the MCP-1 level did not change. We further treated the mice with GdCl3, a selective liver macrophage toxicant (27), during mAb treatment to inhibit the activity of macrophages in vivo. The level of TNF-α was significantly reduced, and IL-6 was nearly undetectable in liver homogenates (Fig. 8*B*). GdCl3 treatment also ameliorated the liver injury, as indicated by serum ALT levels, and hepatic fibrosis, as shown by Sirius red staining (Supplemental Fig. 7).

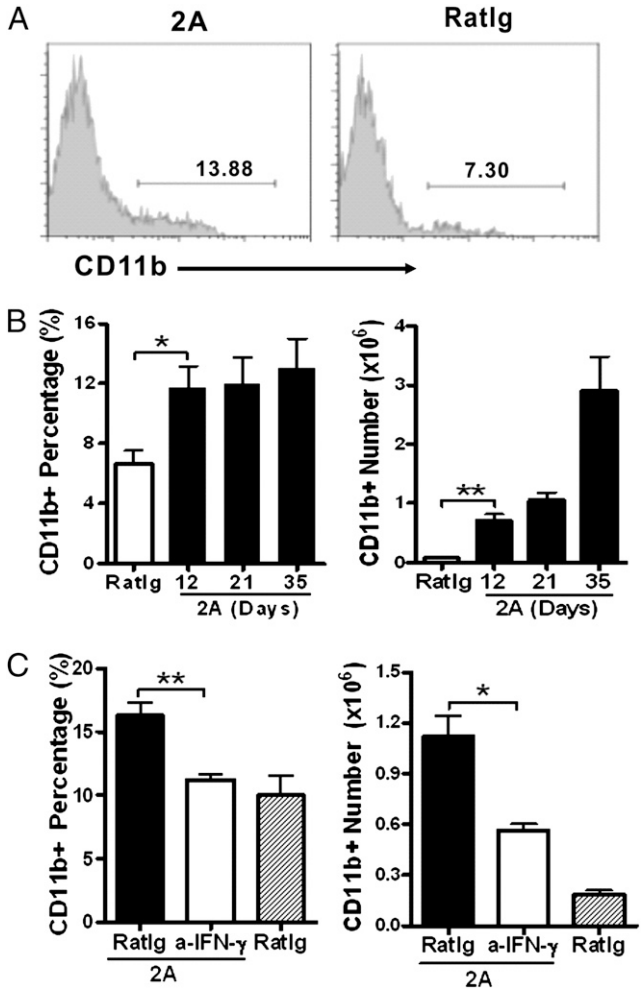

FIGURE 7. IFN-γ stimulates hepatic infiltration of $CD11b^+$ macrophages. HBV-transgenic mice were treated as in Fig. 2. At the indicated time points, IHLs were analyzed by flow cytometry. *A*, Representative graphs of anti-CD11b staining on day 12. *B*, Percentage (*left panel*) or total number (*right panel*) of the $CD11b^+$ liver macrophages at indicated time points. The liver was harvested on day 35 in the RatIg group. *C*, Anti–IFN-γ neutralization Ab or RatIg was injected twice a week from day 4 onward. On day 12, the percentage (*left panel*) and total number (*right panel*) of liver $CD11b^+$ macrophages were determined. $*p < 0.05$; $**p < 0.01$.

To address whether $CD8^+$ T cell-derived IFN-γ could directly induce fibrosis-promoting cytokine/chemokine production from liver macrophages, purified hepatic $CD11b^+$ macrophages from untreated naive mice were cultured with culture supernatants of intrahepatic $CD8^+$ T cells from HBV-transgenic mice. The supernatants of $CD8^+$ T cells from anti-CD137–treated mice dramatically increased TNF-α and IL-6 production of hepatic macrophages. Importantly, inclusion of neutralizing Ab for IFN-γ in the culture significantly decreased the levels of TNF-α and IL-6 (Fig. 8*C*). Our results indicate that activation of $CD8^+$ T cells by CD137 produces IFN-γ that subsequently stimulates the production of proinflammatory cytokines/chemokines by macrophages.

Discussion

A major finding from this study is that persistent CD137 signaling elicits chronic hepatitis, which subsequently progresses to liver fibrosis and HCC in a HBV-transgenic mouse strain. We demonstrated that anti-CD137 treatment induced rapid intrahepatic infil-

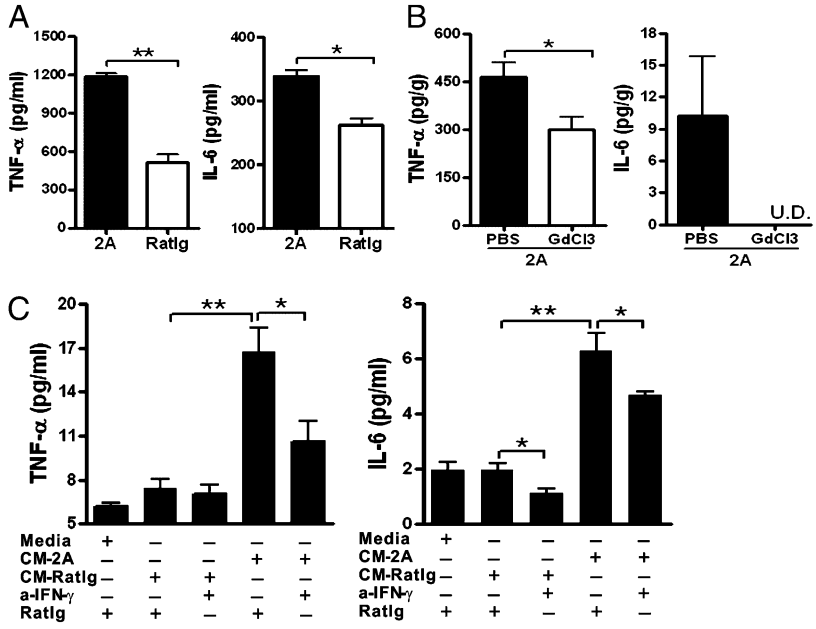

FIGURE 8. T cell-derived IFN-γ stimulates production of fibrosis-promoting cytokines by CD11b$^+$ macrophages. *A*, HBV-transgenic mice were treated as in Fig. 2. On day 12, purified CD11b$^+$ liver macrophages were cultured without any stimulation for 48 h. Cytokines in the culture media were evaluated. *B*, Mice were treated weekly with anti-CD137 mAb on day 0, followed by injections of GdCl3 or PBS twice a week from day 4 onward. On day 12, the cytokines in the liver homogenates were examined. *C*, Ten microliters of cultured media (CM) from CD8$^+$ cells, stimulated as in Fig. 6*A*, or control media was added to cultures of purified liver macrophages (1×10^5 cells/200 μl/well) from untreated mice, or followed by application of 10 μg/ml neutralizing anti–IFN-γ or control RatIg. The supernatants were harvested after 48 h of culture for cytokine analysis by cytometric bead array. Each group was performed in triplicate. Representative data from at least two independent experiments with at least three mice per group are shown. *$p < 0.05$; **$p < 0.01$. U.D., undetected.

tration of CD8$^+$ T cells, and the IFN-γ produced by CD8$^+$ T cells is a major pathogenic factor for liver disease progression through recruitment of early intrahepatic influx of inflammatory cells and induction of macrophages to produce fibrosis-promoting cytokines and chemokines. Because IFN-γ is a major cytokine that mediates control of HBV infection by a noncytolytic mechanism (2), this finding provides molecular evidence that the host can use redundant mechanisms for defense against viral infection and for pathogenic processes of liver diseases. Our findings also implicate a potential mechanism of liver disease progression in patients with CHB; our preliminary study indicated that CD137L is significantly upregulated on circulating monocytes, which may lead to persistent CD137 stimulation in CHB patients with liver cirrhosis.

HBV-specific CD8$^+$ T cell responses are greatly dampened in chronic infection, and CD8$^+$ T cells capable of recognizing HBV epitopes are barely detectable in patients with CHB (4, 5). The CD137 signaling pathway is a potent costimulator for Ag-specific CD8$^+$ T cell responses, which are pivotal in the control of viral infection (10, 11). One would expect that CD137 mAb treatment would stimulate expansion of HBV-specific CTL responses. However, we could not detect such T cell responses in HBV-transgenic mice, even after vigorous immunization with HBV Ag in combination with CD137 mAb. It is possible that HBV-specific T cells are deleted from the transgenic mice as a result of thymic negative selection. Alternatively, HBV-specific T cells may be present but remain immunologically tolerant or exhausted (28). This could explain our observation of the lack of specific CD8$^+$ CTL responses in HBV-transgenic mice after CD137 mAb treatment. Instead, CD137 mAb stimulates memory CD8$^+$ T cells nonspecifically, leading to accumulation of T cells with effector/memory (CD44^{high}CD62L^{low}) phenotypes in lymphoid organs, as shown in our previous studies (12, 29). It is tempting to speculate that elevated CD137L in patients with CHB might similarly stimulate T cells with memory phenotypes to promote inflammation as a compensatory response with lack of HBV-specific T cell responses. CD137-mediated proliferation of memory T cells occurs directly through CD137 on T cells and does not require other cell types or cytokines of IL-7 and IL-15. CD137 signaling has a more profound effect on the growth of CD8$^+$ T cells than CD4$^+$ T cells. In the HBV-transgenic mice, triggering of CD137 by agonist Ab also induced more vigorous growth of CD8$^+$ T cells in liver, especially in the first 2 wk (Fig. 3*B*). The increase in CD11b$^+$ cells in liver was mediated by IFN-γ produced by CD8$^+$ T cells (Fig. 7*C*). Thus, the major targets of anti-CD137 mAb seem to be CD8$^+$ T cells with memory phenotype in this HBV-transgenic model. We also studied the role of CD137 signaling in the Con A-induced hepatitis model, which is dependent on CD4$^+$ T cells, but not CD8$^+$ T cells. There was no significant difference in survival between CD137-deficient mice and WT mice treated with multiple dosage of Con A.

It was shown that triggering of CD137 signaling by agonistic Ab led to immunological anomalies in multiple organs of WT mice (21). We also found that CD137 stimulation induced accumulation of T cells in liver, lymph nodes, spleen, and lung in HBV-transgenic mice (data not shown). The HBV-transgenic mice have a similar T cell distribution in liver as the normal syngeneic mice. Even after anti-CD137 mAb treatment, there was no significant difference in the percentages and numbers of CD4$^+$ and CD8$^+$ T cells, as well as cytokine production, in liver between the HBV-transgenic and WT mice (Supplemental Fig. 1). Although treatment with anti-CD137 mAb induced similar inflammatory responses in the livers of HBV-transgenic and wide-type mice, only the transgenic mice developed severe liver injury and fibrogenesis (Fig. 2*A*, 2*B*, Supplemental Fig. 3). It was reported that the livers of HBV-transgenic mice are highly sensitive to toxin or immune-mediated cell death because of the retained HBsAg expression in hepatocytes (14, 30). These results suggested that HBV-transgenic hepatocytes are susceptible to the damage mediated by T cell responses induced by anti-CD137 mAb treatment, which may help to explain our observation.

Although our findings suggest that an enhanced CD137 signal may participate in triggering memory T cells to induce pathogenic processes of liver diseases in humans, these implications need to be evaluated carefully. CD137 mAb used in this study mimics CD137L to deliver an agonist signal. However, it is unknown whether the strength of this Ab signal is equivalent to that of CD137L in the patient with CHB. Nevertheless, our observation raises a possible mechanism of liver disease progression during HBV infection and encourages further study in patients.

Using an extensive cytokine and chemokine array, we demonstrated that CD137 stimulation elicits production of IFN-γ, TNF-α, MCP-1, and IL-6. Blocking of IFN-γ completely inhibited liver disease mediated by CD8 T cells. The role of IFN-γ was found

to be due, at least in part, to the induction of several fibrosis-promoting cytokines and chemokines, including TNF-α, MCP-1, and IL-6. Although TGF-β is the most potent cytokine for enhancing hepatic fibrogenesis, by stimulating the activation of hemopoietic stem cells, there is no significant increase in hepatic TGF-β levels after injections of 2A. Although we can identify that the major producing cells of TNF-α and IL-6 are macrophages upon stimulation by IFN-γ in supernatant-transfer experiments, macrophages did not secret MCP-1, a chemokine that is critical in the stimulation of fibrogenesis (31), under this experimental setting. However, we found that the production of MCP-1 dramatically increased in the supernatant of cocultures of hepatic macrophages and activated CD8 T cells (Supplemental Fig. 8). Therefore, macrophages might still be capable of producing MCP-1, but this process may be dependent on factors other than IFN-γ. Nevertheless, our results highlight a central role for IFN-γ in the pathogenesis of liver diseases because, in addition to stimulation of fibrosis-promoting cytokines and chemokines, it is important in the hepatic infiltration of inflammatory cells. Therefore, hosts may use mechanisms that are redundant, at least in part, to control viral infection, which, if not well controlled, will cause liver diseases. These findings may have important implications in the prevention of liver disease progression during chronic viral hepatitis.

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Disclosures

The authors have no financial conflicts of interest.

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