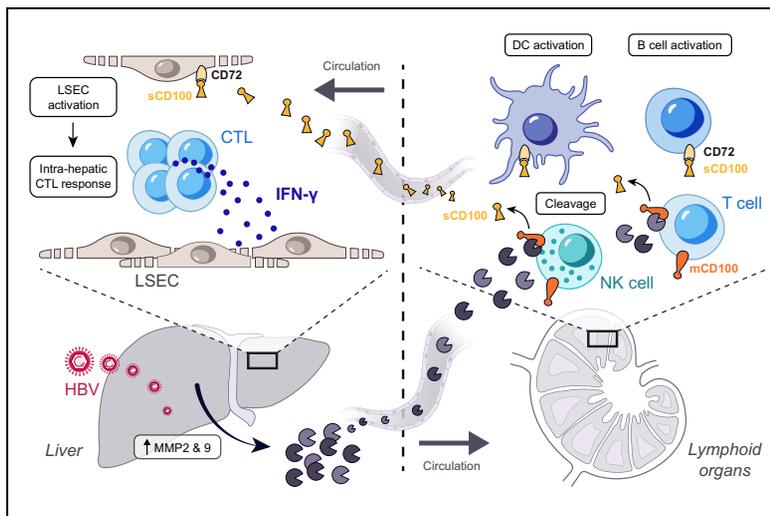# MMP2/MMP9-mediated CD100 shedding is crucial for inducing intrahepatic anti-HBV CD8 T cell responses and HBV clearance

Graphical abstract

Authors

Shangqing Yang, Lu Wang, Wen Pan, ...,
Mengji Lu, Dongliang Yang, Jia Liu

Correspondence

jialiu77@hust.edu.cn
(J. Liu)

Lay summary

Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide. The clearance of HBV relies largely on an effective T cell immune response, which usually becomes dysregulated in chronic HBV infection. Our study provides a new mechanism to elucidate HBV persistence and a new target for developing immunotherapy strategies in patients chronically infected with HBV.

Highlights

- HBV infection results in altered mCD100 expression and serum sCD100 levels.

- sCD100 can increase anti-HBV CTL response and accelerate HBV clearance.

- mCD100 shedding and sCD100 formation is mediated by MMP2 and MMP9.

- CHB patients show decreased serum MMP2 and sCD100 levels.

- MMP2/9 inhibition suppresses anti-HBV CTL response and delays HBV clearance.

MMP2/MMP9-mediated CD100 shedding is crucial for inducing intrahepatic anti-HBV CD8 T cell responses and HBV clearance

Shangqing Yang[1,†], Lu Wang[1,†], Wen Pan[1], Wibke Bayer[2], Christine Thoens[3], Kathrin Heim[4,5],
Ulf Dittmer[2], Joerg Timm[3], Qin Wang[1], Qing Yu[1], Jinzhuo Luo[1], Yanan Liu[1], Maike Hofmann[4,5],
Robert Thimme[4,5], Xiaoyong Zhang[6], Hongtao Chen[7], Hua Wang[1], Xuemei Feng[1], Xuecheng Yang[1],
Yinping Lu[1], Mengji Lu[2], Dongliang Yang[1,†], Jia Liu[1,*,†]

[1]Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022,
China; [2]Institute for Virology, University Hospital of Essen, University of Duisburg-Essen, Essen 45147, Germany; [3]Institute for Virology,
Heinrich-Heine-University, University Hospital, Duesseldorf 40225, Germany; [4]Department of Medicine II, University Hospital Freiburg,
Freiburg 79110, Germany; [5]Faculty of Medicine, University of Freiburg, Freiburg 79110, Germany; [6]Hepatology Unit and Key Laboratory
for Organ Failure Research, Department of Infectious Diseases, Nanfang Hospital, Southern Medical University, Guangzhou 510551, China;
[7]Department of Infectious Diseases, The Second Clinical Medical College, Jinan University, Shenzhen 510632, China

Background & Aims: CD100 is constitutively expressed on T cells and can be cleaved from the cell surface by matrix metalloproteases (MMPs) to become soluble CD100 (sCD100). Both membrane-bound CD100 (mCD100) and sCD100 have important immune regulatory functions that promote immune cell activation and responses. This study investigated the expression and role of mCD100 and sCD100 in regulating antiviral immune responses during HBV infection.

Methods: mCD100 expression on T cells, sCD100 levels in the serum, and MMP expression in the liver and serum were analysed in patients with chronic HBV (CHB) and in HBV-replicating mice. The ability of sCD100 to mediate antigen-presenting cell maturation, HBV-specific T cell activation, and HBV clearance were analysed in HBV-replicating mice and patients with CHB.

Results: Patients with CHB had higher mCD100 expression on T cells and lower serum sCD100 levels compared with healthy controls. Therapeutic sCD100 treatment resulted in the activation of DCs and liver sinusoidal endothelial cells, enhanced HBV-specific CD8 T cell responses, and accelerated HBV clearance, whereas blockade of its receptor CD72 attenuated the intrahepatic anti-HBV CD8 T cell response. Together with MMP9, MMP2 mediated mCD100 shedding from the T cell surface. Patients with CHB had significantly lower serum MMP2 levels, which positively correlated with serum sCD100 levels, compared with healthy controls. Inhibition of MMP2/9 activity resulted in an attenuated anti-HBV T cell response and delayed HBV clearance in mice.

Conclusions: MMP2/9-mediated sCD100 release has an important role in regulating intrahepatic anti-HBV CD8 T cell responses, thus mediating subsequent viral clearance during HBV infection.

Lay summary: Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide. The clearance of HBV relies largely on an effective T cell immune response, which usually becomes dysregulated in chronic HBV infection. Our study provides a new mechanism to elucidate HBV persistence and a new target for developing immunotherapy strategies in patients chronically infected with HBV.

Introduction

Chronic HBV infection continues to be a major public health burden worldwide. The persistence of HBV infection increases the risk of end-stage liver diseases, such as liver cirrhosis and hepatocellular carcinoma.[1] Exposure to HBV in neonates usually leads to viral persistence, whereas most infected adults spontaneously clear the virus.[2] The clearance of HBV relies largely on a potent and diverse T cell immune response, which usually becomes dysregulated in chronic HBV infection.[3–5] However, the mechanism by which a favourable anti-HBV T cell response is generated in an infected individual remains largely unknown.[6]

The semaphorin family, which includes more than 30 secreted or transmembrane proteins, was initially found to be essential for proper neuronal development.[7] However, several semaphorins are also expressed by immune cells, among which semaphorin 4D (also called CD100) has attracted particular attention because of its extensive functions in the immune system.[8] In lymphoid tissues, membrane CD100 (mCD100) is expressed abundantly on the surface of resting T cells and weakly on antigen-presenting cells (APCs), such as B cells and dendritic cells (DCs).[9] The expression of mCD100 on immune cells increases upon cell activation, which subsequently induces

shedding of the CD100 extracellular domain via proteolytic cleavage by certain matrix metalloproteinases (MMPs). This gives rise to a 120-kDa soluble form of CD100 (sCD100).[10–12] CD100 functions as a ligand and binds to different receptors, including CD72 on lymphoid tissues and plexin-B1/B2 on non-lymphoid tissues.[13–15] Through binding with CD72, which is expressed on most professional APCs, CD100 exerts essential functions in DC maturation, B cell proliferation, and antibody production, and, subsequently, in T cell priming and activation during the course of the T cell–APC interaction.[8] DCs from CD100-deficient mice display poor immunogenicity and are defected in costimulatory molecule expression and IL-12 production upon CD40 stimulation.[16] Treatment with exogenous sCD100 restored the normal functions of CD100-deficient DCs and further enhanced the functionality of wild-type DCs.[16] Although CD100-deficient T cells showed normal proliferative responses to anti-CD3 or concanavalin A stimulation *in vitro*, the *in vivo* generation of antigen-specific T cells was impaired in CD100-deficient mice.[17] This defective T cell priming was rescued by the administration of recombinant sCD100 protein.[17] In line with these findings, numerous studies have demonstrated the involvement of CD100 in inflammation and infectious diseases.[10] For example, CD100-deficient mice were protected from developing experimental autoimmune encephalomyelitis (EAE)[16] and glomerulonephritis,[18] whereas serum sCD100 levels positively correlated with autoantibody levels in mice that developed systemic autoimmune disease.[19] Levels of sCD100 were elevated in both serum and synovial fluid from patients with rheumatoid arthritis (RA) and were positively correlated with disease activity markers.[20] Altered mCD100/sCD00 expression has also been reported in viral infections. Serum sCD100 levels were increased and positively correlated with alanine aminotransferase (ALT) levels in patients with acute HCV infection.[21] Similarly, during the acute phase of haemorrhagic fever with renal syndrome (HFRS) caused by Hantan virus infection, plasma sCD100 levels were elevated and positively correlated with disease severity.[22] In patients with HIV, decreased mCD100 expression on CD4 and CD8 T cells in peripheral blood has been observed, and the loss of mCD100 expression on T cells was related to the immune activation status and viral loads.[23]

Despite extensive studies on the physiological and pathological functions of CD100 in different human diseases, the potential role of CD100 in immunity and viral clearance during HBV infection has not yet been reported. In this study, we used the HBV hydrodynamic injection mouse model and patients with chronic HBV infection to explore the role of CD100 in promoting anti-HBV T cell responses and viral clearance. The mechanisms of HBV infection-induced mCD100 shedding and sCD100 formation were also investigated.

Materials and methods

Preparation of mouse and human sCD100 recombinant protein

CD100 protein sequences were obtained from the NCBI database (murine CD100: NP_001268809.1; human CD100: NP_006369.3) and reverse translated. Codon-optimized sequences coding for the murine or human sCD100, comprising the CD100 signal peptide and extracellular domain, were synthesized by GeneArt (Thermo Fisher Scientific, Darmstadt, Germany) and cloned into the plasmid pcDNA3.1. For DNA immunization, plasmids were prepared by cesium chloride gradient ultracentrifugation. For protein purification, a sequence coding for 6 histidines (6-His tag) was added to the 3′ end of the sCD100 coding sequence by site-directed mutagenesis. Plasmids encoding the 6-His-tagged mouse or human sCD100 were transfected into 293T cells and supernatants were collected 3 days later. sCD100 protein was purified from supernatants by nickel affinity chromatography using Profinity IMAC resin (Bio-Rad Laboratories, Munich, Germany), followed by ultrafiltration using Amicon Ultra-4 30K centrifugal filter devices (Millipore, Merck, Darmstadt, Germany) for concentration and changed to physiological buffer. The purity of the protein was examined by SDS-PAGE and was over 90% (Fig. S1). The absence of endotoxin contamination in plasmids and purified proteins was verified by End-point Chromogenic TAL Endotoxin Testing Assay (detection limit 0.1 EU/μg; Bioendo Technology, Xiamen, China). No cellular cytotoxicity of the purified protein was detected (data not shown).

Detection of serum sCD100, MMP2 and MMP9

Serum sCD100, MMP2 and MMP9 levels were measured by the corresponding ELISA kits according to the manufacturer's instructions. Mouse sCD100 and human MMP2/MMP9 ELISA kits were purchased from Elabscience (Wuhan, China). The Human sCD100 ELISA kit was purchased from CUSABIO (Wuhan, China).

CD72 blockade and MMP2/9 inhibition in mice

For CD72 blockade, mice were intravenously injected twice with anti-CD72 antibody (80 μg per mouse, clone 10.1.D2, Southern Biotech, USA) at the indicated time points. For MMP2/9 inhibition, mice were intravenously injected with CTT peptide (CTTHWGFTLC, 200 μg per mouse) at the indicated time points.

Statistical analysis

Statistical analyses were performed using the SPSS statistical software package (version 22.0, SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk method was used to test for normality. Parametric analysis methods were used when the data were normally distributed; otherwise, non-parametric tests were employed. Unpaired *t* test, paired *t* test, one-way ANOVA, Pearson product-moment correlation coefficient, log-rank test, and analysis of covariance (ANCOVA) were used where appropriate. All reported *p* values were two-sided, and a *p* value less than 0.05 was considered statistically significant.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Membrane CD100 expression is increased on T cells and serum sCD100 level is decreased during chronic HBV infection

To explore the possible influence of HBV infection on CD100 expression in patients, we first analysed mCD100 expression on different cell populations of peripheral blood mononuclear cells (PBMCs) from healthy controls (HC) and patients with chronic hepatitis B (CHB) (Table S1) by flow cytometry. As shown in Fig. S2A, CD4 and CD8 T cells and natural killer (NK) cells were the main populations of CD100+ PBMCs in both HC and patients with CHB. The CD100 expression on T cells and

NK cells was abundant, whereas it was weak on B cells, DCs, and monocytes (Fig. S2A). The percentages and absolute numbers of CD100+ T cells (both CD4+ and CD8+) and NK cells were significantly higher in patients with CHB than in HC (Fig. 1A,B). By contrast, decreased absolute numbers of CD100+ B cells were observed in patients with CHB compared with HC, and no significant difference in mCD100 expression on DCs and monocytes was observed between HC and patients with CHB (Fig. S2B). Furthermore, the mean fluorescence intensities (MFIs) of CD100 on both CD4 and CD8 T cells were significantly increased in patients with CHB compared with HC (Fig. 1A). Next, we examined whether CD100 expression differed on T cells with distinct differentiation status. As shown in Fig. 1C, naïve, central memory (TCM), effector memory (TEM), and terminally differentiated effector memory (TEMRA) T cells were distinguished using CCR7 and CD45RA. We showed that the percentages of CD100+ TCM and TEM CD4 and CD8 T cells, as well as of TEMRA

CD8 T cells were significantly higher in patients with CHB than in HC. Naïve CD4 and CD8 T cells as well as TEMRA CD4 T cells showed no significant differences in CD100 expression between patients with CHB and HC (Fig. 1C). These results suggest that memory but not naïve T cells are the main T cell subsets upregulating mCD100 expression during chronic HBV infection.

In addition, core18-specific CD8+ T cells from HLA-A*02-positive patients with CHB were enriched through the pMHC I tetramer-based enrichment technique[24] and analysed for their mCD100 expression. No significant difference in mCD100 expression between total and HBV-specific CD8 T cells was observed in patients with CHB (Fig. S3A). In contrast to the increased mCD100 expression on T cells, the serum sCD100 concentration was significantly decreased in patients with CHB compared with HC (Fig. 1D). The gender or status of HBeAg presence of patients with CHB showed no significant impact on the percentage of CD100+ T cells or serum sCD100 levels
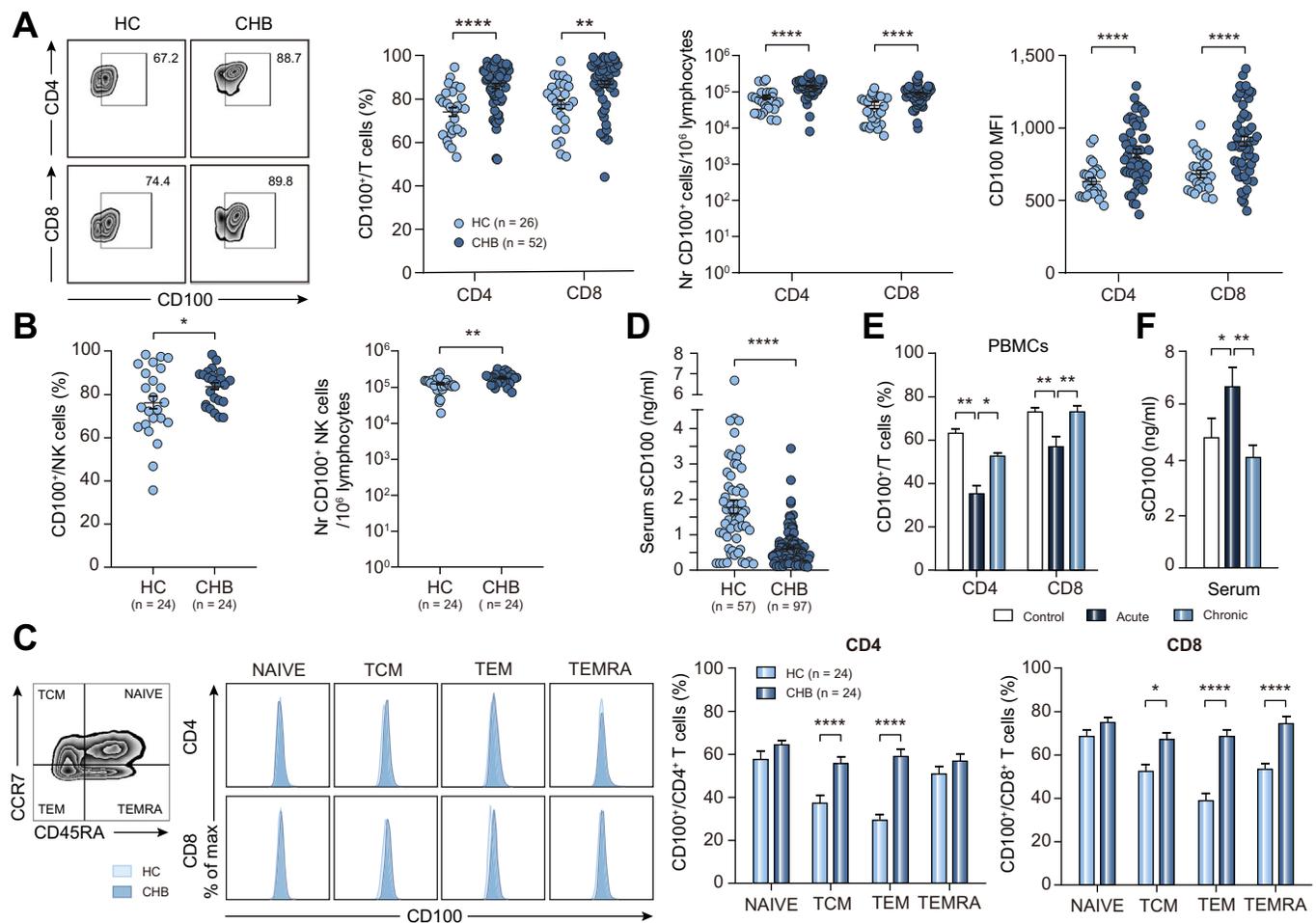

Fig. 1. Characterization of mCD100 expression on T cells and serum sCD100 levels in patients with CHB and in HBV-replicating mice. (A) Frequencies, numbers, and MFIs of CD100+ T cells were detected in HCs (n = 26) and patients with CHB (n = 52) by flow cytometry. (B) Frequencies and numbers of CD100+ NK cells were detected in HC (n = 24) and patients with CHB (n = 24) by flow cytometry. (C) CD100 expression on different T cell memory subsets (TCM, TEM, and TEMRA) was analysed in HC (n = 24) and patients with CHB (n = 24) by flow cytometry. (D) sCD100 levels in the serum were detected in HC (n = 57) and patients with CHB (n = 97) by ELISA. (E) C57BL/6 mice were hydrodynamically injected with either the pSM2 (Acute, n = 5) or pAAV/HBV1.2 (Chronic, n = 5) plasmid. Naïve mice (n = 5) served as a control. The frequencies of CD100+ CD4 or CD8 T cells were detected in PBMCs at 14 days post infection (dpi) by flow cytometry. (F) sCD100 levels in the serum of the mice were detected at 14 dpi by ELISA. Data from mouse experiments are representative of 3 independent experiments. Error bars, mean ± SEM; *p <0.05, **p <0.01, ****p <0.0001; (A–D) unpaired t test; (E,F) one-way ANOVA. CHB, chronic HBV infection; dpi, days post infection; HCs, healthy controls; MFI, mean fluorescence intensities; PBMCs, peripheral blood mononuclear cells; TCM, central memory T cell; TEM, effector memory T cell; and TEMRA, terminally differentiated effector memory T cell.

(Fig. S4A,B). No correlations between mCD100 or serum sCD100 levels with age, HBsAg levels, or ALT levels of patients with CHB were observed (Fig. S4C–E). In addition, no significant differences in mCD100 expression on T cells or serum sCD100 levels were observed among patients with different stages of chronic HBV infection, including HBeAg-positive chronic infection, -positive chronic hepatitis -negative chronic infection, and -negative chronic hepatitis[25] (Fig. S4F).

By using the HBV hydrodynamic injection (HDI) mouse model, we next explored whether the expression of mCD100 on T cells and sCD100 levels in serum were differently regulated during acute-resolving and chronic HBV replication. HDI of different HBV-expressing plasmids results in either acute-resolving (pSM2 plasmid) or chronic (pAAV/HBV1.2 plasmid) HBV replication in male C57BL/6 mice, which mimics different outcomes of HBV exposure in humans.[26–28] Compared with naïve and pAAV/HBV1.2 plasmid-injected mice, pSM2-injected mice showed a significant decrease in mCD100 expression on both CD4 and CD8 T cells in PBMCs and a significant increase in sCD100 levels in the serum at 14 days post HBV exposure (Fig. 1E,F). To further characterize the major source of sCD100 formation during the course of HBV clearance, the mCD100 expression on different populations of lymphocytes in the spleen and liver was analysed in pSM2 HDI mice by flow cytometry at 14 days post infection (dpi). Compared with naïve control mice, HBV-resolving mice showed a significant decrease in mCD100 expression levels on CD4 T cells, CD8 T cells, and NK cells in the spleen, and only on T cells in the liver. No decrease in mCD100 expression on B cells, DCs, or monocytes was observed (Fig. S5A). In addition, although a comparable proportion of NK cells and T cells lost their mCD100 expression in the spleen, CD4 and CD8 T cells presented with significantly higher absolute numbers of cells that lost mCD100 expression compared with NK cells (Fig. S5B). These results suggest that T cell shedding of CD100 is the major source of sCD100 during the course of HBV clearance; however, NK cells shedding CD100 might also contribute to sCD100 accumulation. The mCD100 expression on CD8 T cells and the sCD100 levels were also examined in PBMC and serum samples from patients with acute hepatitis B (AHB) (Table S1). A tendency of decreased mCD100 expression on HBV-specific, but not total CD8 T cells of patients with AHB compared with that of patients with CHB was observed (Fig. S3A). However, patients with AHB showed a significant decrease in serum sCD100 levels compared with patients with CHB (Fig. S3B). Given that all serum samples used in this study were collected from patients with AHB after the onset of hepatitis, we suggest that the serum sCD100 elevation occurred during an early stage of acute HBV infection, before the onset of hepatitis.

Taken together, our data indicate that HBV infection results in altered mCD100 expression on T cells and sCD100 levels in serum in both HBV-replicating mice and patients with CHB.

sCD100 induces DC and liver sinusoidal endothelial cells activation and enhances effector T cell responses

CD100 has an important role in the interaction between APCs and T cells and is essential for the induction of antigen-specific T cell responses. *In vitro* treatment with sCD100 results in enhanced activation of bone marrow-derived DCs.[16] Therefore, we explored how sCD100 treatment influences the activation of APCs and effector T cell responses in the spleen and liver. As expected, *in vitro* treatment with recombinant sCD100 protein significantly increased the expression of the costimulatory molecules CD80 and CD86 on lipopolysaccharide (LPS)-stimulated splenic DCs (Fig. 2A). The production of the inflammatory cytokine IL-12 by sCD100-treated splenic DCs was also significantly higher than by untreated control DCs (Fig. 2A). Liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs) are important liver-resident APCs with crucial roles in maintaining hepatic immune tolerance by suppressing T cell activation.[29–31] Thus, we next investigated the influence of sCD100 stimulation on LSEC- and KC-mediated T cell suppression. LSECs and KCs purified from naïve mice were pretreated with recombinant sCD100 protein and then cocultured with polyclonally T-cell receptor (TCR)-activated T cells (anti-CD3/-CD28 stimulation). In contrast to untreated LSECs, sCD100-pretreated LSECs showed no suppression of the interferon (IFN)-γ production of activated T cells (Fig. 2B). However, sCD100 treatment had no impact on the function of KCs in regulating T cell responses (Fig. S6A). To further explore whether *in vivo* sCD100 treatment also abrogates LSEC-mediated T cell suppression, we performed HDI of the sCD100-expressing plasmid pcDNA3.1-sCD100 into naïve mice. Subsequently, elevation of serum sCD100 concentrations was observed from 1 to 6 days after pcDNA3.1-sCD100 HDI (Fig. S6B). Whereas LSECs separated from control plasmid-injected mice strongly suppressed the IFN-γ production of activated T cells, no suppression of T cell activation was observed for LSECs isolated from the pcDNA3.1-sCD100-injected mice (Fig. 2C).

Endothelial cells express and release CD100.[32] Therefore, we next examined whether sCD100 treatment affects CD100 expression on LSECs. Our data showed that LSECs expressed low levels of CD100 on their surface; however, neither *in vitro* nor *in vivo* sCD100 treatment led to a significant change in mCD100 expression on stimulated LSECs compared with unstimulated controls (Fig. S6C). To further examine the influence of sCD100 treatment on inducing effector T cell responses, total splenocytes (SPLs) from naïve C57BL/6 mice were stimulated with anti-CD3/anti-CD28 in the presence or absence of sCD100 protein. sCD100 treatment significantly increased the IFN-γ concentration in the supernatant in a dose-dependent manner and augmented the intracellular IFN-γ production of both CD4 and CD8 T cells (Figs. 2D and S6D). The IL-2 production of CD4 and CD8 T cells and the concentration of tumour necrosis factor (TNF)-α in the supernatant were also significantly increased in sCD100-treated splenocytes compared with untreated control cells (Fig. S6E). Next, the splenocytes and liver-infiltrating lymphocytes (LILs) were separated from sCD100 plasmid or control plasmid HDI mice and stimulated with anti-CD3/-CD28. The IFN-γ concentrations in the supernatant of both splenocytes and LILs were significantly higher in the cultures with cells from sCD100 HDI mice than in those from control mice (Fig. S6F). Both CD4 and CD8 T cells from the spleen and liver of sCD100-treated mice showed significantly higher intracellular IFN-γ production in response to polyclonal TCR activation compared with control mice (Fig. S6F). We also explored the effect of sCD100 stimulation on augmenting antigen-specific CD8 T cell responses by using Friend virus (FV) TCR transgene CD8 T cells specific for the immunodominant DbGagL epitope (FV-TCR tg mice). Total splenocytes were separated from FV-TCR tg mice and stimulated with the DbGag epitope peptide in the presence or absence of sCD100 protein. sCD100 treatment resulted in significantly increased IFN-γ production by the FV-specific splenocytes (Fig. S6G), demonstrating
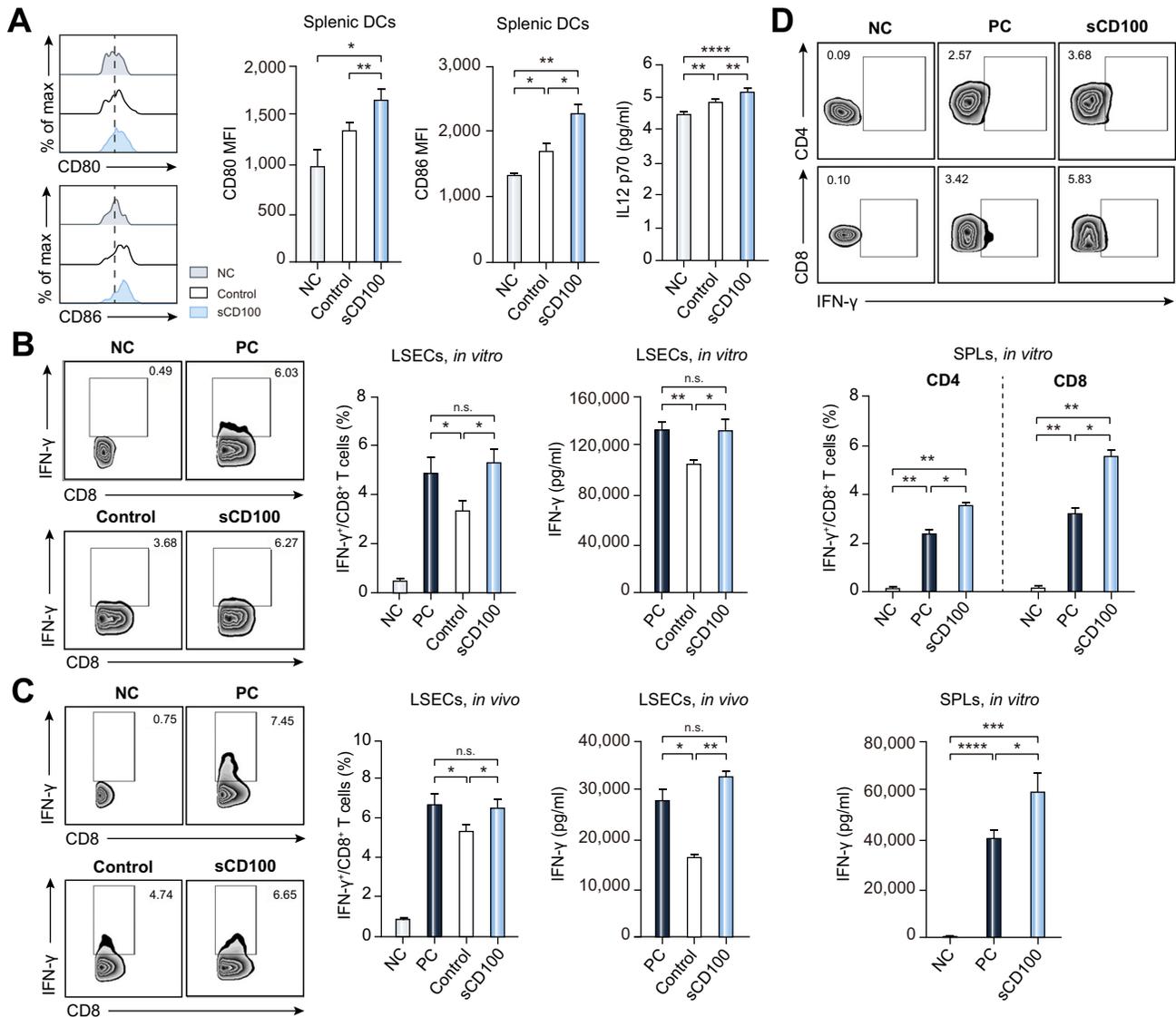
Fig. 2. sCD100 induces DC and LSEC activation and enhances the effector T cell response. DCs were purified from the spleen of C57BL/6 mice and stimulated with LPS (20 ng/ml) for 24 h in the absence or presence of recombinant sCD100 protein (10 μg/ml). (A) CD80 and CD86 expression on DCs was analysed by flow cytometry and IL-12 concentration in the supernatants was measured by ELISA. (B) LSECs were purified from C57BL/6 mice and treated with sCD100 (1 μg/ml) or left untreated for 24 h. (C) C57BL/6 mice were hydrodynamically injected with 20 μg sCD100-expressing plasmid or the control plasmid (Control). LSECs were purified after 48 h. LSECs were then cocultured with anti-CD3/anti-CD28 (1 μg/ml)-stimulated SPLs at a ratio of 1:2 (LSECs:SPLs). IFN-γ production by T cells was measured after 48 h by intracellular cytokine staining (left) and ELISA (right). Unstimulated SPLs were used as a NC and anti-CD3/anti-CD28-stimulated SPLs were used as a PC. (D) SPLs were separated from C57BL/6 mice and stimulated with anti-CD3/anti-CD28 in the absence or presence of sCD100 protein (1 μg/ml). IFN-γ production by T cells was measured after 48 h by intracellular cytokine staining (left) and ELISA (right). Data are representative of 2 to 3 independent experiments. Error bars, mean ± SEM; *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001, n.s., not significant (p >0.05); (A–D) one-way ANOVA. DC, dendritic cell; IFN, interferon; LPS, lipopolysaccharide; LSEC, liver sinusoidal endothelial cell; NC, negative control; PC, positive control; SPL, splenocyte.

the ability of sCD100 to enhance antigen-specific CD8 T cell responses. Taken together, our data suggest that sCD100 induces the maturation and activation of APCs in the spleen and liver, such as splenic DCs and LSECs, thus promoting the induction of effector CD8 T cell responses.

sCD100 treatment accelerates HBV clearance and enhances intrahepatic anti-HBV CD8 T cell responses
Our previous data indicated that chronic HBV infection is associated with an insufficient serum sCD100 level; therefore, we investigated the influence of sCD100 treatment on HBV clearance in the pAAV/HBV1.2 HDI mouse model, which mimics persistent HBV infection in humans. C57BL/6 mice were

hydrodynamically injected with pAAV/HBV1.2 in combination with either the sCD100-expressing plasmid or the pcDNA3.1 control plasmid, and monitored for HBV viraemia. The clearance of serum HBsAg and HBeAg was significantly accelerated in mice injected with the sCD100-expressing plasmid compared with those injected with the control plasmid (Fig. S7A). Eighty percent of sCD100 HDI mice became serum HBsAg negative at 56 dpi and HBeAg negative at 49 dpi, whereas the control mice remained 75% positive for HBsAg and 100% positive for HBeAg at the corresponding time points (Fig. S7B). To further validate our findings, we also treated HBV-persistent mice with purified sCD100 protein, as shown schematically in Fig. 3A. Given that the time required for inducing immune tolerance toward HBV
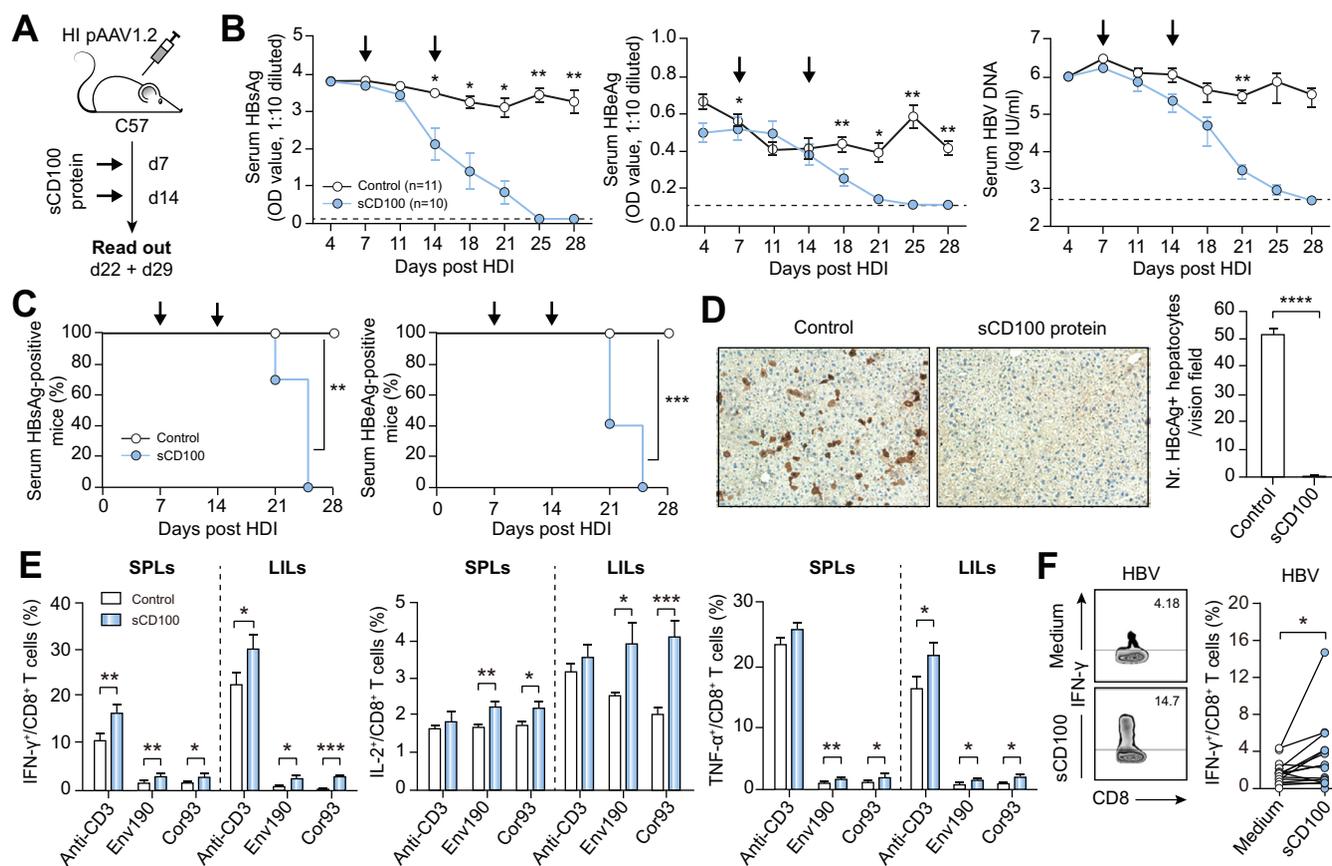
Fig. 3. sCD100 treatment accelerates HBV clearance and enhances the intrahepatic anti-HBV CD8 T cell response. (A) Experimental scheme of sCD100 treatment in the HDI mouse model. C57BL/6 mice were hydrodynamically injected with the pAAV/HBV1.2 plasmid and intravenously injected with sCD100 protein (50 μg per mouse, n = 10) or normal saline (Control, n = 11) twice, on 7 and 14 dpi. (B) The kinetics of serum HBsAg, HBeAg, and HBV DNA levels were monitored by ELISA or real-time PCR. (C) The percentages of HBsAg-positive mice (left) and HBeAg-positive mice (right) were analysed. (D) Immunohistochemical staining of HBV core protein in the livers of control and sCD100-treated mice at 29 dpi. Left: representative staining of a liver section (original magnification: 200×); Right: calculation of the average numbers of HBcAg-positive hepatocytes per vision field. (E) Splenocytes and LILs were separated from control and sCD100-treated mice at 22 dpi. Cells were stimulated with anti-CD3, HBsAg epitope peptide (Env190) or HBcAg epitope peptide (Cor93) together with anti-CD28 for 5 h, and the production of IFN-γ, IL-2 and TNF-α of CD8 T cells was detected by intracellular cytokine staining. (F) PBMCs from 16 patients with HLA-A*02-positive chronic hepatitis B were stimulated with HLA-A*02 HBV-specific peptide (FLPSDFFPSV) in the absence or presence of human sCD100 protein (1 μg/ml) for 10 days. Cells were analysed for IFN-γ production by intracellular cytokine staining. Error bars, mean ± SEM; *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001; (B) ANCOVA; (C) log-rank test; (D,E) unpaired t test; (F) paired t test. Cor93, core93-100; dpi, days post infection; Env190, env190-197; HDI, hydrodynamic injection; IFN, interferon; LIL, liver-infiltrating lymphocytes; PBMC, peripheral blood mononuclear cell; TNF, tumour necrosis factor.

antigens is 7 days after pAAV/HBV1.2 HDI,[33] we started to treat the mice with purified sCD100 protein from 7 dpi to examine its ability to break HBV immune tolerance. Two intravenous injections of sCD100 resulted in a substantial reduction in HBsAg, HBeAg, and viral DNA levels in the serum (Fig. 3B). All sCD100-treated mice became serum HBsAg and HBeAg negative at 25 dpi (Fig. 3C). The treated mice also cleared HBcAg from the liver at 29 dpi, whereas untreated mice harboured high levels of HBcAg in the liver (Fig. 3D). Next, we examined the impact of sCD100 protein treatment on the splenic and intrahepatic anti-HBV cytotoxic T cell (CTL) response. Significantly higher percentages of CD8 T cells from the spleens and livers of sCD100-treated mice were capable of producing IFN-γ, IL-2, and TNF-α in response to HBV epitope peptide (Env190) and core peptide (Cor93) stimulation compared with cells from control mice (Fig. 3E). An improved functionality of CD8 T cells following sCD100 treatment was also seen after polyclonal anti-CD3/anti-CD28 stimulation (Fig. 3E). To further confirm that the sCD100-mediated HBV clearance in HBV-persistent

mice was CD8 T cell dependent, CD8 T cells were depleted in sCD100-treated mice by intraperitoneal injection of purified anti-CD8 mAbs from 6 to 20 dpi. Significant decreases in CD8 T cells in terms of both frequency and number in the spleen and liver were observed in the CD8 T cell-depleted mice compared with the only sCD100-treated mice at 22 dpi (Fig. S7C). The serum HBsAg and HBV DNA levels of CD8 T cell-depleted mice were significantly higher than those of only sCD100-treated mice from 14 to 21 dpi, indicating that CD8 T cell depletion abolished the antiviral effect of sCD100 treatment in HBV-persistent mice (Fig. S7D). These results demonstrate that the sCD100 treatment-mediated HBV clearance in mice is CD8 T cell dependent.

Next, we investigated the potency of sCD100 in enhancing HBV-specific CD8 T cell responses in patients with chronic HBV infection or HC. The PBMCs from 16 patients with HLA-A*0201-positive CHB were stimulated with an HLA-A*0201 HBV-specific peptide (FLPSDFFPSV) to induce antigen-specific CD8 T cell expansion in the presence or absence of purified

recombinant human sCD100. sCD100 treatment resulted in significantly increased frequencies of IFN-γ-producing HBV-specific CD8 T cells in patients with CHB (Fig. 3F). It also resulted in significantly increased frequencies of IFN-γ-producing cytomegalovirus (CMV)-specific CD8 T cells in PBMCs from 6 HLA-A*0201-positive HC (Fig. S7E). In addition, we observed that sCD100 treatment enhanced T cell INF-γ production in a dose-dependent manner in patients with HBV and weak CMV-specific CD8 T cell responses, but not in patients with existing strong CMV-specific CD8 T cell responses (Fig. S7F).

Taken together, these results demonstrate that sCD100 treatment enhances intrahepatic anti-HBV CD8 T cell responses *in vivo*, thus accelerating HBV clearance in the liver.

CD72 blockade attenuates anti-HBV CD8 T cell responses in the spleen and liver

In lymphoid tissue, CD100 exerts its functions mainly through interaction with the receptor CD72.[15] CD72 is expressed by APCs, such as B cells, macrophages, and DCs, and the interaction between CD72 on APCs with CD100 is crucial for the priming and activation of T cells.[8] Therefore, we next examined the role of the CD72–CD100 interaction in anti-HBV CD8 T cell responses by performing a CD72 antibody blockade in the pSM2 HDI mouse model, as schematically shown in Fig. 4A. In this model of resolved HBV infection, the serum sCD100 levels are high and the anti-HBV immune responses are sufficiently activated. The CD8 T cells in the spleens and livers of HBV HDI mice showed a less-activated phenotype with lower CD43 and higher
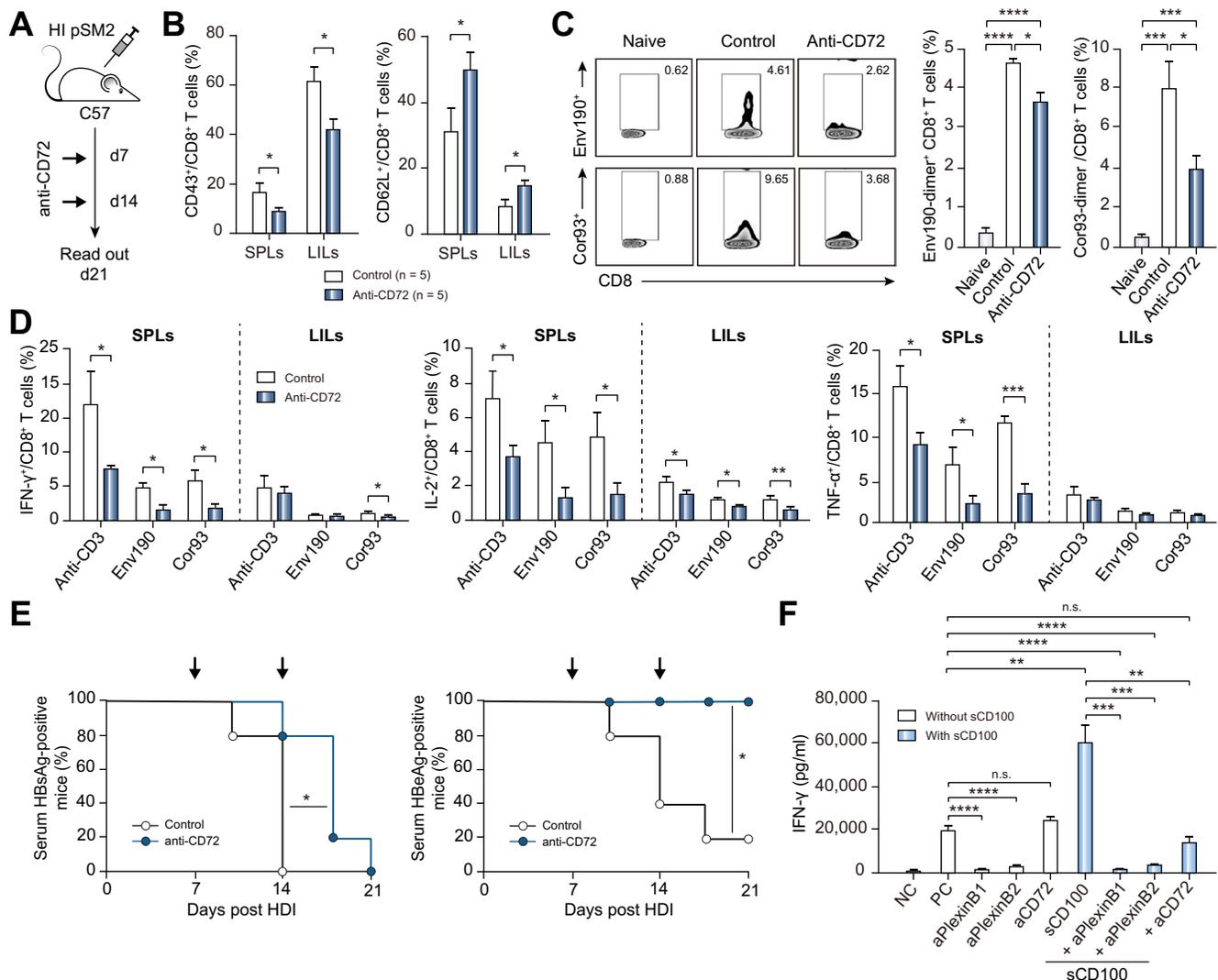

Fig. 4. CD72 blockade attenuates anti-HBV CD8 T cell responses in the spleen and liver. (A) Experimental scheme of CD72 blockade in the HDI mouse model. C57BL/6 mice were hydrodynamically injected with the pSM2 plasmid and intravenously injected with anti-CD72 antibody (80 μg per mouse, n = 5) or isotype control antibody (Control, n = 5) twice, on 7 and 14 dpi. (B–E) SPLs and liver-infiltrating lymphocytes (LILs) were separated from mice at 21 dpi. (B) CD43 and CD62L expression on CD8 T cells in the spleen and liver was detected by flow cytometry. (C) The frequencies of HBV Env190- and Cor93-specific CD8 T cells were determined by flow cytometry. (D) SPLs and LILs were stimulated with anti-CD3, Env190 peptide, or Cor93 peptide together with anti-CD28 for 5 h, and the production of IFN-γ, IL-2, and TNF-α of CD8 T cells was detected by intracellular cytokine staining. (E) The percentages of HBsAg-positive mice (left) and HBsAb-negative mice (right) were analysed. (F) SPLs were separated from naïve C57BL/6 mice and stimulated with anti-CD3/anti-CD28 in the absence or presence of recombinant sCD100 protein. Blocking antibodies against plexin-B1, plexin-B2, or CD72 were added to the cell culture as indicated. IFN-γ production by T cells was measured after 48 h by ELISA. Data were replicated in at least 2 independent experiments. Error bars, mean ± SEM; *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001; (B-D) unpaired t test; (E) log-rank test; (F) one-way ANOVA. Cor93, core93-100; dpi, days post infection; Env190, env190-197; HDI, hydrodynamic injection; IFN, interferon; LIL, liver-infiltrating lymphocytes; SPL, splenocyte; TNF, tumour necrosis factor.

CD62L expression after CD72 blockade compared with control mice (Fig. 4B). The frequencies of intrahepatic HBsAg- and HBcAg-specific CD8 T cells were significantly lower in the CD72 blockade group than in control mice (Fig. 4C). Moreover, CD72 blockade resulted in significant decreases in the percentages of IFN-γ-, IL-2-, and TNF-α-producing CD8 T cells in the spleen in response to anti-CD3, HBsAg, or HBcAg epitope peptide restimulation compared with control mice (Figs. 4D and S8A–C). Significant decreases in IFN-γ production by HBcAg-specific CD8 T cells and IL-2 production by HBcAg- and HBsAg-specific CD8 T cells in the liver were also observed following CD72 blockade (Fig. 4D). Moreover, CD72 blockade resulted in a significant delay in serum HBsAg clearance and anti-HBsAb development in mice with acute HBV replication (Fig. 4E). Next, we further characterized the possible role of the other 2 receptors of CD100, plexin-B1/B2, in modulating the effector CD8 T cell response. Splenocytes were separated from naïve C57BL/6 mice and stimulated with anti-CD3/anti-CD28 in the absence or presence of recombinant sCD100 protein. Blocking antibodies against plexin-B1, plexin-B2, or CD72 were added to the cell culture as indicated. IFN-γ production by T cells was measured after 48 h by ELISA. In TCR-activated T cells without sCD100 treatment, CD72 blockade showed no significant effect on the regulation of IFN-γ production. However, whereas sCD100 treatment significantly increased IFN-γ production by T cells, the effect was totally abolished by CD72 blockade (Fig. 4F). Interestingly, both plexin-B1 and -B2 blockade also reduced IFN-γ production by TCR-activated T cells to low levels irrespective of the status of additional sCD100 presence (Fig. 4F). These results suggest that plexin-B1 and -B2 perform a more fundamental role in the induction of effector T cell response, given that blockade of the function of these receptors fully abolished IFN-γ production by TCR-activated T cells. By contrast, the effect of enhancing T cell activation by sCD100 is mainly mediated through interacting with receptor CD72. These data indicate that the interaction between CD100 and its receptor CD72 participates in the induction of anti-HBV CD8 T cell responses in the spleen and liver during the course of acute HBV clearance.

MMP2 and MMP9 induce mCD100 cleavage on T cells and sCD100 formation during HBV infection

Increased mCD100 expression on T cells and decreased serum sCD100 levels suggested insufficient mCD100 cleavage from T cell membranes in patients with chronic HBV infection. The processes of mCD100 cleavage and sCD100 formation are believed to be mediated by MMPs.[11] Thus far, different MMPs, including MMP14, ADAM17, ADAMTS4, and MMP9 (also known as gelatinase B), have been reported to mediate mCD100 shedding from different types of cell in several diseases.[20,34–36] Therefore, we first examined the intrahepatic mRNA expression of MMP14, ADAM17, ADAMTS4, MMP9, and MMP2 (the other member of gelatinase, gelatinase A) in the naïve mouse. The mouse liver showed medium levels of MMP14 and ADAMTS4, but very low levels of ADAM17 and MMP9 expression. Interestingly, the expression of MMP2 was significantly higher than that of MMP14, ADAM17, ADAMTS4, and MMP9 in the liver (Fig. 5A). Next, we explored how HBV replication influences the intrahepatic expression of these MMPs in the HBV HDI mouse model. A sustained and robust upregulation of intrahepatic MMP2 mRNA was observed during HBV clearance in mice with acute-resolving HBV replication compared with control mice

(Fig. 5B), but no MMP2 upregulation was observed in the livers of HBV-persistent mice (Fig. 5B). By contrast, mice with acute-resolving HBV replication or persistent HBV showed only limited MMP14, ADAM17 and MMP9 upregulation in the liver, mostly at early time points after HBV plasmid challenge, whereas no upregulation of ADAMTS4 was observed (Fig. 5B). Next, we examined the expression of MMP2 in different liver cell populations and found that hepatocytes and LSECs expressed higher levels of MMP2 mRNA compared with KCs or the rest of the liver cells in naïve mice (Fig. 5C). LSECs of mice with acute-resolving HBV replication showed profound upregulation of MMP2 expression compared with their naïve, HDI control, or HBV-persistent counterparts, whereas hepatocytes only showed a slight increase in MMP2 expression (Fig. 5C). To further explore whether HBV infection influences MMP2 and MMP9 expression in humans, we next determined serum MMP2 and MMP9 levels in HC and patients with CHB. Serum MMP2 but not MMP9 concentration was significantly lower in patients with CHB than in HC (Fig. 5D). Consistent with this result, a positive correlation between serum sCD100 and MMP2 but not MMP9 levels was observed in patients with CHB (Fig. 5E).

Next, we further investigated the roles of MMP2 and MMP9 in mediating mCD100 cleavage on T cells and sCD100 formation during HBV infection. T cells of PBMCs from patients with CHB spontaneously lost mCD100 expression during the course of in vitro cultivation (Fig. 6A). This process was significantly inhibited when SB-3CT, a specific MMP2 and MMP9 inhibitor,[37,38] was added to the cell culture (Fig. 6A). Adding a combination of recombinant MMP2 and MMP9 to the cell culture of PBMCs from patients with CHB resulted in a significant decrease in mCD100 expression on both CD4 and CD8 T cells (Fig. 6B) and simultaneously increased the sCD100 concentration in the supernatant (Fig. 6C). Interestingly, the effect was not observed when MMP2 and MMP9 were used separately (Fig. 6B,C).

Taken together, these data suggest that both MMP2 and MMP9 are required for mCD100 cleavage on T cells and subsequent sCD100 formation during HBV infection; however, MMP2 expression appears to be selectively suppressed in chronic HBV infection

Specific MMP2 and MMP9 inhibition *in vivo* delays HBV clearance and suppresses intrahepatic anti-HBV CD8 T cell responses

Next, we explored the functions of MMP2 and MMP9 in mediating HBV clearance and anti-HBV CD8 T cell responses *in vivo*. Mice were hydrodynamically injected with pSM2 to establish acute-resolving HBV replication and treated with cyclic CTTHWGFTLC-peptide (CTT) to specifically inhibit the activities of MMP2 and MMP9 (Fig. 7A).[39] The CTT-treated HBV-replicating mice showed significantly lower serum sCD100 concentrations than their control peptide-treated counterparts (Fig. 7B). The CTT-treated mice also showed significantly higher serum HBeAg levels at 14 dpi and HBV DNA levels at 21 dpi compared with the control peptide-treated mice, whereas no difference in HBsAg was observed (Fig. 7C). At 21 dpi, CTT-treated mice remained 20% positive for serum HBsAg and 40% positive for HBeAg, whereas all of their control counterparts cleared the virus (Fig. 7D). We also found that significantly lower percentages of CD8 T cells from the livers of CTT-treated mice were capable of producing IL-2
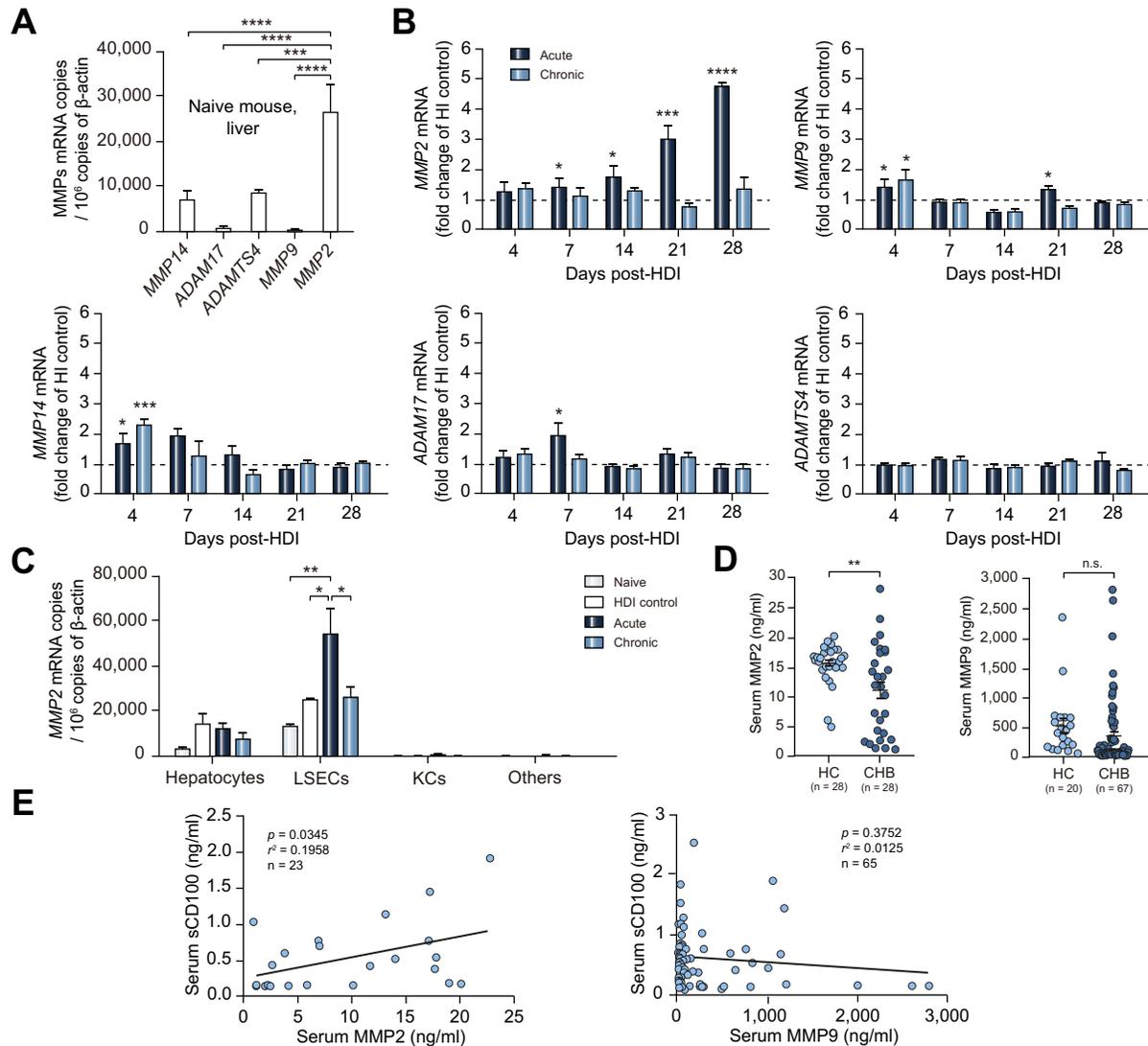
Fig. 5. Characterization of MMP2 and MMP9 expression in patients with CHB and in HBV-replicating mice. (A) RNA levels of *MMP14, ADAM17, ADAMTS4, MMP9,* and *MMP2* in the livers of naïve C57BL/6 mice were determined by real-time PCR. (B) C57BL/6 mice were hydrodynamically injected with either the pSM2 (Acute, n = 5) or pAAV/HBV1.2 (Chronic, n = 5) plasmid. Normal saline HDI mice (n = 5) served as a HDI control. The RNA levels of *MMP14, ADAM17, ADAMTS4, MMP9* and *MMP2* in the liver were determined by real-time PCR at the indicated time points. (C) Hepatocytes, LSECs, KCs, and the remaining liver cells were separated from the livers of naïve, HDI control, pSM2 HDI, and pAAV/HBV1.2 HDI mice at 7 dpi. The *MMP2* RNA levels in different liver cells were determined by real-time PCR. (D) MMP2 and MMP9 levels in the serum of healthy controls and patients with CHB were determined by ELISA. (E) Correlation analysis between serum sCD100, MMP2 (left) and MMP9 (right) levels was performed in patients with CHB. Error bars, mean ± SEM; *p <0.05, **p <0.01, ****p <0.0001, n.s., not significant (p >0.05); (A–C) one-way ANOVA; (D) unpaired t test; (E) Pearson product-moment correlation coefficient. CHB, chronic hepatitis B infection; dpi, days post infection; HDI, hydrodynamic injection; KC, Kupffer cells; LIL, liver-infiltrating lymphocytes; MMP, matrix metalloprotease.

and TNF-α in response to anti-CD3, HBsAg, or HBcAg epitope peptide restimulation than those from mice receiving the control peptide (Figs. 7E and S8D,E). No significant effect on cytokine production by CD8 T cells from the spleen was mediated by CTT treatment. In addition, no difference in IFN-γ production by CD8 T cells from the liver or spleen was observed between the two groups (data not shown). Taken together, our data indicate that inhibition of MMP2 and MMP9 activities during the course of HBV clearance results in decreased serum sCD100 levels, which delays HBV clearance, possibly by impairing intrahepatic anti-HBV T cell responses.

Discussion

Many molecules have been reported to be involved in the complex immune response against HBV. In this study, our findings revealed that HBV infection affected mCD100 expression on T cells and serum sCD100 levels. sCD100 stimulation induced DC and LSEC activation and enhanced effector T cell responses. Moreover, upregulation of sCD100 expression accelerated HBV clearance and increased intrahepatic anti-HBV CTL response, probably through the interaction between CD100 and CD72. mCD100 shedding on T cells and sCD100 formation were mediated by MMP2 and MMP9, whereas HBV infection affected liver and serum MMP2 expression. Specific MMP2 and MMP9
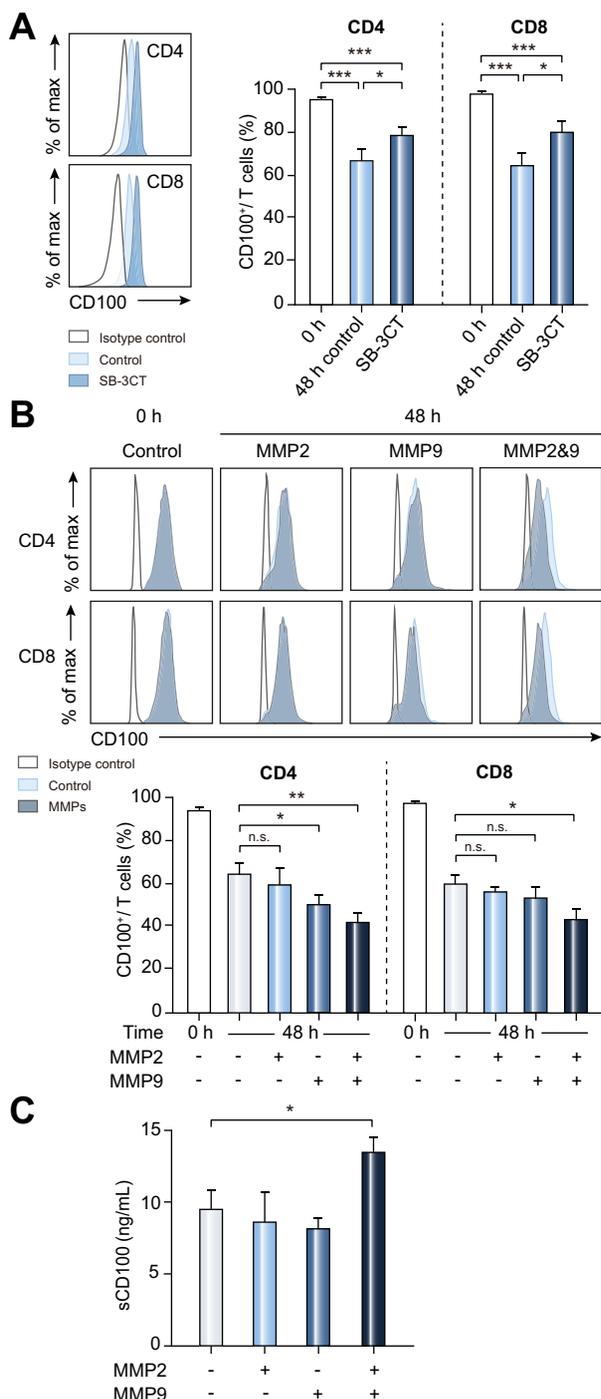
Fig. 6. MMP2 and MMP9 mediate CD100 shedding from the surface of T cells in patients with CHB. (A) Peripheral blood mononuclear cells (PBMCs) of patients with CHB (n = 6) were cultured in the absence or presence of the specific MMP2/9 inhibitor SB-3CT (0.1 μM) for 48 h. CD100 expression on CD4 and CD8 T cells was determined by flow cytometry. (B) PBMCs of patients with CHB (n = 6) were cultured in the absence or presence of MMP2 (1 μg/ml) and/or MMP9 (1 μg/ml) for 48 h. CD100 expression on CD4 and CD8 T cells was determined by flow cytometry. (C) sCD100 levels in the supernatants were measured by ELISA. Error bars, mean ± SEM; *p <0.05, **p <0.01, ***p <0.001, n.s., not significant (p >0.05); (A–C) one-way ANOVA. CHB, chronic hepatitis B infection; MMP, matrix metalloprotease; PBMC, peripheral blood mononuclear cell.

inhibition decreased sCD100 levels, delayed HBV clearance, and suppressed intrahepatic anti-HBV CD8 T cell responses. Based on these novel findings, we propose a model whereby, during

acute-resolving HBV infection, the liver produces increased amounts of MMP2, which, together with MMP9, mediate mCD100 shedding from the surface of T cells and NK cells in secondary lymphoid organs and increase serum sCD100 levels. By interacting with CD72, sCD100 induces the activation of APCs in the spleen and liver, thus promoting the intrahepatic anti-HBV CD8 T cell response. By contrast, during chronic HBV infection, decreased MMP2 expression results in insufficient sCD100 formation, which could lead to a compromised anti-HBV CD8 T cell response and viral persistence (Fig. S9). However, the role of mCD100/sCD100 in acute-resolving HBV infection versus clearance of already-established chronic HBV infection might differ. Given the limited access to samples from patients with acute HBV infection, our hypothesis of the function of CD100 in acute HBV infection was made mainly based on observations in the HBV HDI mouse model. Studies should be conducted in the future in natural hepatitis virus infection animal models, such as acute/chronic woodchuck hepatitis virus-infected woodchucks, to thoroughly examine the kinetics and functions of mCD100/sCD100.

Communication between immune cells is crucial for the proper coordination of immune responses. The interplay between cells of different organs is, to a great extent, achieved by the interaction of soluble mediators with their respective receptors or ligands.[10] It remains largely unclear how local cells in the liver and immune cells in the secondary lymphoid organs communicate with each other during HBV infection. Previous studies demonstrated the essential role of CD100 in the interaction of T cells and APCs during immune responses.[17] Importantly, after shedding from the cell surface upon cell activation, the extracellular domain of CD100 remains biologically active to induce the maturation and activation of professional APCs, such as DCs and B cells.[16,40,41] In line with these findings, we demonstrated, for the first time, that sCD100 also induces the activation of LSCEs, one of the most important populations of liver-resident APCs.[42] These properties make sCD100 a possible key factor that mediates the communication of immune cells between the liver and the lymphoid organs during a liver infection. These data are supported by the observation that serum sCD100 levels were increased and positively correlated with ALT levels in patients with acute HCV infection.[21,43]

An increasing number of studies have reported elevated sCD100 levels during infectious and inflammatory diseases.[16,19–22,44,45] In some of these diseases, such as RA and HFRS, significantly reduced CD100 expression on the surface of PBMCs or T cells has been observed, suggesting that elevated sCD100 levels are a result of enhanced shedding of CD100 from the surface of these cells. In the current study, elevated serum sCD100 levels and decreased surface CD100 expression on T cells were observed during the course of acute-resolving but not persistent HBV replication, suggesting that enhanced membrane CD100 shedding is an important immunological event related to HBV clearance. mCD100 shedding and subsequent sCD100 formation are considered consequences of the cellular activation of immune cells.[19,40,46,47] The loss of CD100 expression is correlated with the immune activation status of CD8 T cells in patients with HIV.[23] Thus, it is likely that the elevated sCD100 levels reflect the activation status of the adaptive immune system. Along this line, we observed significantly decreased sCD100 levels and increased mCD100 expression on T cells in patients with chronic HBV, suggesting a lower activation status of T cells in these patients. This result is supported by
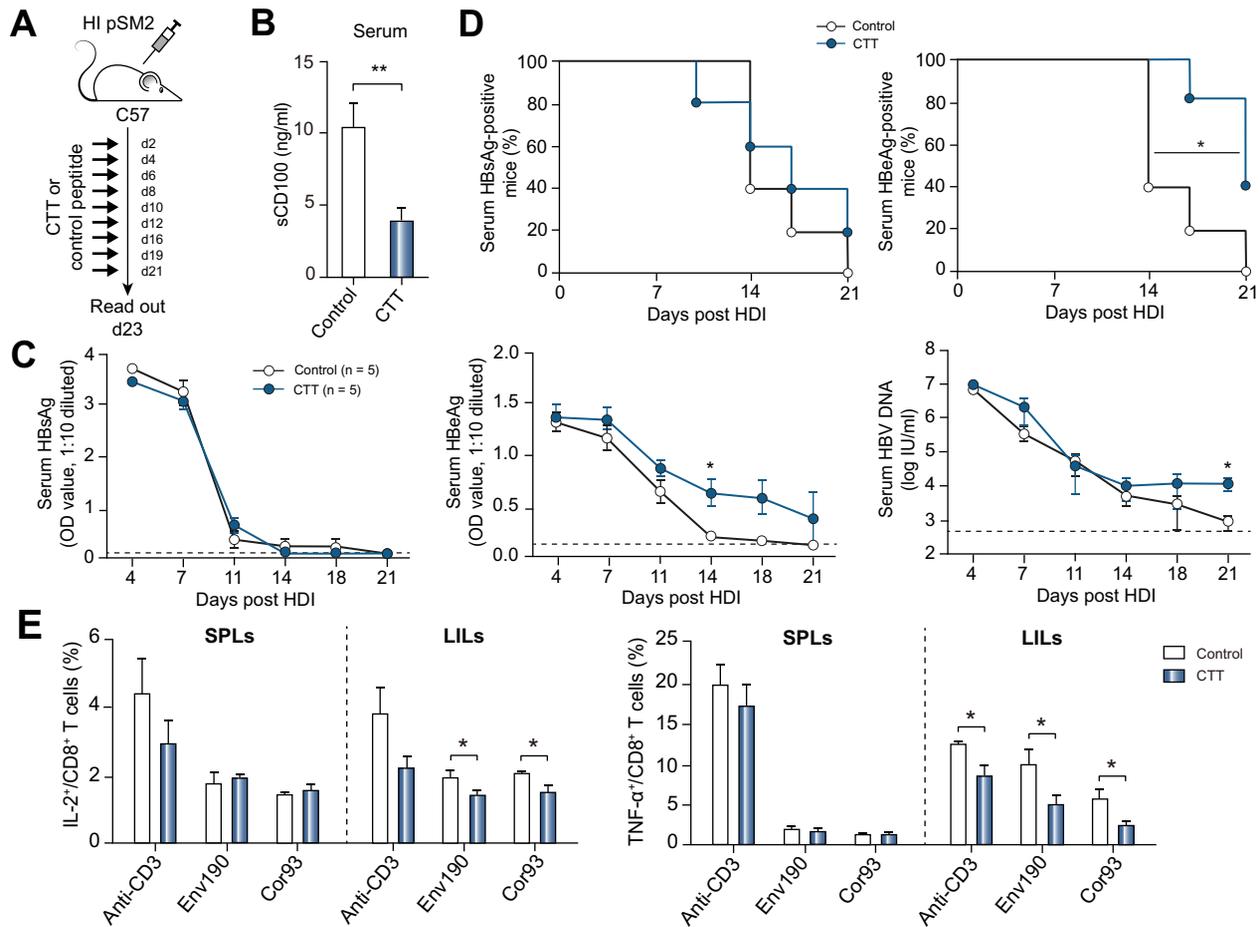
Fig. 7. MMP2 and MMP9 inhibition delays HBV clearance and suppresses the intrahepatic anti-HBV CD8 T cell response. (A) Experimental scheme of MMP2/9 inhibition in the HDI mouse model. C57BL/6 mice were hydrodynamically injected with the pSM2 plasmid and intravenously injected with CTT peptide (200 μg per mouse, n = 5) or control peptide (Control, n = 5) at the indicated time points. (B) The sCD100 levels in the serum of mice were detected at 14 dpi by ELISA. (C) The kinetics of serum HBsAg, HBeAg and HBV DNA levels were monitored by ELISA or real-time PCR. (D) The percentages of HBsAg-positive mice (left) and HBeAg-positive mice (right) were analysed. (E) SPLs and LILs were separated from treated mice at 23 dpi. Cells were stimulated with anti-CD3, Env190 peptide, or Cor93 peptide together with anti-CD28 for 5 h, and the production of IL-2 and TNF-α of CD8 T cells was detected by intracellular cytokine staining. Data were replicated in at least 2 independent experiments. Error bars, mean ± SEM; *p <0.05, **p <0.01; (B,E) unpaired t test; (C) ANCOVA; (D) log-rank test. CHB, chronic hepatitis B infection; Cor93, core93-100; dpi, days post infection; Env190, env190-197; HDI, hydrodynamic injection; LIL, liver-infiltrating lymphocyte; MMP, matrix metalloprotease; PBMC, peripheral blood mononuclear cell; SPL, splenocyte; TNF, tumour necrosis factor.

the observation that T cells from these patients produced significantly less IFN-γ in response to TCR stimulation compared with HC (data not shown).

The mechanism by which peripheral sCD100 levels are regulated during infectious and inflammatory diseases remains largely unknown. MMP14, ADAM17, and ADAMTS4, which trigger CD100 cleavage from cell membranes, are involved in tumour-induced angiogenesis, platelet activation, and RA progression, respectively.[20,34,35] MMP9 was recently identified as a new proteolytic enzyme for CD100 cleavage from T cells in oral keratinocytes.[36] Interestingly, whereas ADAMTS4 can induce CD100 shedding from the membrane of THP-1 cells,[20] MMP9 failed to do so, suggesting that proteolytic mediators of CD100 shedding vary between cell types.[36] However, in our current study, MMP9 alone showed only limited effects on mediating CD100 cleavage from T cells in patients with CHB, whereas both MMP2 and MMP9 were required for this process. Furthermore, the levels of MMP2 but not MMP9 consistently correlated with sCD100 levels in patients with CHB. Therefore, we speculate that proteolytic mediators of CD100 shedding among different diseases also vary.

Dysregulation of MMP2 and MMP9 expression has been previously reported in chronic virus infection diseases, such as HCV and HIV, and is believed to be associated with disease progression. The protein levels and enzymatic activities of MMP2 and MMP9 were increased in patients with HIV-1 compared with HIV-1-negative controls.[48] The enzymatic activity of MMP-2, but not MMP-9, was further increased in plasma samples of patients with HIV-1-associated neurocognitive disorders (HAND) than in patients with HIV-1 without HAND.[48] The polymorphisms of MMP2 and MMP9 were associated with progression and assessment of prognosis for HIV-associated non-Hodgkin lymphoma, periodontitis, and the development of HAND and its severity.[49] The intrahepatic expression level of MMP2 and MMP9 was also significantly higher in patients with HCV than in uninfected controls, and the overexpression of MMP2 and MMP9 was associated with progressive hepatic fibrosis, suggesting their pathogenic role in fibrogenesis.[50] MMP9, but not MMP2, was found to be associated with intrahepatic HCV mRNA levels,[51] and upregulation of MMP9 expression was observed during the transition from mild to moderate fibrosis in patients.[51,52] However, studies in animal models

suggest that the increased MMP9 expression contributes to liver fibrosis resolution.[53] For example, MMP9 expression was increased in carbon tetrachloride (CCL4)-induced liver fibrosis mice, whereas adoptive transfer of microencapsulated human mesenchymal stem cells decreased liver fibrosis in mice and led to further significant increases in MMP9 expression.[54] MMP9 is synthesized and released by human and rat KCs during liver fibrogenesis.[55] Adoptive transfer of KCs from wild-type mice to thioacetamide-induced liver fibrosis mice promoted liver fibrosis resolution, whereas the transfer of KCs from MMP9−/− mice failed to improve fibrosis regression.[56] Similar results have been observed when DCs were used as donor cells for the treatment of liver fibrosis in a CCL4-induced fibrosis mouse model.[57]

Over the past year, a growing number of non-matrix substrates for MMPs have been identified, many of which are related to the modulation of inflammatory processes.[58,59] By regulating the availability and activity of inflammatory mediators, such as cytokines and chemokines, MMPs have important roles in the pathogenesis of inflammatory diseases.[60] It has also been shown that inhibition of MMP activity reduced the intrahepatic recruitment of non-specific inflammatory cells and ameliorated the severity of liver disease in HBV-transgenic mice adoptively transferred with HBV-specific CTL.[61] In this model, the recruitment of non-specific inflammatory cells into the liver is believed to be associated with the upregulation of MMP8 and MMP9 expression in neutrophils after CTL transfer.[61] Interestingly, we also observed a slight increase in liver MMP9 expression at 21 days post HBV challenge in mice, which is the time point of the peak infiltration of HBV-specific CTLs (data not shown). Importantly, we demonstrated the sustained and profound upregulation of liver MMP2 expression during the course of HBV clearance, and inhibition of MMP2 and MMP9 activity reduced intrahepatic HBV-specific CTL responses. Taken together, these findings suggest that, in addition to participating in the immunopathology of HBV infection, MMPs also have a pivotal role in mediating intrahepatic anti-HBV CTL responses by inducing CD100 cleavage. Although we showed that HBeAg triggers MMP2 expression in LSECs, it remains unclear how chronic HBV infection results in decreased MMP2 levels in patients. Further studies are required to explore the mechanism of MMP regulation during HBV infection.

Previous studies have shown that CD100 also exerts crucial functions in regulating B cell[15,40,62] and platelet activation.[35,63] CD100 stimulation enhanced CD40-dependent B cell aggregation, proliferation, and differentiation.[15,40,62] CD100 expression on platelets increases upon platelet activation and promotes platelet aggregate formation, which is followed by CD100 shedding.[35] Activated platelets are essential for the intrahepatic accumulation of HBV-specific CTLs.[64] The circulating HBV-specific CTLs arrest within liver sinusoids by docking onto platelet aggregates previously adhered to sinusoidal hyaluronan.[65] Therefore, whether increased sCD100 levels have a role in regulating the humoral immune response and intrahepatic CTL adhesion during the course of HBV clearance requires further investigation.

The immunostimulatory properties of CD100, together with its association with disease severity, make it a potential therapeutic target or prognostic biomarker in certain autoimmune diseases and cancers.[10] *In vivo* CD100 blockade reduces disease severity in the experimental EAE animal model.[66] Recently, a phase I clinical trial demonstrated that the anti-CD100 antibody VX15/2503 was safe and well tolerated at various doses in patients with multiple sclerosis (MS).[67] However, in EAE and MS, enhanced production of sCD100 was observed, whereas we found insufficient sCD100 production in chronic HBV infection. Importantly, increasing the sCD100 concentration enhanced anti-HBV immune responses and accelerated HBV clearance. Therefore, our study raises the questions of whether sCD100 represents a useful biomarker for evaluating the immune activation status in patients with CHB and whether it could serve as a potential therapeutic target for the treatment of chronic HBV infection, both of which should be addressed in future studies.

Conflict of interest

The authors disclose no conflicts of interest. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceived and designed the experiments: SQY, LW, DLY, JL. Performed the experiments: SQY, LW, WP, CT, KH, QW, QY, JZL, YNL, HW. Analysed and interpreted the data: SQY, WL, JL. Contributed reagents/materials/analysis tools: WB, UD, JT, MH, RT, XYZ, HTC, XMF, XCY, YPL, MJL, DLY, JL. Drafted the manuscript: SQY, UD, DLY, JL.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.05.013.

References

Author names in bold designate shared co-first authorship

[1] Dienstag JL. Hepatitis B virus infection. N Engl J Med 2008;359:1486–1500.
[2] Ganem D, Prince AM. Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 2004;350:1118–1129.
[3] Thimme R, Wieland S, Steiger C, Ghrayeb J, Reimann KA, Purcell RH, et al. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. J Virol 2003;77:68–76.
[4] Jung MC, Spengler U, Schraut W, Hoffmann R, Zachoval R, Eisenburg J, et al. Hepatitis B virus antigen-specific T-cell activation in patients with acute and chronic hepatitis B. J Hepatol 1991;13:310–317.

[5] Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection. Nat Rev Immunol 2005;5:215–229.

[6] Chisari FV, Isogawa M, Wieland SF. Pathogenesis of hepatitis B virus infection. Pathol Biol 2010;58:258–266.

[7] Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. Science 1996;274:1123–1133.

[8] Kikutani H, Kumanogoh A. Semaphorins in interactions between T cells and antigen-presenting cells. Nat Rev Immunol 2003;3:159–167.

[9] Delaire S, Elhabazi A, Bensussan A, Boumsell L. CD100 is a leukocyte semaphorin. Cell Mol Life Sci 1998;54:1265–1276.

[10] Maleki KT, Cornillet M, Bjorkstrom NK. Soluble SEMA4D/CD100: a novel immunoregulator in infectious and inflammatory diseases. Clin Immunol 2016;163:52–59.

[11] Elhabazi A, Delaire S, Bensussan A, Boumsell L, Bismuth G. Biological activity of soluble CD100. I. The extracellular region of CD100 is released from the surface of T lymphocytes by regulated proteolysis. J Immunol 2001;166:4341–4347.

[12] Bougeret C, Mansur IG, Dastot H, Schmid M, Mahouy G, Bensussan A, et al. Increased surface expression of a newly identified 150-kDa dimer early after human T lymphocyte activation. J Immunol 1992;148:318–323.

[13] Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, et al. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 1999;99:71–80.

[14] Witherden DA, Watanabe M, Garijo O, Rieder SE, Sarkisyan G, Cronin SJ, et al. The CD100 receptor interacts with its plexin B2 ligand to regulate epidermal gammadelta T cell function. Immunity 2012;37:314–325.

[15] Kumanogoh A, Watanabe C, Lee I, Wang X, Shi W, Araki H, et al. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. Immunity 2000;13:621–631.

[16] Kumanogoh A, Suzuki K, Ch'ng E, Watanabe C, Marukawa S, Takegahara N, et al. Requirement for the lymphocyte semaphorin, CD100, in the induction of antigen-specific T cells and the maturation of dendritic cells. J Immunol 2002;169:1175–1181.

[17] Shi W, Kumanogoh A, Watanabe C, Uchida J, Wang X, Yasui T, et al. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. Immunity 2000;13:633–642.

[18] Li M, O'Sullivan KM, Jones LK, Semple T, Kumanogoh A, Kikutani H, et al. CD100 enhances dendritic cell and CD4+ cell activation leading to pathogenetic humoral responses and immune complex glomerulonephritis. J Immunol 2006;177:3406–3412.

[19] Wang X, Kumanogoh A, Watanabe C, Shi W, Yoshida K, Kikutani H. Functional soluble CD100/Sema4D released from activated lymphocytes: possible role in normal and pathologic immune responses. Blood 2001;97:3498–3504.

[20] Yoshida Y, Ogata A, Kang S, Ebina K, Shi K, Nojima S, et al. Semaphorin 4D contributes to rheumatoid arthritis by inducing inflammatory cytokine production: pathogenic and therapeutic implications. Arthritis Rheumatol 2015;67:1481–1490.

[21] He Y, Li B, Zhou Y, Zhang Y, Guo Y, Ji G, et al. Alteration of CD100 expression on natural killer cells in chronic patients with hepatitis C virus before and after initiation of antiviral treatment. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2014;30:856–860 (in Chinese).

[22] Liu B, Ma Y, Yi J, Xu Z, Zhang YS, Zhang C, et al. Elevated plasma soluble Sema4D/CD100 levels are associated with disease severity in patients of hemorrhagic fever with renal syndrome. PLoS ONE 2013;8 e73958.

[23] Eriksson EM, Milush JM, Ho EL, Batista MD, Holditch SJ, Keh CE, et al. Expansion of CD8+ T cells lacking Sema4D/CD100 during HIV-1 infection identifies a subset of T cells with decreased functional capacity. Blood 2012;119:745–755.

[24] Schuch A, Salimi Alizei E, Heim K, Wieland D, Kiraithe MM, Kemming J, et al. Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load. Gut 2019;68:905–915.

[25] European Association of the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017;67:370–398.

[26] Dietze KK, Schimmer S, Kretzmer F, Wang J, Lin Y, Huang X, et al. Characterization of the Treg response in the hepatitis B virus hydrodynamic injection mouse model. PLoS ONE 2016;11 e0151717.

[27] Huang LR, Wu HL, Chen PJ, Chen DS. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. Proc Natl Acad Sci U S A 2006;103:17862–17867.

[28] Wang Q, Pan W, Liu Y, Luo J, Zhu D, Lu Y, et al. Hepatitis B virus-specific CD8+ T cells maintain functional exhaustion after antigen reexposure in an acute activation immune environment. Front Immunol 2018;9:219.

[29] Diehl L, Schurich A, Grochtmann R, Hegenbarth S, Chen L, Knolle PA. Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+ T cell tolerance. Hepatology 2008;47:296–305.

[30] Liu J, Jiang M, Ma Z, Dietze KK, Zelinskyy G, Yang D, et al. TLR1/2 ligand-stimulated mouse liver endothelial cells secrete IL-12 and trigger CD8+ T cell immunity in vitro. J Immunol 2013;191:6178–6190.

[31] Liu J, Yu Q, Wu W, Huang X, Broering R, Werner M, et al. TLR2 stimulation strengthens intrahepatic myeloid-derived cell-mediated T cell tolerance through inducing Kupffer cell expansion and IL-10 production. J Immunol 2018;200:2341–2351.

[32] Mou P, Zeng Z, Li Q, Liu X, Xin X, Wannemacher KM, et al. Identification of a calmodulin-binding domain in Sema4D that regulates its exodomain shedding in platelets. Blood 2013;121:4221–4230.

[33] Xu L, Yin W, Sun R, Wei H, Tian Z. Kupffer cell-derived IL-10 plays a key role in maintaining humoral immune tolerance in hepatitis B virus-persistent mice. Hepatology 2014;59:443–452.

[34] Basile JR, Holmbeck K, Bugge TH, Gutkind JS. MT1-MMP controls tumor-induced angiogenesis through the release of semaphorin 4D. J Biol Chem 2007;282:6899–6905.

[35] Zhu L, Bergmeier W, Wu J, Jiang H, Stalker TJ, Cieslak M, et al. Regulated surface expression and shedding support a dual role for semaphorin 4D in platelet responses to vascular injury. Proc Natl Acad Sci U S A 2007;104:1621–1626.

[36] Ke Y, Dang E, Shen S, Zhang T, Qiao H, Chang Y, et al. Semaphorin4D drives CD8(+) T-cell lesional trafficking in oral lichen planus via CXCL9/CXCL10 upregulations in oral keratinocytes. J Invest Dermatol 2017;137:2396–2406.

[37] Cui J, Chen S, Zhang C, Meng F, Wu W, Hu R, et al. Inhibition of MMP-9 by a selective gelatinase inhibitor protects neurovasculature from embolic focal cerebral ischemia. Mol Neurodegener 2012;7:21.

[38] Kruger A, Arlt MJ, Gerg M, Kopitz C, Bernardo MM, Chang M, et al. Antimetastatic activity of a novel mechanism-based gelatinase inhibitor. Cancer Res 2005;65:3523–3526.

[39] Koivunen E, Arap W, Valtanen H, Rainisalo A, Medina OP, Heikkilä P, et al. Tumor targeting with a selective gelatinase inhibitor. Nat Biotechnol 1999;17:768–774.

[40] Hall KT, Boumsell L, Schultze JL, Boussiotis VA, Dorfman DM, Cardoso AA, et al. Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. Proc Natl Acad Sci U S A 1996;93:11780–11785.

[41] Watanabe C, Kumanogoh A, Shi W, Suzuki K, Yamada S, Okabe M, et al. Enhanced immune responses in transgenic mice expressing a truncated form of the lymphocyte semaphorin CD100. J Immunol 2001;167:4321–4328.

[42] Knolle PA, Bottcher J, Huang LR. The role of hepatic immune regulation in systemic immunity to viral infection. Med Microbiol Immunol 2015;204:21–27.

[43] He Y, Guo Y, Zhou Y, Zhang Y, Fan C, Ji G, et al. CD100 up-regulation induced by interferon-alpha on B cells is related to hepatitis C virus infection. PLoS ONE 2014;9 e113338.

[44] Besliu A, Banica L, Predeteanu D, Vlad V, Ionescu R, Pistol G, et al. Peripheral blood lymphocytes analysis detects CD100/SEMA4D alteration in systemic sclerosis patients. Autoimmunity 2011;44:427–436.

[45] Giraudon P, Vincent P, Vuaillat C, Verlaeten O, Cartier L, Marie-Cardine A, et al. Semaphorin CD100 from activated T lymphocytes induces process extension collapse in oligodendrocytes and death of immature neural cells. J Immunol 2004;172:1246–1255.

[46] Delaire S, Billard C, Tordjman R, Chédotal A, Elhabazi A, Bensussan A, et al. Biological activity of soluble CD100. II. Soluble CD100, similarly to H-SemaIII, inhibits immune cell migration. J Immunol 2001;166:4348–4354.

[47] Herold C, Elhabazi A, Bismuth G, Bensussan A, Boumsell L. CD100 is associated with CD45 at the surface of human T lymphocytes. Role in T cell homotypic adhesion. J Immunol 1996;157:5262–5268.

[48] Xing Y, Shepherd N, Lan J, Li W, Rane S, Gupta SK, et al. MMPs/TIMPs imbalances in the peripheral blood and cerebrospinal fluid are associated with the pathogenesis of HIV-1-associated neurocognitive disorders. Brain Behav Immun 2017;65:161–172.

[49] Singh H, Nain S, Krishnaraj A, Lata S, Dhole TN. Genetic variation of matrix metalloproteinase enzyme in HIV-associated neurocognitive disorder. Gene 2019;698:41–49.

[50] Nunez O, Fernandez-Martinez A, Majano PL, Apolinario A, Gómez-Gonzalo M, Benedicto I, et al. Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. Gut 2004;53:1665–1672.

[51] Bieche I, Asselah T, Laurendeau I, Vidaud D, Degot C, Paradis V, et al. Molecular profiling of early stage liver fibrosis in patients with chronic hepatitis C virus infection. Virology 2005;332:130–144.

[52] Asselah T, Bieche I, Laurendeau I, Paradis V, Vidaud D, Degott C, et al. Liver gene expression signature of mild fibrosis in patients with chronic hepatitis C. Gastroenterology 2005;129:2064–2075.

[53] Higashiyama R, Inagaki Y, Hong YY, Kushida M, Nakao S, Niioka M, et al. Bone marrow-derived cells express matrix metalloproteinases and contribute to regression of liver fibrosis in mice. Hepatology 2007;45:213–222.

[54] Meier RP, Mahou R, Morel P, Meyer J, Montanari E, Muller YD, et al. Microencapsulated human mesenchymal stem cells decrease liver fibrosis in mice. J Hepatol 2015;62:634–641.

[55] Winwood PJ, Schuppan D, Iredale JP, Kawser CA, Docherty AJ, Arthur MJ. Kupffer cell-derived 95-kd type IV collagenase/gelatinase B: characterization and expression in cultured cells. Hepatology 1995;22:304–315.

[56] Feng M, Ding J, Wang M, Zhang J, Zhu X, Guan W. Kupffer-derived matrix metalloproteinase-9 contributes to liver fibrosis resolution. Int J Biol Sci 2018;14:1033–1040.

[57] Jiao J, Sastre D, Fiel MI, Lee UE, Ghiassi-Nejad Z, Ginhoux F, et al. Dendritic cell regulation of carbon tetrachloride-induced murine liver fibrosis regression. Hepatology 2012;55:244–255.

[58] Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 2004;4:617–629.

[59] Butler GS, Overall CM. Matrix metalloproteinase processing of signaling molecules to regulate inflammation. Periodontol 2000;2013 (63):123–148.

[60] Nissinen L, Kahari VM. Matrix metalloproteinases in inflammation. Biochim Biophys Acta 2014;1840:2571–2580.

[61] Sitia G, Isogawa M, Iannacone M, Campbell IL, Chisari FV, Guidotti LG. MMPs are required for recruitment of antigen-nonspecific mononuclear cells into the liver by CTLs. J Clin Invest 2004;113:1158–1167.

[62] Pan C, Baumgarth N, Parnes JR. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. Immunity 1999;11:495–506.

[63] Zhu L, Stalker TJ, Fong KP, Jiang H, Tran A, Crichton I, et al. Disruption of SEMA4D ameliorates platelet hypersensitivity in dyslipidemia and confers protection against the development of atherosclerosis. Arterioscler Thromb Vasc Biol 2009;29:1039–1045.

[64] Iannacone M, Sitia G, Isogawa M, Marchese P, Castro MG, Lowenstein PR, et al. Platelets mediate cytotoxic T lymphocyte-induced liver damage. Nat Med 2005;11:1167–1169.

[65] Guidotti LG, Inverso D, Sironi L, Di Lucia P, Fioravanti J, Ganzer L, et al. Immunosurveillance of the liver by intravascular effector CD8(+) T cells. Cell 2015;161:486–500.

[66] Smith ES, Jonason A, Reilly C, Veeraraghavan J, Fisher T, Doherty M, et al. SEMA4D compromises blood-brain barrier, activates microglia, and inhibits remyelination in neurodegenerative disease. Neurobiol Dis 2015;73:254–268.

[67] LaGanke C, Samkoff L, Edwards K, Jung Henson L, Repovic P, Lynch S, et al. Safety/tolerability of the anti-semaphorin 4D antibody VX15/2503 in a randomized phase 1 trial. Neurol Neuroimmunol Neuroinflamm 2017;4 e367.