

## Supplementary Information

### Hepatitis B virus promotes liver cancer by modulating the immune response to environmental carcinogens

Mei Huang<sup>1,2,3,8</sup>, Dongyao Wang<sup>1,2,4,8</sup>, Jiao Huang<sup>1,2</sup>, An-Na Bae<sup>5</sup>, Yun Xia<sup>1,2</sup>, Xutu Zhao<sup>1,2</sup>, Mahsa Mortaja<sup>1,2</sup>, Marjan Azin<sup>1,2</sup>, Michael R. Collier<sup>6</sup>, Yevgeniy R. Semenov<sup>6,7</sup>, Jong Ho Park<sup>1,2,5\*</sup>, and Shadmehr Demehri<sup>1,2,9\*</sup>

<sup>1</sup>Center for Cancer Immunology, Krantz Family Center for Cancer Research, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

<sup>2</sup>Cutaneous Biology Research Center, Department of Dermatology, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

<sup>3</sup>Department of General Surgery, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China

<sup>4</sup>Department of Hematology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, China

<sup>5</sup>Department of Anatomy, School of Medicine, Keimyung University, Daegu, South Korea.

<sup>6</sup>Department of Dermatology, Massachusetts General Hospital and Harvard Medical School. Boston, MA, USA.

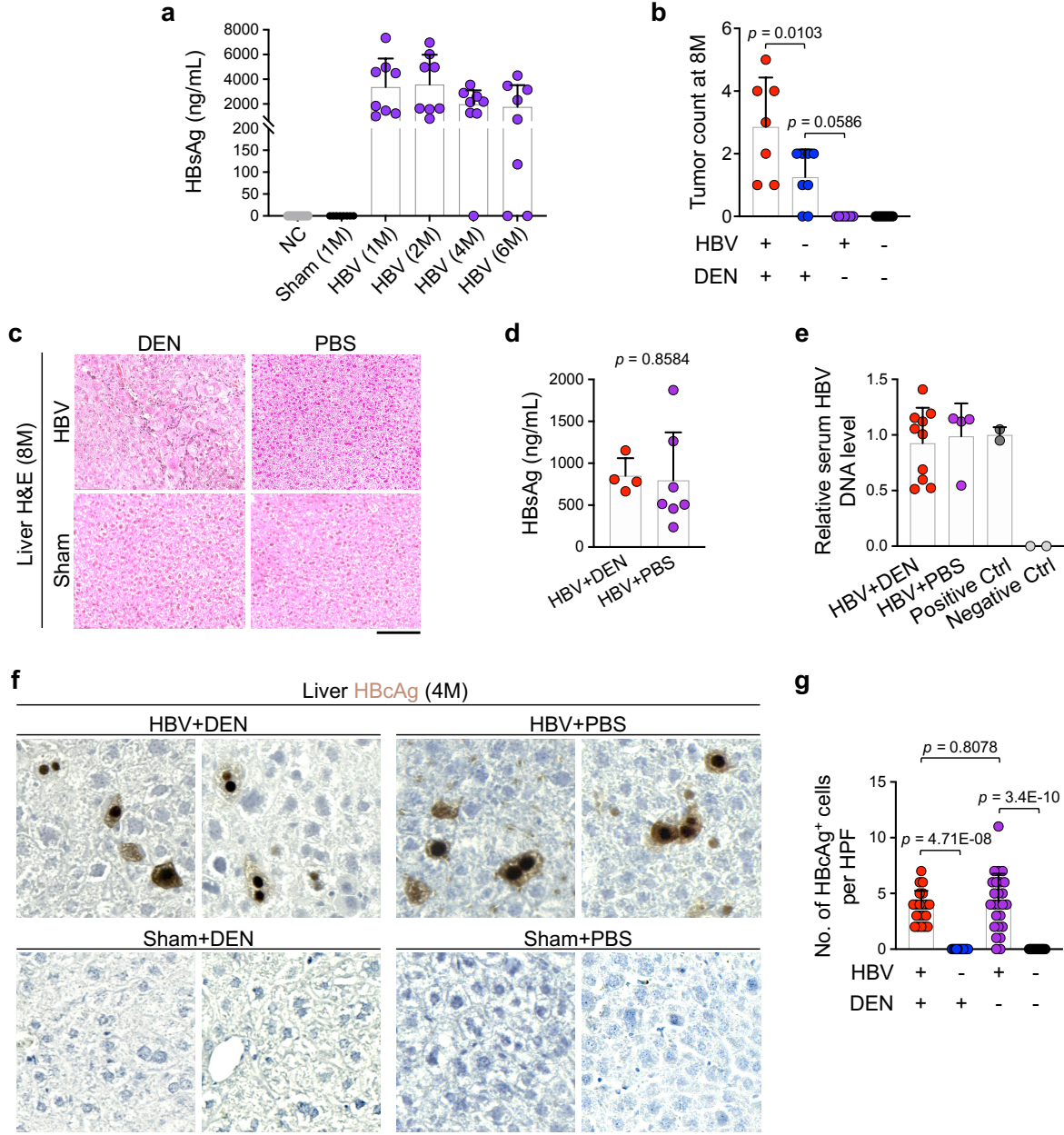
<sup>7</sup>Laboratory of Systems Pharmacology, Harvard Program in Therapeutic Science, Harvard Medical School, Boston, USA.

<sup>8</sup>These authors contributed equally

<sup>9</sup>Lead author

\* Authors for correspondence: [jpark@dsmc.or.kr](mailto:jpark@dsmc.or.kr) (JHP), [sdemehri1@mgh.harvard.edu](mailto:sdemehri1@mgh.harvard.edu) (SD)

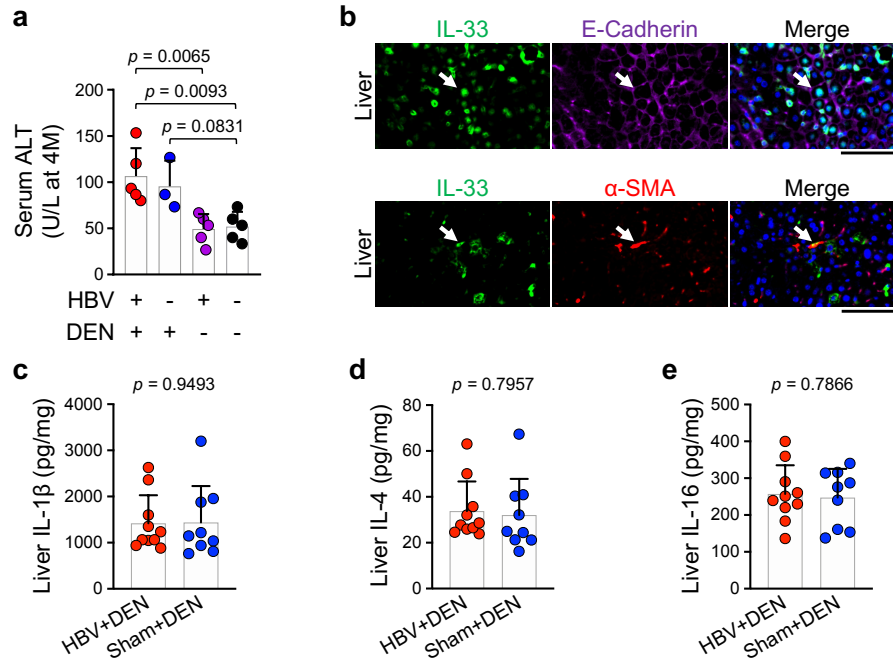
## Supplementary Figures



**Supplementary Fig. 1, related to Fig. 1.**

**Persistent liver infection after pAAV-HBV hydrodynamic injection and liver cancer induced by HBV+DEN treatment.** **a** Serum HBsAg levels in WT mice at 1, 2, 4, and 6 months after hydrodynamic injection of pAAV-HBV plasmid. NC, negative control; pAAV-sham was used as Sham control (n = 8 mice per group). Each dot represents a mouse. **b** Liver tumor counts in WT

mice that underwent liver carcinogenesis protocol at 8 months post-infection. n = 7 mice in HBV+DEN group, n = 8 mice in Sham+DEN group, n = 7 mice in HBV+PBS group, and n = 7 mice in Sham+PBS group. Each dot represents a mouse. **c** Representative images of H&E stained liver from WT mice that underwent liver carcinogenesis protocol at 8 months post-infection. **d** Serum HBsAg levels in HBV+DEN- (n = 4) and HBV+PBS-treated (n = 7) WT mice at 8 months after hydrodynamic injection of pAAV-HBV plasmid. Each dot represents a mouse. **e** HBV DNA levels in the serum of mice treated with HBV+DEN (n = 10) and HBV+PBS (n = 4) at 4 months post-hydrodynamic pAAV-HBV1.2 injection. HBV DNA positive and negative control samples are provided in the detection kit. Each dot represents a mouse. **f** Representative images of hepatitis B core antigen (HBcAg) stained liver tissues from HBV+DEN-, HBV+PBS-, Sham+DEN-, and Sham+PBS-treated mice at 4 months post-hydrodynamic pAAV-HBV1.2 injection. **g** Quantification of HBcAg<sup>+</sup> cells per high power field (HPF) in WT mice at 4 months after the hydrodynamic injection of pAAV-HBV plasmid. HBcAg<sup>+</sup> cells were counted in 5 to 10 randomly selected HPFs per liver. n = 4 mice in HBV+DEN group, n = 3 mice in Sham+DEN group, n = 3 mice in HBV+PBS group, and n = 3 mice in Sham+PBS group. Each dot represents one HPF. Graphs show mean + SD, **b**, **g**: one-way ANOVA with Tukey's multiple comparison test, **d**: two-sided unpaired *t*-test, scale bar: 100  $\mu$ m. Source data are provided as a Source Data file.

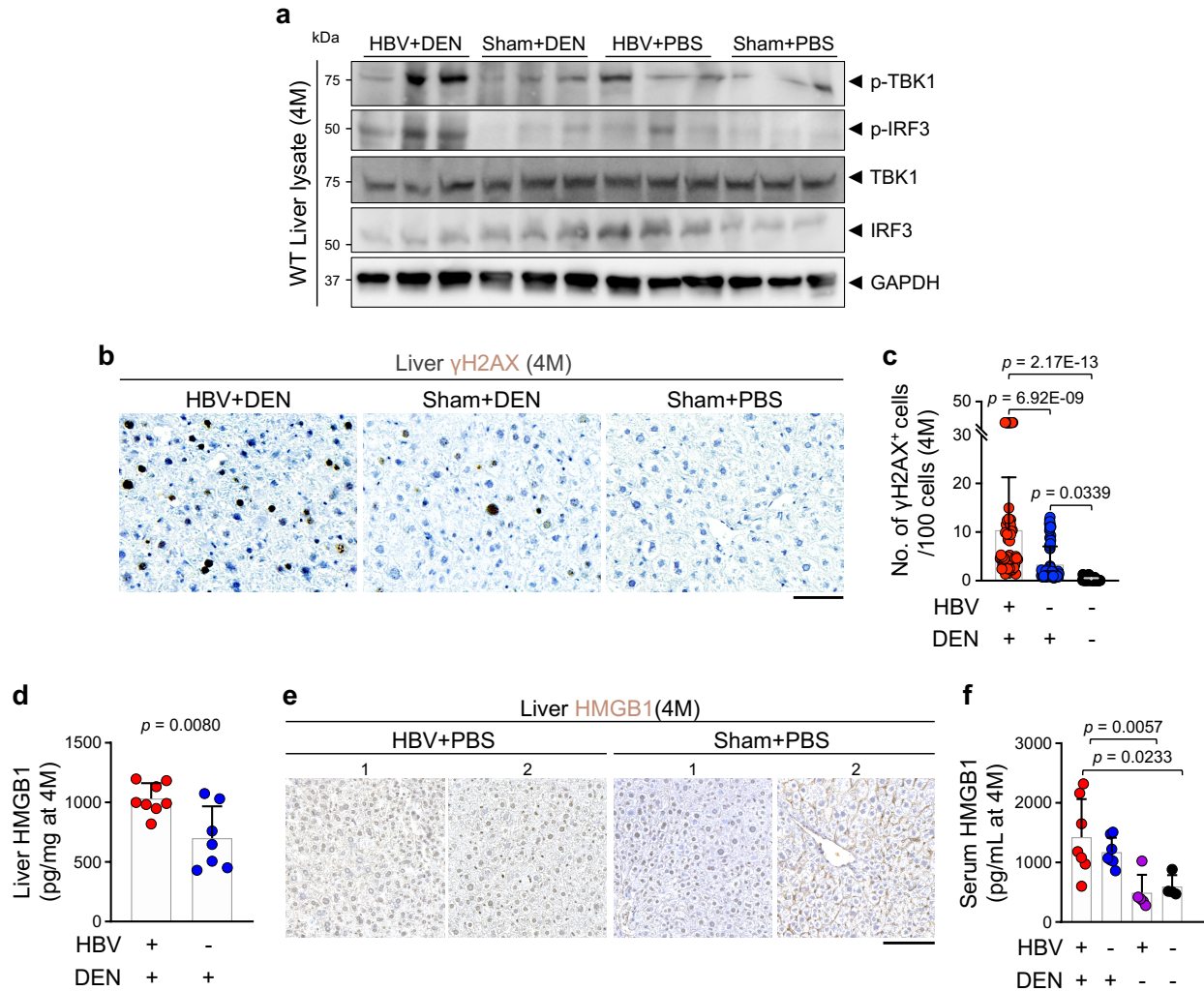


**Supplementary Fig. 2, related to Fig. 2.**

**IL-33 cytokine is upregulated upon liver inflammation induced by HBV plus carcinogen**

**treatment. a** Quantification of serum ALT levels in WT mice that underwent liver carcinogenesis protocol at 4 months post-infection.  $n = 5$  mice in HBV+DEN group,  $n = 3$  mice in Sham+DEN group,  $n = 5$  mice in HBV+PBS group, and  $n = 5$  mice in Sham+PBS group. Each dot represents a mouse. **b** (top) Representative image of IL-33 and E-cadherin stained liver treated with carcinogenesis protocol at 4 months post-infection. The arrow points to an IL-33-expressing hepatocyte. (bottom) Representative image of IL-33 and  $\alpha$ -SMA stained liver treated with carcinogenesis protocol at 4 months post-infection. The arrow points to an IL-33-expressing hepatic stellate cell. **c** IL-1 $\beta$  protein levels in HBV+DEN- ( $n = 10$  mice) versus Sham+DEN-treated liver ( $n = 9$  mice) at 4 months post-infection. Each dot represents a mouse. **d** IL-4 protein levels in HBV+DEN- ( $n = 10$  mice) versus Sham+DEN-treated liver ( $n = 9$  mice) at 4 months post-infection. Each dot represents a mouse. **e** IL-16 protein levels in HBV+DEN- ( $n = 10$  mice) versus Sham+DEN-treated liver ( $n = 9$  mice) at 4 months post-infection. Each dot represents a mouse.

Graphs show mean + SD, **a**: one-way ANOVA with Tukey's multiple comparison test, **c, d, e**: two-sided unpaired *t*-test. Source data are provided as a Source Data file.

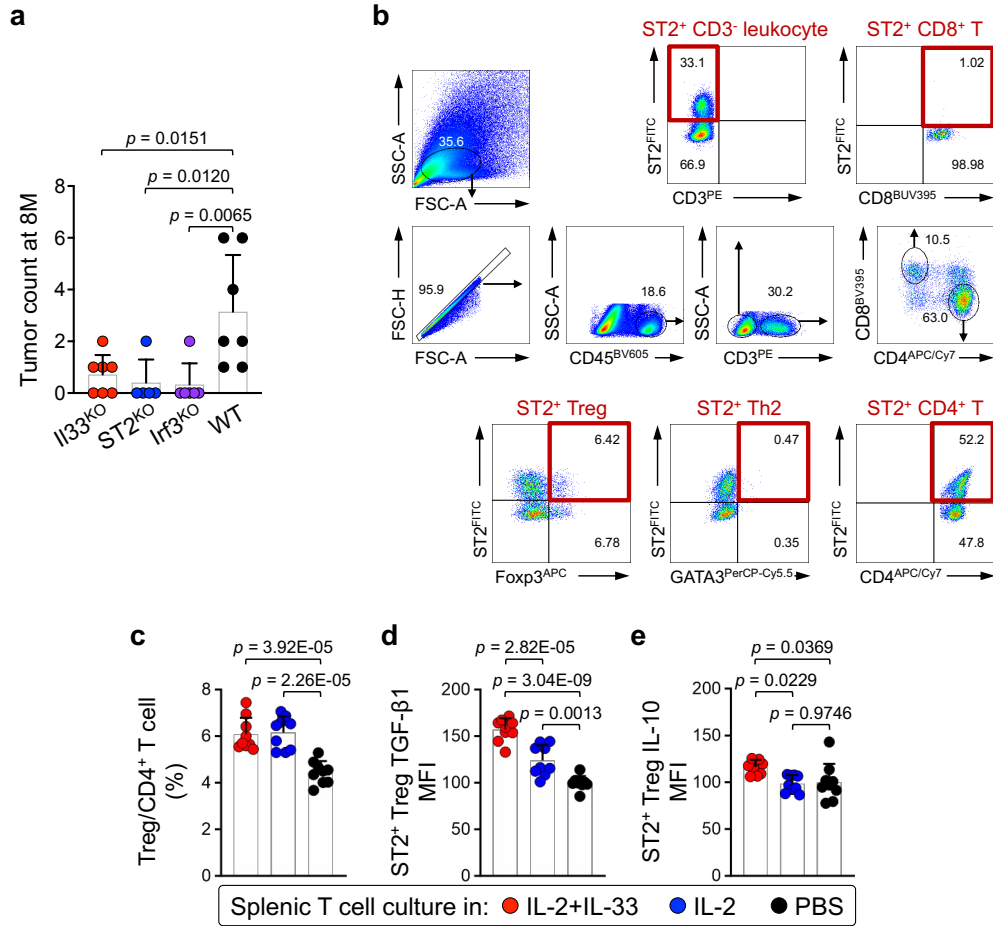


**Supplementary Fig. 3, related to Fig. 2.**

**Hepatocytes experience increased DNA damage and immunogenic cell death after HBV plus carcinogen treatment.**

**a** Immunoblot of p-TBK1, p-IRF3, TBK1, IRF3, and GAPDH proteins in WT liver that received liver carcinogenesis protocol at 4 months post-infection (n = 3 mice in each group). The samples were derived from the same experiment but different gels for p-TBK1, another for p-IRF3, another for TBK1, another for IRF3, and another for GAPDH were processed in parallel. **b** Representative images of  $\gamma$ H2AX stained HBV+DEN-, Sham+DEN-, and Sham+PBS-treated liver at 4 months post-infection. **c** Quantification of  $\gamma$ H2AX<sup>+</sup> cells per 100 cells in the HBV+DEN (n = 7 mice) compared with Sham+DEN- (n = 8 mice) and Sham+PBS-treated

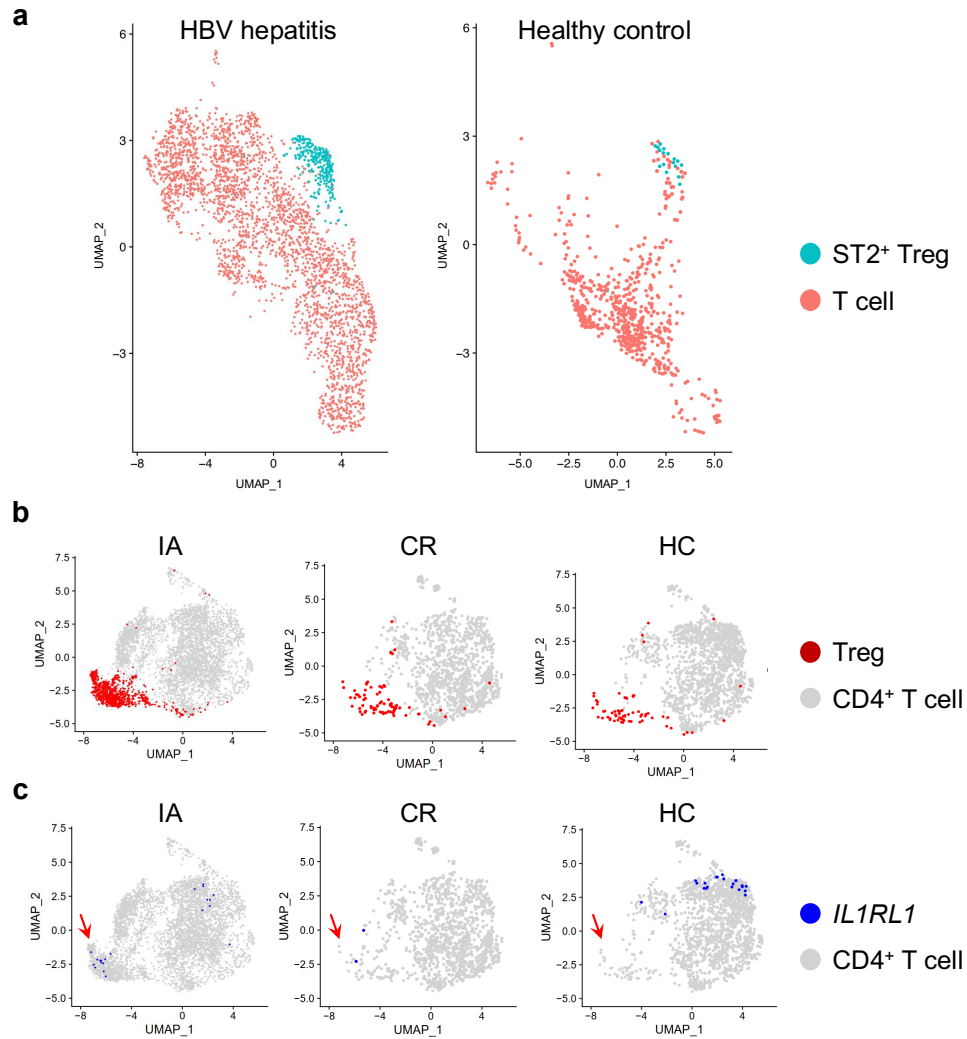
liver (n = 6 mice) at 4 months post-infection.  $\gamma$ H2AX<sup>+</sup> cells per 100 cells were counted in five to ten randomly selected HPF images per liver. Each dot represents an HPF image. **d** HMGB1 levels in the liver of HBV+DEN- (n = 8) versus Sham+DEN-treated (n = 7) WT mice at 4 months post-infection. Each dot represents a mouse. **e** Representative images of HMGB1 stained HBV+PBS- and Sham+PBS-treated liver at 4 months post-infection. **f** Serum HMGB1 levels in WT mice that underwent liver carcinogenesis protocol at 4 months post-infection. n = 7 mice in HBV+DEN group, n = 7 mice in Sham+DEN group, n = 5 mice in HBV+PBS group, and n = 4 mice in Sham+PBS group. Each dot represents a mouse. Graphs show mean + SD, **c** and **f**: one-way ANOVA with Tukey's multiple comparison test, **d**: two-sided unpaired *t*-test, scale bars: 100  $\mu$ m. Source data are provided as a Source Data file.



**Supplementary Fig. 4, related to Fig. 3.**

**Tumor counts, flow gating strategy, and cytokine expression in splenic Tregs.** **a** Liver tumor counts in Il33<sup>KO</sup> (n = 7), ST2<sup>KO</sup> (n = 5), Irf3<sup>KO</sup> (n = 6), and WT (n = 7) mice that received HBV+DEN at 8 months post-infection. Each dot represents a mouse. **b** Flow gating strategy for immune cell types in mouse tissues (refer to Fig. 3e, 3f, and Supplementary Fig. 4c). Numbers on the flow plots represent % cells in the gate. **c** Splenic Treg frequency as % total CD4<sup>+</sup> T cells in WT spleen after incubation with IL-2 plus IL-33, IL-2, versus no cytokine treatment (PBS) control (n = 9 culture wells per condition). Each dot represents a culture well. **d** TGF-β1 MFI of IL-2 plus IL-33, IL-2, versus PBS-treated ST2<sup>+</sup> Tregs (n = 9 culture wells per condition). Each dot represents a culture well. **e** IL-10 MFI of IL-2 plus IL-33, IL-2, versus PBS-treated ST2<sup>+</sup> Tregs (n = 9 culture wells per

condition). Each dot represents a culture well. Graphs show mean + SD, **a**, **c**, **d**, **e**: one-way ANOVA with Tukey's multiple comparison test. Source data are provided as a Source Data file.



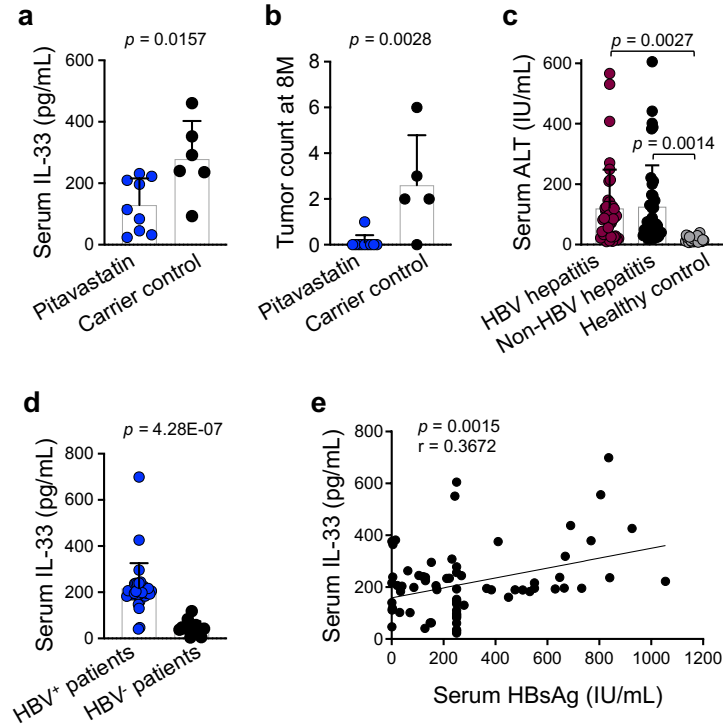
**Supplementary Fig. 5, related to Fig. 3.**

**ST2<sup>+</sup> Tregs are increased in HBV-associated chronic hepatitis in humans.** **a** scRNA-seq UMAP plot of ST2<sup>+</sup> Tregs (cyan dots) among T cells in human liver tissues from HBV-associated chronic hepatitis (n = 13) and healthy controls with no viral markers (n = 3). There are 175 ST2<sup>+</sup> Tregs out of 16021 T cells in HBV hepatitis (1.1%) and 18 ST2<sup>+</sup> Treg cells out of 2178 T cells in healthy control (0.8%). **b** scRNA-seq UMAP plots of Tregs (red dots) among CD4<sup>+</sup> T cells in human liver tissues from HBV-associated hepatitis (immune active or IA, n = 5), chronic resolved hepatitis (CR, HBV DNA level is below the detection limit, n = 3), and HBV-free healthy controls

(HC, n = 6). **c** *IL1RL1* expression highlights ST2<sup>+</sup> CD4<sup>+</sup> T cells (blue dots). The arrow points to the Treg cluster.



cocktail (unstimulated control) from HBV+DEN-treated TregST2<sup>CKO</sup> and WT liver at 4 months post-infection. **e** Representative images of CD44 and CD8 stained TregST2<sup>CKO</sup> and WT liver treated with HBV+DEN at 4 months post-infection. **f** Quantification of CD44<sup>+</sup>CD8<sup>+</sup> T cells in TregST2<sup>CKO</sup> (n = 4) and WT (n = 3) liver treated with HBV+DEN at 4 months post-infection. Note that all CD8<sup>+</sup> cells are CD3<sup>+</sup> T cells. T cells were counted in four randomly selected HPF images per liver sample. Each dot represents an HPF image. Graphs show mean + SD, two-sided unpaired *t*-test, scale bar: 100  $\mu$ m. Source data are provided as a Source Data file.



**Supplementary Fig. 7, related to Fig. 5.**

**IL-33 levels in HBV<sup>+</sup> and HBV<sup>-</sup> patients.** **a** Serum IL-33 levels in pitavastatin- (n = 9) and PBS-treated (n = 6) WT mice that received HBV+DEN at 8 months post-infection. Each dot represents a mouse. **b** Liver tumor counts in pitavastatin- (n = 10) and PBS-treated (n = 5) WT mice that received HBV+DEN at 8 months post-infection. Each dot represents a mouse. **c** Serum ALT levels in HBV<sup>+</sup> hepatitis patients (n = 40), HBV<sup>-</sup> hepatitis patients (n = 40), and healthy controls (n = 24). Each dot represents a patient serum sample. **d** Serum IL-33 levels in HBV<sup>+</sup> (n = 32) and HBV<sup>-</sup> patients (n = 15) with liver diseases (including HCC, benign tumor, hepatic cyst, and liver lipoma). Each dot represents a patient serum sample. **e** The correlation between serum IL-33 and HBsAg levels in HBV<sup>+</sup> patients (n = 72) with liver diseases, including hepatitis, HCC, benign tumors, hepatic cysts, and liver lipoma. Each dot represents a patient. Graphs show mean + SD, **a, b, d**: two-sided unpaired *t*-test, **c**: one-way ANOVA with Tukey's multiple comparison test, **e**: two-sided *t*-test for Pearson correlation coefficient. Source data are provided as a Source Data file.

**Supplementary Tables**

**Supplementary Table 1. Characteristics of pitavastatin and ezetimibe treatment cohorts.**

<b>Baseline Characteristics: Matched Cohorts</b>				
	Pitavastatin		Ezetimibe	
Number of patients	242,645		242,645	
Age at index (years)	Mean	SD	Mean	SD
	63.8	10.9	63.4	11.7
Gender	N	%	N	%
Male	102,968	42.436	101,989	42.032
Female	139,588	57.528	140,591	57.941
Unknown	89	0.037	65	0.027
Race	Mean	%	Mean	%
White	79,571	32.793	76,501	31.528
Black	6,103	2.515	5,829	2.402
Asian	1,624	0.669	876	0.361
American Indian or Native Hawaiian and	0	0	0	0
Unknown	155,347	64.022	159,439	65.709
Ethnicity	Mean	%	Mean	%
Hispanic or Latino	4,433	1.827	4,500	1.855
Not Hispanic or Latino	84,913	34.995	80,960	33.366
Unknown	153,299	63.178	157,185	64.78