International Immunopharmacology 70 (2019) 252–259

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Elevated interleukin-35 suppresses liver inflammation by regulation of T helper 17 cells in acute hepatitis B virus infection

Deng-Ke Teng[a,1], Yi Liu[b,1], Yi-Fei Lv[c], Li Wang[d], Wei Zhang[e], Jiu-Ping Wang[e,*], Yu Li[f,**]

[a] Department of Ultrasound, China-Japan Union Hospital of Jilin University, Changchun, Jilin Province 130033, China
[b] Department of Oncology, Shaanxi Provincial People's Hospital and The Affiliated Hospital of Xi'an Medical University, Xi'an, Shaanxi Province 710068, China
[c] Department of Gastroenterology, Shaanxi Provincial People's Hospital and The Affiliated Hospital of Xi'an Medical University, Xi'an, Shaanxi Province 710068, China
[d] Department of Neurology, Shaanxi Provincial People's Hospital and The Affiliated Hospital of Xi'an Medical University, Xi'an, Shaanxi Province 710068, China
[e] Department of Infectious Diseases, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province 710032, China
[f] Department of Infectious Diseases, Shaanxi Provincial People's Hospital and The Affiliated Hospital of Xi'an Medical University, Xi'an, Shaanxi Province 710068, China

ARTICLE INFO

Keywords:
Hepatitis B virus
Interleukin-35
T helper 17 cells
Liver inflammation
Acute infection

ABSTRACT

Interleukin (IL)-35 is a responsive anti-inflammatory cytokine implicated in different diseases processes. It has been reported that elevated IL-35 contributed to immunosuppression in chronic hepatitis by modulation of T helper 17 (Th17) and regulatory T cells. However, the role of IL-35 in acute hepatitis B (AHB) was still not completely elucidated. Thus, in the present study, we analyzed the expression and regulatory activity of IL-35 to Th17 cells and inflammatory response during acute hepatitis B virus (HBV) infection in both peripheral blood cells isolated from AHB patients and in hydrodynamic induced HBV-infected mouse model. Plasma IL-35 level and circulating HBV peptides-induced Th17 frequency was significantly elevated in AHB patients, and IL-35 expression negatively correlated with liver inflammation. *In vitro* IL-35 stimulation to $CD4^+$ T cells purified from AHB patients down-regulated HBV peptides-induced Th17-phenotype, which presented as reduced IL-17 and IL-22 production. *In vivo* IL-35 administration dampened liver inflammation in HBV plasmid injected mice, however, did not affect HBV antigens production. This process was accompanied by suppression of natural killer cells and down-regulation of HBV peptides-induced Th17 cells in the liver, but did not affect total intrahepatic lymphocytes and other cell subsets numbers or chemokines expression in the liver. In conclusion, the current data indicated that IL-35 might be a novel mediator associated with hepatocytes damage and liver inflammation by regulating HBV peptides-induced Th17 cells during acute HBV infection. The potential anti-inflammatory property of IL-35 might be pivotal for developing new therapeutic approaches for hepatitis B.

1. Introduction

Hepatitis B virus (HBV) infection is one of the major global health problems, with approximate 2 billion current and past exposure to HBV all over the world [1]. The clinical outcome of HBV infection is mainly affected by the interaction between viral replication and host immune response [2–4]. Cellular and humoral immune response is weak or undetectable in chronic hepatitis B, leading to a state of relative collapse of HBV specific adaptive immunity which was responsible for viral persistence [5]. In contrast, acute HBV infection could activate the immune system and induce extensive inflammatory response [6]. Multi-specific T cell responses and cytokine-mediated immune responses contribute to controlling of infection and accelerating hepatocytes injury [7,8]. However, the roles of various cytokines in different phase of HBV infection and cytokine-related mechanisms for viral inhibition and liver damage are still not fully elucidated.

Interleukin (IL)-35 is a novel member of IL-12 cytokine family, and is composed of IL-12 p35 subunit and Epstein-Barr virus-induced gene 3 [9]. IL-35 is reported as an anti-inflammatory cytokine [10,11] and an immunomodulator in autoimmune disorders [12] with therapeutic effect through expansion of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) and suppression of T helper 17 (Th17) cells [13]. Chronic HBV

* Correspondence to: J.-P. Wang, Department of Infectious Diseases, Xijing Hospital, Fourth Military Medical University, 15 West Changle Rd, Xi'an, Shaanxi Province 710032, China.
** Correspondence to: Y. Li, Department of Infectious Diseases, Shaanxi Provincial People's Hospital and The Affiliated Hospital of Xi'an Medical University, 256 West Youyi Rd, Xi'an, Shaanxi Province 710068, China.
E-mail addresses: jpwang@fmmu.edu.cn (J.-P. Wang), drlee2810@126.com (Y. Li).
[1] Deng-Ke Teng and Yi Liu contributed equally to this work.

https://doi.org/10.1016/j.intimp.2019.02.048
Received 14 January 2019; Received in revised form 10 February 2019; Accepted 26 February 2019
Available online 06 March 2019
1567-5769/

infection always results in the elevation of serum IL-35 and IL-35 mRNA expression in $CD4^+$ T cells [14–16]. Importantly, IL-35 not only regulates HBV replication [16,17] but also plays an immunosuppressive function through increasing suppressive activity of Tregs and dampening $CD8^+$ T cell response during chronic hepatitis B [14,18]. However, few studies focused on the expression and function of IL-35 in acute hepatitis B (AHB) and IL-35 activity on liver microenvironment. Th17 cells and the secreting cytokines (IL-17 and IL-22) contribute to liver inflammation during HBV infection, especially in AHB patients [19,20]. Based on the modulatory mechanism of IL-35 on Th17 cells, we hypothesized that IL-35 performed an important regulatory role for Th17 cells to suppress hepatic injury in acute HBV infection. To test this possibility, we examined the non-specific and HBV peptides-induced Th17 response and IL-35 expression as well as their relationship to liver inflammation *in vitro* and *in vivo*.

2. Materials and methods

2.1. Enrolled subjects

This study examined 21 cases of acute HBV infected patients as well as 21 sex- and age-matched healthy controls. All acute hepatitis B patients were positive for HBV core antibody IgM, and were negative for HBV surface antigen (HBsAg) six months post baseline enrollments. All patients were excluded for other chronic virus infection (hepatitis C virus, hepatitis D virus, and human immunodeficiency virus), other chronic liver diseases, or autoimmune disorders. The clinical and virological characteristics of enrolled subjects were shown in Table 1. The study protocol was approved by the Clinical Research Ethics Committee of Shaanxi Provincial People's Hospital and Xijing Hospital. Informed consent was obtained from each subjects.

2.2. Plasma and peripheral blood mononuclear cells (PBMCs) preparation

20 ml of EDTA anti-coagulant peripheral bloods were collected from each subject. Peripheral bloods were centrifugated at 1000 ×*g* for 10 min for plasma isolation. PBMCs were then isolated by using Ficoll paque Premium (GE Healthcare Bio-Sciences AB, Bjokgatan, Uppsala, Sweden).

2.3. $CD4^+$ T cells purification

$CD4^+$ T cells were purification from PBMCs by using $CD4^+$ T Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany). The purity of enriched $CD4^+$ T cells was > 95% by flow cytometry determination. Enriched human $CD4^+$ T cells were stimulated with recombinant human IL-35 (1 ng/ml; Peprotech, Rocky Hill, NJ, USA) in combination with HBV envelope peptide pool (15 amino acids of each peptide with 5 amino acids overlapping, 2.5 μg/ml) for 12 h [20,21]. The HBV envelope peptide pool was kindly provided by Dr. Jian-Qi Lian and Dr. Ye Zhang (Center for Infectious Diseases, Tangdu Hospital, Fourth Military Medical University).

Table 1
Clinical and virological characteristics of enrolled subjects.

	Healthy control (n = 21)	AHB (n = 21)
Age, years	29.86 ± 4.84	28.57 ± 9.69
Male/female	13/8	14/7
HBcAb IgM positive	0	21
HBV DNA, $\log_{10}$IU/ml	Not detectable	5.09 ± 0.87
ALT, IU/l	25.33 ± 6.75	975 [773, 1497]*
T-BIL, mmol/l	16.99 ± 5.09	216.7 [124.7, 268.6]*

* $P < 0.0001$.

2.4. Mice and challenges

Six to eight week-old male BALB/c ($H\text{-}2^d$) mice were purchased from Experimental Animal Center of China-Japan Union Hospital of Jilin University. The animal experiment protocols were performed in accordance with the procedures approved by Animal Care and Use Committee of Jilin University. Mice were challenged using a hydrodynamic transfection protocol, whereby a total of 10 μg pHBV1.3 plasmid (kindly provided by Dr. Michael D. Robek, Albany Medical College) with recombinant mouse IL-35 (AdipoGen, Liestal, Switzeland; 0.75 μg per mouse) or mouse IL-17 antibody (R&D systems, Minneapolis, MN, USA, Catalog# MAB421; 100 μg per mouse), was injected into veins in a volume of PBS equal to 9% of mouse body mass [21,22]. Mouse IL-17 antibody was confirmed to present neutralizing activity in previous study [23]. Blood samples were collected at different time points post-transfection. Livers were also harvested for further experiments.

2.5. Intrahepatic lymphocytes (IHLs) preparation

The IHLs were purified from the liver of mouse as described previously [21,22].

2.6. Virological and biochemical assessments

HBV DNA was quantified using a real-time PCR kit (Da'an Gene, Guangzhou, Guangdong Province, China) with a detection limit of 2 $\log_{10}$IU/ml. HBsAg and HBeAg was determined by commercial enzyme linked immunosorbent assay (ELISA) kits (Kehua Biotech, Shanghai, China). Alanine aminotransferase (ALT) and total bilirubin (T-BIL) level was measured using an automatic analyzer (Hitachi 7170A, Hitachi, Tokyo, Japan).

2.7. ELISA

Levels of cytokine production in plasma/serum and in cultured supernatants were determined by commercial ELISA kits (ColorfulGene, Wuhan, Hubei Province, China; R&D systems) according to the manufacturer's instructions.

2.8. Flow cytometry

PBMCs were stimulated with either phorbol myristate acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml) for 6 h or HBV envelope peptide pool (2.5 μg/ml) in the presence of monensin (10 μg/ml) for 12 h. Anti-human CD3 FITC (BD Bioscience, San Jose, CA, USA) and anti-human CD4 PerCP (BD Bioscience) were used for surface staining, and anti-human IL-17 PE (BD Bioscience) was used for intracellular staining. Purified IHLs were stimulated with HBV envelope peptide pool (2.5 μg/ml) in the presence of monensin (10 μg/ml) for 12 h. Anti-mouse CD3 PerCP Cy5.5 (eBioscience, San Diego, CA, USA), anti-mouse CD4 FITC (eBioscience), anti-mouse CD8 PE Texas Red (eBioscience), anti-mouse NK1.1 PE Cy7 (eBioscience) were used for surface staining, and anti-mouse IL-17 APC (eBioscience) was used for intracellular staining. Data were acquired using a FACS Aria II flow cytometer (BD Bioscience), and were analyzed using FlowJo software version 8.6 (Tree Star Inc., Ashland, OR, USA).

2.9. Cellular proliferation assay

Cellular proliferation was performed using Cell Counting Kit-8 (CCK-8; Beyotime, Wuhan, Hubei Province, China). The known number of $CD4^+$ T cells was used for setting up standard curve, and cell number was then calculated.

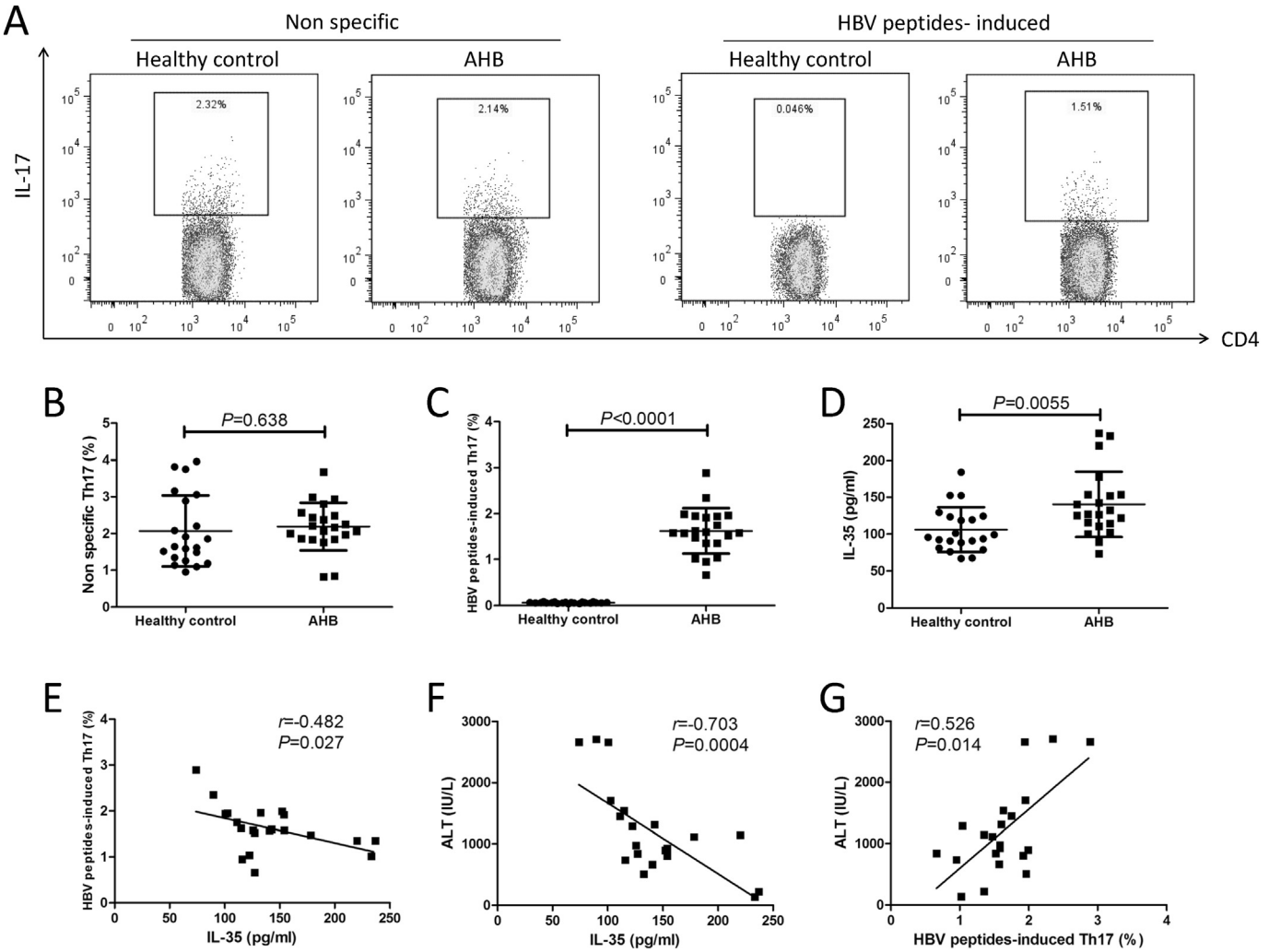

Fig. 1. Th17 cells and IL-35 expression in AHB and healthy controls. PBMCs and plasma were isolated from AHB patients (n = 21) and healthy controls (n = 21). PBMCs were stimulated with either PMA + inonmycin (for non-specific analysis) or HBV envelope peptide pool (for HBV peptides-induced analysis), and the percentage of $CD3^+CD4^+IL\text{-}17^+$ Th17 subset was investigated by flow cytometry. IL-35 concentration in the plasma was measured by ELISA. (A) The representative flow dots of non-specific and HBV peptides-induced Th17 cells of healthy control and AHB patients were shown. The percentage of (B) non-specific Th17 cells and (C) HBV peptides-induced Th17 cells, as well as (D) plasma IL-35 expression was compared between healthy controls and AHB patients. Horizontal bars indicated the mean values, while error bars indicated the SD. The individual level for each subject was shown. Significance was calculated using Student t-test. Correlation analysis of (E) IL-35 with HBV peptides-induced Th17, (F) IL-35 with serum ALT, and (G) HBV peptides-induced Th17 with serum ALT was calculated using Pearson or Spearman correlation analysis in 21 patients with AHB.

2.10. Histopathological examination

Livers were fixed in 10% neutral buffered formalin, processed, embedded in paraffin, sliced at 5 μm, and stained with hematoxylin and eosin (H&E) using routine methods.

2.11. Real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from liver of the mice using RNeasy Minikit (Qiagen, Hilden, Germany). RNA was reversely transcribed with random hexamers using PrimeScript RT Master Mix (TaKaRa, Beijing, China). Real-time PCR was performed using TB Green Premix Ex *Taq* II (TaKaRa). Relative CXCL9 and CXCL10 gene expression was semi-quantified by $2^{-\Delta\Delta CT}$ method using ABI 7500 System Sequence Detection Software (Applied Biosystems, Foster, CA, USA). The sequences of the primers were used as described previously [21].

2.12. Statistical analyses

All data were analyzed using SPSS version 19.0 for Windows (Chicago, IL, USA). Shapiro-Wilk test was used for normal distribution assay. Variables following normal distribution were presented as mean ± standard deviation (SD), and statistical significance was determined by Student t-test, paired t-test, One-way ANOVA, or SNK-q test. Variables following skewed distribution were presented as median [Q1, Q3]. Pearson or Spearman correlation analysis was performed for correlation analysis. P values of < 0.05 were considered to indicate significant differences.

3. Results

3.1. Elevated plasma IL-35 expression negatively correlated with HBV peptides-induced Th17 cells in AHB patients

The non-specific (PMA and ionomycin stimulation) and HBV peptides-induced (HBV envelope peptide pool stimulation) Th17 cell

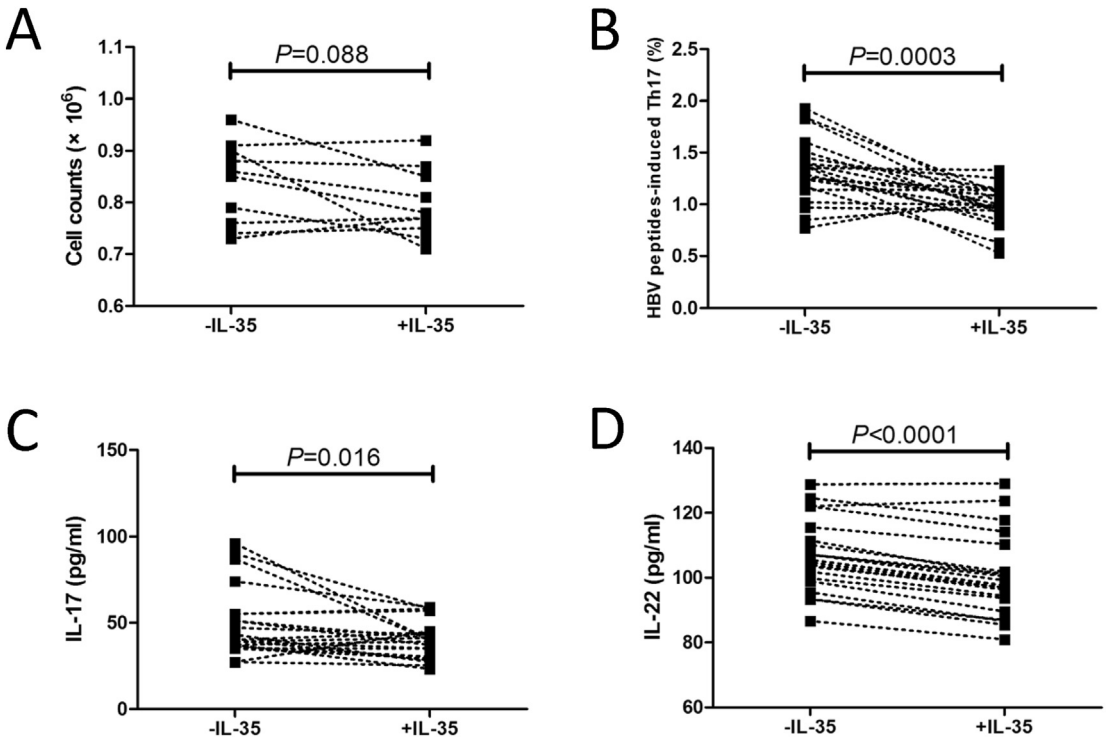

Fig. 2. Th17 cell percentage and Th17 cytokine production by $CD4^+$ T cells from AHB patients. $CD4^+$ T cells were purified from AHB patients (n = 21), and were stimulated with HBV envelope peptide pool in the presence or absence of recombinant human IL-35. (A) Cellular proliferation was assessed by CCK-8 in ten of AHB patients. (B) HBV peptides-induced Th17 cell frequency was investigated by flow cytometry, and was compared between cells with and without IL-35 stimulation. (C) IL-17 and (D) IL-22 level in the cultured supernatants was measured by ELISA, and was compared between cells with and without IL-35 stimulation. The individual level for each subject was shown. Significance was calculated using paired *t*-test.

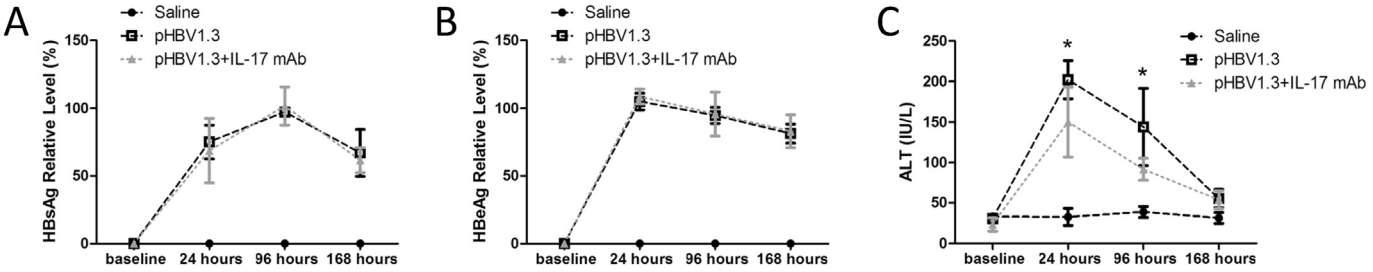

Fig. 3. IL-17 neutralizing antibody reduces liver inflammation in pHBV1.3 plasmid hydrodynamically injected mouse model. BALB/c mice (3 per group) were injected with saline, pHBV1.3, or pHBV1.3 + mouse IL-17 antibody under hydrodynamic conditions. Blood samples were collected at baseline, 24 h, 96 h, and 168 h post-transfection. (A) HBsAg, (B) HBeAg, and (C) ALT levels were measured and compared among groups. The value for each time point was shown as mean and SD. Significance was calculated using SNK-*q* test.

subsets in peripheral bloods and IL-35 level in the plasma was determined by flow cytometry and ELISA, respectively. The representative flow dots of non-specific and HBV peptides-induced Th17 cells of healthy control and AHB patients were shown in Fig. 1A. After stimulation with PMA and ionomycin, the percentage of circulating non-specific $CD3^+CD4^+IL\text{-}17^+$ Th17 cells in stimulated $CD4^+$ T cells from AHB patients was comparable with that in healthy controls (2.19 ± 0.65% *vs.* 2.07 ± 0.97%, Student *t*-test, $P = 0.638$, Fig. 1B). However, after stimulation with HBV envelope peptide pool, the percentage of HBV peptides-induced Th17 cells in stimulated $CD4^+$ cells from AHB patients was significantly higher than that in healthy controls (1.62 ± 0.49% *vs.* 0.06 ± 0.01%, Student *t*-test, $P < 0.0001$, Fig. 1C). Moreover, IL-35 concentration in the plasma was also notably elevated in AHB patients than that in healthy controls (140.5 ± 44.34 pg/ml *vs.* 106.1 ± 30.43 pg/ml, Student *t*-test, $P = 0.0055$, Fig. 1D).

Both ALT and T-BIL was significantly elevated in AHB patients when compared with healthy individuals ($P < 0.0001$, Table 1). Thus, the correlations between Th17/IL-35 and liver injury were analyzed. IL-35 level was negatively correlated with HBV peptides-induced Th17 cell frequency in AHB patients ($r = -0.482$, Pearson correlation analysis, $P = 0.027$, Fig. 1E). Furthermore, IL-35 level was also negatively correlated with serum ALT ($r = -0.703$, Spearman correlation analysis, $P = 0.0004$, Fig. 1F), and HBV peptides-induced Th17 percentage was positively correlated with serum ALT ($r = 0.526$, Spearman correlation analysis, $P = 0.014$, Fig. 1G). However, T-BIL level did not notably correlate with either IL-35 ($r = -0.143$, Spearman correlation analysis, $P = 0.536$) or HBV peptides-induced Th17 cells ($r = -0.139$, Spearman correlation analysis, $P = 0.548$). There was also no remarkable correlation between IL-35 and HBV viral load ($r = -0.160$, Pearson correlation analysis, $P = 0.487$).

3.2. *In vitro* IL-35 stimulation reduced Th17 cell subset and Th17 cytokine production in $CD4^+$ T cells from AHB patients

$CD4^+$ T cells were purified from all AHB patients, and 5×10^5 of

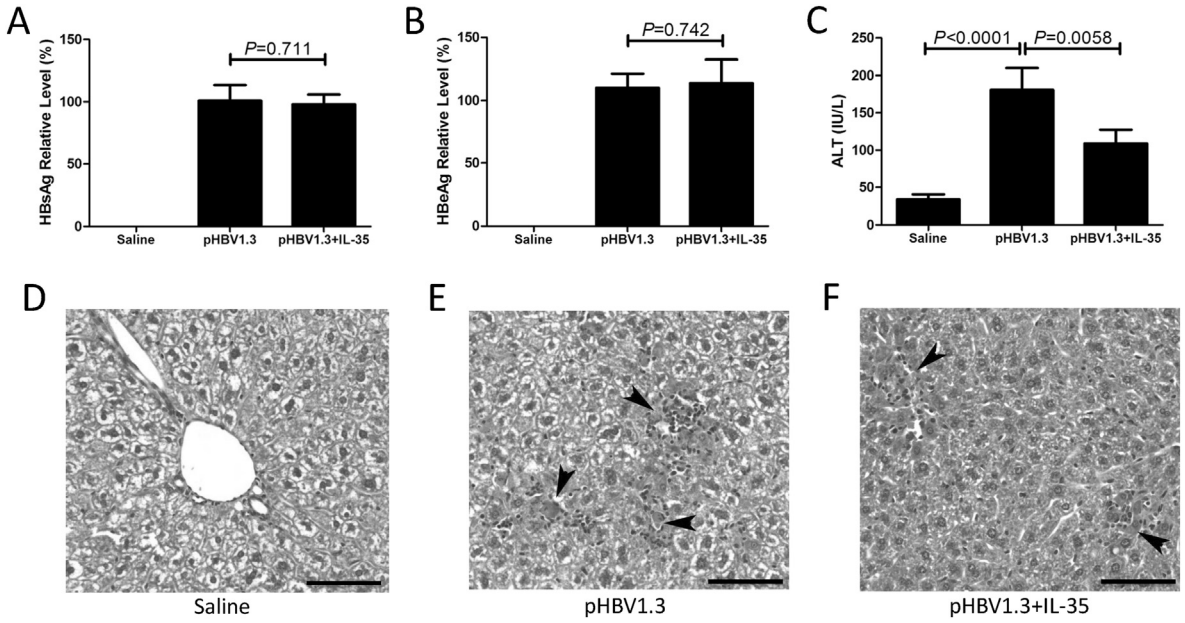

Fig. 4. IL-35 administration did not affect HBV antigen production but reduced liver inflammation and pathology in pHBV1.3 plasmid hydrodynamically injected mouse model. BALB/c mice (4 per group) were injected with saline, pHBV1.3, or pHBV1.3 + recombinant mouse IL-35 under hydrodynamic conditions. Mice were scarified 96 h post injection. Serum and livers were harvested for further analyses. (A) HBsAg, (B) HBeAg, and (C) ALT levels were measured and compared among groups. The columns indicated the mean values, while error bars indicated the SD. Significance was calculated using SNK-*q* test. Representative sections of liver from mice injected with (D) saline, (E) pHBV1.3, or (F) pHBV1.3 + recombinant mouse IL-35 were shown. Scale bars = 100 μm.

$CD4^+$ T cells were stimulated with HBV envelope peptide pool in the presence or absence of recombinant human IL-35. Cells and supernatants were harvested 12 h post-stimulation. CCK-8 results showed that IL-35 did not affect cellular proliferation of HBV peptides induced $CD4^+$ T cells ($[0.83 \pm 0.08] \times 10^6$ *vs.* $[0.80 \pm 0.07] \times 10^6$, paired *t*-test, $P = 0.088$, Fig. 2A). HBV peptides-induced Th17 cells frequency was notably down-regulated in response to IL-35 stimulation ($0.99 \pm 0.19\%$ *vs.* $1.33 \pm 0.30\%$, paired *t*-test, $P = 0.0003$, Fig. 2B). Moreover, Th17 cytokines production by $CD4^+$ T cells, including IL-17 and IL-22, was also decreased in response to IL-35 stimulation (IL-17: 39.71 ± 11.08 pg/ml *vs.* 49.57 ± 20.31 pg/ml, paired *t*-test, $P = 0.016$, Fig. 2C; IL-22: 100.2 ± 12.62 pg/ml *vs.* 106.8 ± 11.02 pg/ml, paired *t*-test, $P < 0.0001$, Fig. 2D).

3.3. *In vivo IL-17 neutralization reduced liver inflammation in HBV plasmid hydrodynamically injected mouse model*

All mice received intravenous injections of saline with or without pHBV1.3 under hydrodynamic conditions, allowing for transfection of hepatocytes *in vivo* [21,22]. A group of mice also received hydrodynamic injection combined with pHBV1.3 and 100 μg of mouse IL-17 antibody. Blood samples were collected at baseline, 24 h, 96 h, and 168 h post-transfection. HBV plasmid transfection induced high level of HBsAg and HBeAg productions at 24 h and 96 h post-transfection (Fig. 3A and B), however, IL-17 neutralizing antibody did not affect HBV antigens production in the serum (SNK-*q* test, $P > 0.05$, Fig. 3A and B). HBV plasmid transfection induced moderate increase of serum ALT at 24 h and 96 h post-transfection (202.3 ± 23.46 IU/L and 144.0 ± 47.82 IU/L). IL-7 neutralizing antibody reduced HBV-induced ALT elevation (150.0 ± 43.21 IU/L and 91.67 ± 13.65 IU/L, SNK-q test, $P < 0.05$, Fig. 3C).

3.4. *In vivo IL-35 treatment did not affect HBV antigen production but reduced liver damage in HBV plasmid hydrodynamically injected mouse model*

Mice received hydrodynamic injection with pHBV1.3 in the presence or absence of recombinant mouse IL-35, and were sacrificed 96 h post-transfection. Administration of IL-35 did not alter the release of the secreted viral antigens into the serum (SNK-*q* test, $P > 0.05$, Fig. 4A and B). Liver damage in mice which received HBV plasmid transfection was moderate as measurement of serum ALT (34.00 ± 6.48 IU/L *vs.* 180.8 ± 29.07 IU/L, SNK-*q* test, $P < 0.0001$, Fig. 4C), while IL-35 administration diminished severity of liver disease with down-regulation of serum ALT in comparison with pHBV1.3 transfection only (108.8 ± 18.55 IU/L, SNK-*q* test, $P = 0.0058$, Fig. 4C). Consistent with the ALT levels, in mice received saline injection, there was no evidence of significant hepatitis in the liver (Fig. 4D). In contrast, histological examination of the liver from the mice received HBV plasmid transfection showed scattered foci of necrotic hepatocyte and recruitment of inflammatory cells in parenchyma (Fig. 4E). IL-35 administration reduced the necrosis of hepatocytes and inflammation (Fig. 4F).

3.5. *In vivo IL-35 treatment reduced hepatic NK cells and HBV peptides-induced Th17 cells in HBV plasmid hydrodynamically injected mouse model*

The absolute number IHLs were quantified 96 h post hydrodynamic injection, and cell marker expression and IL-17 production in the recruited inflammatory cells was determined by flow cytometry. The total numbers of IHLs was increased in the liver of mice injected with pHBV1.3 ($[1.41 \pm 0.34] \times 10^5$ *vs.* $[0.55 \pm 0.16] \times 10^5$, SNK-*q* test, $P = 0.0035$, Fig. 5A), however, IL-35 administration did not significantly reduced the number of total IHLs ($[1.32 \pm 0.15] \times 10^5$, SNK-*q* test, $P = 0.653$, Fig. 5A). There were no significant differences of cell subsets percentages corresponding to Th cells ($CD3^+CD4^+$), CTLs ($CD3^+CD8^+$), NK cells ($CD3^-NK1.1^+$), or NKT cells ($CD3^+NK1.1^+$) among three groups (One-way ANOVA, $P > 0.05$). The cell numbers corresponding to Th cells (Fig. 5B), CTLs (Fig. 5C), NK cells (Fig. 5D), and NKT cells (Fig. 5E) was increased in the liver of mice injected with HBV plasmid (SNK-*q* test, $P < 0.01$). IL-35 administration only reduced NK cell number with slightly higher number than control mice (SNK-*q* test, $P = 0.0068$, Fig. 5D). Hepatic HBV peptides-induced Th17 cells were also investigated. HBV plasmid hydrodynamic injection

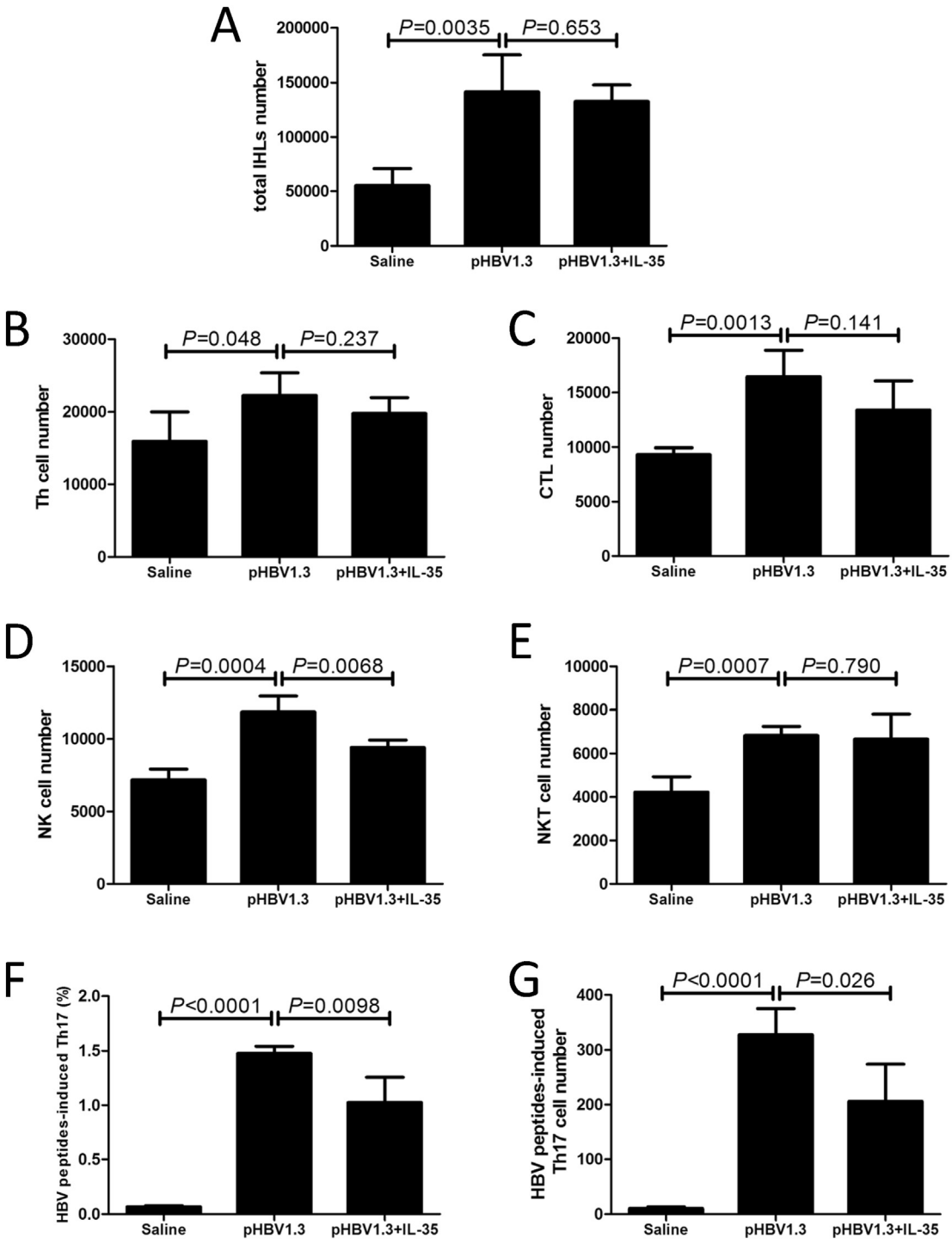

Fig. 5. IL-35 administration reduced NK cells and HBV peptides-induced Th17 cells in the liver. Lives from BALB/c mice (4 per group) receiving saline, pHBV1.3, or pHBV1.3 + recombinant mouse IL-35 hydrodynamic injections were weighed at the time the mice were scarified, and IHLs were isolated from 2 liver lobes of a similar weight and analyzed by flow cytometry. (A) Total IHL number, (B) $CD3^+CD4^+$ Th cells, (C) $CD3^+CD8^+$ CTLs, (D) $CD3^-NK1.1^+$ NK cells, (E) $CD3^+NK1.1^+$ NKT cells represented the absolute numbers in the liver, respectively. (F) Percentage and (G) absolute number of HBV peptides-induced Th17 cells in the liver was also investigated. The columns indicated the mean values, while error bars indicated the SD. Significance was calculated using SNK-*q* test.

induced high level of hepatic HBV peptides-induced Th17 cells in comparison with control mice (SNK-*q* test, $P < 0.0001$, Fig. 5F and G). IL-35 administration notably down-regulated both percentage and absolute number of HBV peptides-induced Th17 cells in the liver (SNK-*q* test, $P = 0.0098$, Fig. 5F; $P = 0.026$, Fig. 5G). Moreover, we also examined the influence of IL-35 on Th17 cytokines and intrahepatic chemokine mRNA expression. IL-35 administration reduced IL-17 (Fig. 6A) and IL-22 (Fig. 6B) mRNA expression in the liver (SNK-*q* test, $P < 0.05$). However, CXCL9 (Fig. 6C) and CXCL10 (Fig. 6D) was comparable in the presence of IL-35 in pHBV1.3 injected mice (SNK-*q* test, $P > 0.05$).

4. Discussion

Cytokine-mediated host reactions play a crucial role in viral control and virus-induced hepatic injury due to the non-cytopathic property of

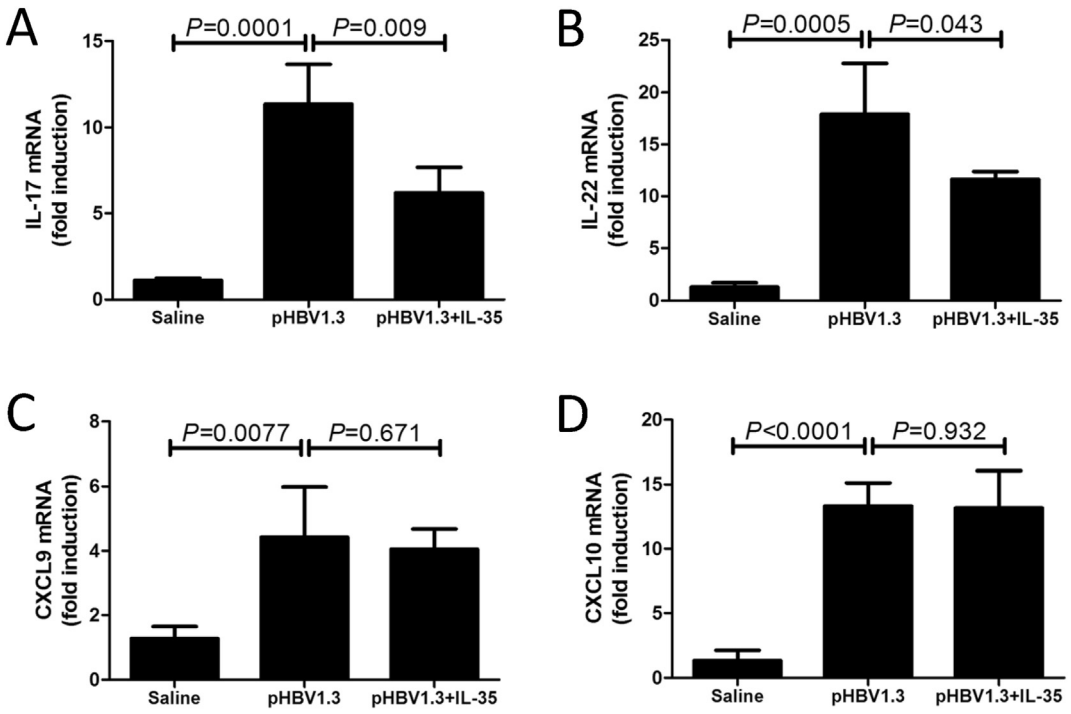

Fig. 6. IL-35 administration reduced IL-17 and IL-22 mRNA, but did not affect chemokine expression in the liver. (A) IL-17, (B) IL-22, (C) CXCL9, and (D) CXCL10 mRNA expression in the liver of BALB/c mice (4 per group) receiving saline, pHBV1.3, or pHBV1.3 + recombinant mouse IL-35 hydrodynamic injections was semi-quantified by real-time PCR. The results were shown as fold differences relative to control group, normalized to GAPDH. The columns indicated the mean values, while error bars indicated the SD. Significance was calculated using SNK-*q* test.

HBV. IL-12 cytokine family consists of IL-12, IL-23, IL-27, and IL-35 [24], which play important roles in infection, inflammation, and autoimmune disorders. Up-regulation of serum IL-12 in chronic HBV infection boosted HBeAg seroconversion [25,26]. HBV infection also promoted IL-23 production in antigen presenting cells, which induced liver damage through IL-23/Th17 axis in hepatitis B [27,28]. Elevated IL-27 positively correlated with Th17 cells, which indicated liver injury and predicted spontaneous HBeAg seroconversion in HBV-infected patients [29,30]. The current results revealed that the new member of IL-12 family IL-35 was significantly elevated in peripheral bloods of AHB patients, which was consistent with the findings in chronic HBV infection [14,16]. This was also in accordance with the reports on other acute infections, including influenza virus infection, secondary bacterial pneumonia [31] and sepsis [32]. Thus, high level of IL-35 might take part in the immunopathogenesis of acute HBV infection.

Acute HBV infection always leads to severe hepatic damage, which is presented as > 10-fold elevation of serum ALT. We showed that plasma IL-35 expression negatively correlated with ALT level in AHB patients, however, no remarkable correlation was found between IL-35 level and HBV DNA. This indicated a potential anti-inflammatory activity to liver injury without affecting viral replication. To further confirm this finding, we performed *in vivo* experiments using an acute HBV infected mouse model, which were hydrodynamically injected with HBV plasmid [33]. Previous studies revealed contradictory influence of IL-35 to HBV replication. Cheng et al. showed that *in vitro* IL-35 stimulation inhibited HBV replication and decreased HBsAg/HBeAg secretion by HepAD38 cells [16]. In contrast, Tao et al. revealed that IL-35 was able to reinforce HBV replication both *in vitro* and *in vivo* using HBV transgenic mouse model [17]. Our current results showed that IL-35 administration *in vivo* did not affect HBV antigens production 96 h post HBV plasmid transfection, suggesting that IL-35 did not reveal direct antiviral activity against virus. Moreover, IL-35 decreased the HBV-induced liver injury, which presented as reduced necrosis of hepatocytes and liver inflammation, and down-regulation of serum ALT. It was well accepted that hepatic necrosis, liver inflammation, and subsequent ALT elevation were potentiated by recruitment of inflammatory cells into the liver, which required specific mediators such as chemokines and neutrophils [20–22]. However, total inflammatory cell numbers which recruited into the liver and elevated hepatic CXCL9/10 expression following HBV plasmid transfection did not change significantly in response to IL-35 administration. Only NK cells were slightly reduced following IL-35 treatment. Liver-resident and conventional NK cells played dual functions in liver immunity and in viral hepatitis [34,35]. However, there was no evidence supporting the regulatory activity of IL-35 to NK cells. Thus, there might be other mechanisms which account for the anti-inflammatory effect of IL-35 in acute HBV infected mouse model.

Circulating Th17 cells elevation and IL-17 production was closely associated with disease aggravation and pathogenesis of liver injury in HBV infected patients [36,37]. Non-specific Th17 cells frequency in peripheral bloods significantly correlated with aminotransferase levels in AHB patients [20]. In this study, we found that HBV envelope peptides-induced Th17 cells, but not non-specific Th17 cells were increased in AHB patients. This elevation also positively correlated with serum ALT, however, negatively correlated with plasma IL-35 expression. However, even certain cytokines were increased in the context of inflammation, it did not necessary mean those cytokines were critically responsible for the pathogenesis. Thus, we verified the role of IL-17 in acute HBV infection by treating the hydrodynamic injection mouse model with IL-17 neutralizing antibody. The *in vivo* results confirmed the proinflammatory role of IL-17 in acute HBV infection. More importantly, although IL-35 did not affect cultured $CD4^+$ T cells proliferation *in vitro*, IL-35 stimulation not only suppressed Th17 differentiation from $CD4^+$ T cells *in vitro*, but also reduced HBV peptides-induced Th17 cells in the liver *in vivo*. It was well accepted that IL-35 played an immunosuppressive effect on inflammation through induction and expansion of Tregs and suppression of Th1/Th17 cells [13,38]. Our current data reiterated that elevated IL-35 could directly inhibit Th17 activity during acute HBV infection, which might partly reduce the subsequent liver inflammation. IL-35 administration could also

inhibit the HBV peptides-induced Th17 cells in the liver in acute HBV infection mouse model, confirming the *in vitro* results in cultured $CD4^+$ T cells and providing a novel mechanism corresponding to immunosuppression of IL-35 in regulation of liver inflammation. However, down-regulation of HBV peptides-induced Th17 cells might not be due to the reduced recruitment of Th17 cells into the liver, because the IL-35 did not affect the total IHLs numbers recruited into the liver following HBV plasmid hydrodynamic injection. Further experiments using adaptive transfer of HBV transgenic mouse model [20] were needed for distinguishing changes of recruited or resident Th17 cells in the liver.

In conclusion, we found that IL-35 was dispensable for HBV antigens secretion, but crucial for regulation of peripheral and hepatic HBV peptides-induced Th17 cells *in vitro* and *in vivo*, which might subsequently modulated hepatocytes damage and liver inflammation during acute HBV infection. The potential anti-inflammatory property of IL-35 might be pivotal for development new therapeutic approaches for hepatitis B.

Acknowledgements

We thank Drs. Jian-Qi Lian, Ye Zhang, Xin Wei (Center for Infectious Diseases, Tangdu Hospital, Fourth Military Medical University) and Dr. Michael D. Robek (Albany Medical College) for providing HBV envelope peptide pool and pHBV1.3 plasmid, and for the technical assistance. This work was supported by the grant from Health Commission of Shaanxi Province (No. 2018A005) and Tianqing Liver Disease Research Program of Chinese Foundation for Hepatitis Prevention and Control (No. TQGB20180096).

Disclosure statement

No competing financial interests exist.

References

[1] European Association for the Study of the Liver, EASL 2017 clinical practice guidelines on the management of hepatitis B virus infection, J. Hepatol. 67 (2017) 370–398.
[2] L.L. Boeijen, R.C. Hoogeveen, A. Boonstra, G.M. Lauer, Hepatitis B virus infection and the immune response: the big questions, Best Pract. Res. Clin. Gastroenterol. 31 (2017) 265–272.
[3] C. Ferrari, HBV and the immune response, Liver Int. 35 (Suppl. 1) (2015) 121–128.
[4] L.G. Guidotti, M. Isogawa, F.V. Chisari, Host-virus interactions in hepatitis B virus infection, Curr. Opin. Immunol. 36 (2015) 61–66.
[5] P.T.F. Kennedy, S. Litwin, G.E. Dolman, A. Bertoletti, W.S. Mason, Immune tolerant chronic hepatitis B: the unrecognized risks, Viruses 9 (2017) E96.
[6] A. Jindal, M. Kumar, S.K. Sarin, Management of acute hepatitis B and reactivation of hepatitis B, Liver Int. 33 (Suppl. 1) (2013) 164–175.
[7] Y. Xia, U. Protzer, Control of hepatitis B virus by cytokines, Viruses 9 (2017) E18.
[8] X. Li, X. Liu, L. Tian, Y. Chen, Cytokine-mediated Immunopathogenesis of hepatitis B virus infections, Clin. Rev. Allergy Immunol. 50 (2016) 41–54.
[9] L.W. Collison, C.J. Workman, T.T. Kuo, K. Boyd, Y. Wang, K.M. Vignali, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function, Nature 450 (2007) 566–569.
[10] D. Hu, Role of anti-inflammatory cytokines IL-35 and IL-37 in asthma, Inflammation 40 (2017) 697–707.
[11] L.C. Su, X.Y. Liu, A.F. Huang, W.D. Xu, Emerging role of IL-35 in inflammatory autoimmune diseases, Autoimmun. Rev. 17 (2018) 665–673.
[12] L.I. Sakkas, A. Mavropoulos, C. Perricone, D.P. Bogdanos, IL-35: a new immunomodulator in autoimmune rheumatic diseases, Immunol. Res. 66 (2018) 305–312.
[13] W. Niedbala, X.Q. Wei, B. Cai, A.J. Hueber, B.P. Leung, I.B. McInnes, et al. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells, Eur. J. Immunol. 37 (2007) 3021–3029.
[14] X. Shao, J. Ma, S. Jia, L. Yang, W. Wang, Z. Jin, Interleukin-35 suppresses antiviral immune response in chronic hepatitis B virus infection, Front. Cell. Infect. Microbiol. 7 (2017) 472.
[15] Y. Zhou, H. Zhang, Y. Li, IL-35 expression in peripheral blood CD4(+) T cells from chronic hepatitis B virus-infected patients directly correlates with virus load, Cytokine 73 (2015) 169–175.
[16] S.T. Cheng, D. Yuan, Y. Liu, Y. Huang, X. Chen, H.B. Yu, et al., Interleukin-35 level is elevated in patients with chronic hepatitis B virus infection, Int. J. Med. Sci. 15 (2018) 188–194.
[17] N.N. Tao, R. Gong, X. Chen, L. He, F. Ren, H.B. Yu, et al. Interleukin-35 stimulates hepatitis B virus transcription and replication by targeting transcription factor HNF4alpha, J. Gen. Virol. 99 (2018) 645–654.
[18] X. Li, L. Tian, Y. Dong, Q. Zhu, Y. Wang, W. Han, et al., IL-35 inhibits HBV antigen-specific IFN-gamma-producing CTLs in vitro, Clin. Sci. (Lond.) 129 (2015) 395–404.
[19] J.Y. Zhang, Z. Zhang, F. Lin, Z.S. Zou, R.N. Xu, L. Jin, et al. Interleukin-17-producing CD4(+) T cells increase with severity of liver damage in patients with chronic hepatitis B, Hepatology 51 (2010) 81–91.
[20] Y. Zhang, M.A. Cobleigh, J.Q. Lian, C.X. Huang, C.J. Booth, X.F. Bai, et al. A proinflammatory role for interleukin-22 in the immune response to hepatitis B virus, Gastroenterology 141 (2011) 1897–1906.
[21] X. Wei, J.P. Wang, C.Q. Hao, X.F. Yang, L.X. Wang, C.X. Huang, et al., Notch signaling contributes to liver inflammation by regulation of Interleukin-22-producing cells in hepatitis B virus infection, Front. Cell. Infect. Microbiol. 6 (2016) 132.
[22] M.A. Cobleigh, L. Buonocore, S.L. Uprichard, J.K. Rose, M.D. Robek, A vesicular stomatitis virus-based hepatitis B virus vaccine vector provides protection against challenge in a single dose, J. Virol. 84 (2010) 7513–7522.
[23] Y. Fan, W. Weifeng, Y. Yuluan, K. Qing, P. Yu, H. Yanlan, Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of coxsackievirus b3-induced viral myocarditis reduces myocardium inflammation, Virol. J. 8 (2011) 17.
[24] D.A. Vignali, V.K. Kuchroo, IL-12 family cytokines: immunological playmakers, Nat. Immunol. 13 (2012) 722–728.
[25] H.W. Wang, H.L. Gao, X.X. Wei, X.H. Wang, Up-regulation of IL-12 expression in patients with chronic hepatitis B is mediated by the PI3K/Akt pathway, Mol. Cell. Biochem. 407 (2015) 135–142.
[26] D. He, G. Yan, Y. Wang, Serum levels of interleukin-12 in various clinical states with hepatitis B virus infection, Cell. Immunol. 272 (2012) 162–165.
[27] Q. Wang, J. Zhou, B. Zhang, Z. Tian, J. Tang, Y. Zheng, et al. Hepatitis B virus induces IL-23 production in antigen presenting cells and causes liver damage via the IL-23/IL-17 axis, PLoS Pathog. 9 (2013) e1003410.
[28] Z. Huang, J.C. van Velkinburgh, B. Ni, Y. Wu, Pivotal roles of the interleukin-23/T helper 17 cell axis in hepatitis B, Liver Int. 32 (2012) 894–901.
[29] G.L. Zhang, D.Y. Xie, Y.N. Ye, C.S. Lin, X.H. Zhang, Y.B. Zheng, et al. High level of IL-27 positively correlated with Th17 cells may indicate liver injury in patients infected with HBV, Liver Int. 34 (2014) 266–273.
[30] J. Li, L.Y. Mak, D.K. Wong, J. Fung, W.K. Seto, C.L. Lai, et al. The role of interleukin-27 in predicting spontaneous HBeAg seroconversion in chronic hepatitis B infection, Liver Int. 37 (2017) 1287–1294.
[31] Y. Chen, C.J. Wang, S.H. Lin, M. Zhang, S.Y. Li, F. Xu, Interleukin-35 is upregulated in response to influenza virus infection and secondary bacterial pneumonia, Cytokine 81 (2016) 23–27.
[32] J. Cao, F. Xu, S. Lin, X. Tao, Y. Xiang, X. Lai, et al. IL-35 is elevated in clinical and experimental sepsis and mediates inflammation, Clin. Immunol. 161 (2015) 89–95.
[33] P.L. Yang, A. Althage, J. Chung, F.V. Chisari, Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13825–13830.
[34] H. Peng, E. Wisse, Z. Tian, Liver natural killer cells: subsets and roles in liver immunity, Cell. Mol. Immunol. 13 (2016) 328–336.
[35] S.F. Wu, W.J. Wang, Y.Q. Gao, Natural killer cells in hepatitis B virus infection, Braz. J. Infect. Dis. 19 (2015) 417–425.
[36] W. Wu, J. Li, F. Chen, H. Zhu, G. Peng, Z. Chen, Circulating Th17 cells frequency is associated with the disease progression in HBV infected patients, J. Gastroenterol. Hepatol. 25 (2010) 750–757.
[37] B. Yang, Y. Wang, C. Zhao, W. Yan, H. Che, C. Shen, et al. Increased Th17 cells and interleukin-17 contribute to immune activation and disease aggravation in patients with chronic hepatitis B virus infection, Immunol. Lett. 149 (2013) 41–49.
[38] Q. Huang, Y. Wang, C. Si, D. Zhao, Y. Wang, Y. Duan, Interleukin-35 modulates the imbalance between regulatory T cells and T helper 17 cells in enterovirus 71-induced hand, foot, and mouth disease, J. Interf. Cytokine Res. 37 (2017) 522–530.