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*Original Article*

# Overproduction of IL-27 may play a pro-inflammatory role in HBV infected patients with severe liver inflammation

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**Abstract:** Our previous study showed that high level of IL-27 may indicate liver injury in HBV infected patients. The aim of this study was to evaluate the level of serum IL-27 in HBV infected patients with severe liver inflammation and to explore how it impacts the course of the disease. We assessed samples from 49 HBV infected patients with severe liver inflammation (29 severe chronic hepatitis B (CHB-S) subjects and 20 acute-on-chronic liver failure (ACLF) subjects), 16 HBV patients with mild chronic hepatitis B (CHB-M), and 18 normal controls (NC). Serum IL-27 levels were examined by enzyme-linked immunosorbent assay. We collected clinical parameters from patients' records and analyzed their associations with IL-27 expression. IL-27 levels were raised in HBV infected patients compared to normal controls (P<0.0001). Furthermore, IL-27 levels progressively increased from CHB-M to CHB-S to maximum in HBV-ACLF patients (P<0.0001). Next, we divided patients with severe liver inflammation into two groups, based on the IL-27 cut-off value of 538 pg/ml. Patients from the elevated IL-27 group had significantly lower levels of albumin, alpha-fetoprotein, cholesterol, and prothrombin time activity than those from normal IL-27 group (P=0.018, 0.047, 0.015 and <0.001, respectively). Additionally, patients with elevated IL-27 levels also had a significantly higher rate of complications and poor outcome than those with normal IL-27 levels (P=0.022 and 0.040, respectively). These results indicate that serum IL-27 levels are significantly elevated in HBV infected patients with severe liver inflammation and may exert a pro-inflammatory effect in the development of liver damage.

**Keywords:** Hepatitis B virus, interleukin-27, immunopathogenesis

## Introduction

Patients with severe liver inflammation due to hepatitis B virus (HBV) infection represent a major source of morbidity and mortality worldwide. China has a high occurrence of HBV infection. A national survey announced in 2006 by the Ministry of Health showed that 30 million people in China suffer from chronic hepatitis B (CHB) [1]. The outcomes of CHB are extremely variable. Approximately 15-40% of all CHB patients will develop cirrhosis, liver failure, or hepatocellular carcinoma (HCC) [2]. The pathogenesis of liver damage during HBV infection is still poorly understood. As HBV is a preferentially hepatotropic but not directly cytopathic virus, increasing evidences show that cytokine-mediated immunity, linking host innate and adaptive immunity, may play a crucial role in determining the outcome of HBV infection [3-6].

Interleukin-27 (IL-27), which belongs to the interleukin-12 (IL-12) cytokine family, was recently identified. Previous studies have demonstrated that members of this family play roles in regulating the differentiation of T helper (TH) cells. IL-27 is a heterodimeric cytokine that is formed by the association between the subunit protein IL-27 p28 with the Epstein Bar virus-induced protein 3 (Ebi3) [7]. Predominantly expressed by myeloid cells, the protein signals through a heterodimeric receptor that consists of IL-27Ra and gp130 [8] and is expressed throughout the immune system. IL-27 is initially described as a pro-inflammatory cytokine that could induce TH1 cell differentiation [7]. Sub-

sequent studies in multiple models of infectious and autoimmune diseases have confirmed an anti-inflammatory role for IL-27 in TH cell responses by producing the anti-inflammatory cytokine IL-10 [9-12]. However, several studies revealed that, in some situations, IL-27 may play a pro-inflammatory role. For example, IL-27ra-/- mice are protected from proteoglycan-induced arthritis [13], and deletion of the IL-27ra gene in the MRL/lpr model of lupus results in lower TH1 cytokine production, diminished anti-dsDNA antibodies, and enhanced survival [14]. Taken together, these results indicate that the role of IL-27 in regulating the immune response is somewhat controversial.

Several studies have focused on the role of IL-27 in the HBV infection. A report showed that serum IL-27 levels were significantly higher in patients with CHB than in healthy controls, and they were associated with the development of CHB [15]. An additional study indicated that IL-27 may trigger immune responses to prevent hepatic injury [16]. However, our previous study showed that High level of IL-27 positively correlated with Th17 cells may indicate liver injury in patients infected with HBV [17]. These studies suggest that IL-27 may play a role in the pathogenesis of HBV-related liver injury, but it is still unclear how it shapes pathogenesis. Therefore, in the present study, we investigated serum IL-27 levels in patients with severe liver inflammation due to HBV infection, and we examined their relationship with disease activity.

## Patients and methods

### *Serum samples*

Peripheral blood samples were collected in our hospital, between Jun 2009 and Jan 2010, from 49 patients with severe liver inflammation due to HBV infection (29 with severe chronic hepatitis B (CHB-S) and 20 with HBV-related acute-on-chronic liver failure (HBV-ACLF)) and 16 patients with mild chronic hepatitis B (CHB-M). All patients had persistent seropositivity for the hepatitis B s antigen (HBsAg) for at least 6 months and exhibited hepatitis symptoms or signs and abnormal hepatic function before enrollment. The CHB-M group included patients who had a history of chronic hepatitis based on compatible laboratory data and ultrasonographic findings, and presented mild to moderate liver disease activities, but did not reach the criteria for CHB-S. CHB-S patients had severe liver disease symptoms, including obvious clinical manifestations and significant alterations in biochemical parameters. Based on the biochemical parameters, the diagnosis of CHB-S had to meet at least one of the following criteria: (1) serum albumin (ALB) level ≤32 g/L; (2) serum total bilirubin (TBIL) level >85.5 umol/L; (3) plasma prothrombin activity (PTA) 40-60%; and (4) serum cholinesterase (CHE) level <4,500 U/L [18]. Patients with HBV-ACLF were diagnosed according to the criteria previously described [19]. The criteria for ACLF have been widely used in China and are similar, but not exactly identical with the newly issued Asian Pacific Association for the Study of the Liver (APASL) criteria [20]. Clinical assessment and blood sampling were performed at admission. Individuals treated with immunomodulatory drugs before enrollment were excluded. No evidence of HCC, other metastatic liver disease, liver cirrhosis, concomitant infection with the hepatitis C/D (HCV/HDV) or human immunodeficiency (HIV) viruses, or autoimmune liver disease were present in any of the patients. Eighteen age- and sex-matched healthy individuals (17 men and 1 woman; age 38.94±1.92 years) were enrolled as normal controls (NC). The study protocol was approved by the ethics committee of our hospital and written informed consent was obtained from each subject. The clinical characteristics of these subjects are listed in **Table 1**.

### *Clinical assessment*

Complete medical histories, physical examinations, and laboratory tests were conducted for all patients. Complications were monitored and diagnosed at admission. Ascites was confirmed by abdominal ultrasound or diagnostic paracentesis as described previously [21]. Spontaneous bacterial peritonitis (SBP) was diagnosed based on the following criteria: (1) ascitic fluid polymorphonuclear count ≥250 cells/mm$^3$ with or without a positive culture (bedside inoculation); and (2) ascitic fluid polymorphonuclear count <250 cells/mm$^3$ but with a positive culture (nonneutrocytic bacterascites) [21]. The criteria for diagnosing hepatorenal syndrome (HRS) were (1) serum creatinine >1.5 mg/dl (133 mmol/l); (2) failure to see improvement in the renal function to improve following diuretic withdrawal and plasma vol-

**Table 1.** Clinical characteristics of the populations enrolled in the study

| Group | NC (n=18) | CHB-M (n=16) | CHB-S (n=29) | HBV-ACLF (n=20) |
|---|---|---|---|---|
| Sex (M/F) | 17/1 | 15/1 | 27/2 | 18/2 |
| Age (years) | 38.94±1.92 | 38.00±2.22 | 37.03±2.01 | 40.25±2.97 |
| ALT (U/L) | 19.05±1.72 | 203.56±20.56 | 270 (34-2424) | 134 (27-1688) |
| TBIL (umol/L) | N.D. | 45.46±3.85 | 255.80 (84.3-957.90) | 508.83 (197.71-1156.96) |
| ALB (g/L) | N.D. | 39.78±0.59 | 38.07±0.80 | 32.40±0.77 |
| PTA% | N.D. | 88.31±2.78 | 71.44±4.02 | 31.35±1.46 |
| CHE (U/L) | N.D. | 6779.13±184.27 | 4140.00±235.09 | 3010.35±260.25 |
| HBV-DNA ($log_{10}$ IU/mL) | N.D. | 5.52±0.12 | 4.88±0.26 | 5.04±0.37 |
| HbeAg (P/N) | 0 | 10/6 | 17/12 | 8/12 |

Data are shown as means and standard error or median and range. ACLF, acute on chronic liver failure; CHB-S, severe chronic hepatitis B; CHB-M, moderate chronic hepatitis B; NC, normal control. P, positive; N, negative; N.D, not determined.

ume expansion; and (3) the absence of an identifiable cause of renal failure [21]. Pneumonia was defined by infiltrates on chest radiography most likely to be associated with pulmonary infection and at least two of the following criteria: fever (temperature ≥38.3°C); leucopenia or leucocytosis (white blood-cell count ≤4 × $10^9$/L or ≥12 × $10^9$/L); purulent tracheal secretions; and the presence of rales or bronchial breath sounds on physical examination [22]. Hepatic encephalopathy was diagnosed according to the West Haven criteria and the existence of hyperammonemia (venous ammonia concentration >50 mmol/L) [23].

*Analysis of cytokines by ELISA*

Serum IL-27 concentrations were quantified by sandwich ELISA using commercial kits (Biolegend, San Diego, USA), according to the manufacturer's protocols. The serum cytokine levels were quantified by using standard samples with known cytokine concentrations expressed as pg/ml that were provided by the manufacturer. The detection sensitivity of the ELISA kit was 11 pg/mL.

*Virological assessment and liver biochemical assays*

HBV DNA levels were quantified by Real-Time quantitative PCR using the ABI7300 thermocycler (AppliedBiosystems, Foster City, USA). The limit of detection of the assay was 100 IU/mL. Serum HBV markers, including HBsAg, hepatitis Bs antibody (HBsAb), hepatitis Be antigen (HBeAg), hepatitis Be antibody (HBeAb), and hepatitis Bc antibody (HBcAb) were determined using the Roche's Elecsys system (Hoffmann-La Roche, Basel, Switzerland). Liver biochemical assays including ALB, PTA, CHE, cholesterol, triglycerides, alanine aminotransferase (ALT), aspartate aminotransferase (AST), TBIL, and creatinine were performed using an autoanalyzer (TBA-30FR Toshiba, Tokyo, Japan).

*Statistical analysis*

All data were analyzed using SPSS version 13.0 software (Chicago, IL, USA) and summarized as means and standard error or median and range. Differences in variables were analyzed using ANOVA and Student's *t* tests (for normally distributed data) or Kruskal-Wallis and Mann-Whitney U tests (for non-normally distributed data), as appropriate. Categorical data were analyzed using the Chi-square test and Fisher's exact test. A two-sided $P<0.05$ was considered statistically significant.

## Results

*Characteristics of patients*

The characteristics of patients from the CHB-M, CHB-S, HBV-ACLF, and NC groups are shown in **Table 1**. There were no significant differences among the 4 groups for the age and gender ratio. Moreover, no statistically significant differences were observed among the CHB-M, CHB-S and HBV-ACLF groups with respect to the presence of HBeAg ($P=0.352$).

*Serum IL-27 level increased in HBV-infected patients with severe liver inflammation*

IL-27 levels in serum samples from patients infected with HBV and in those from the NC

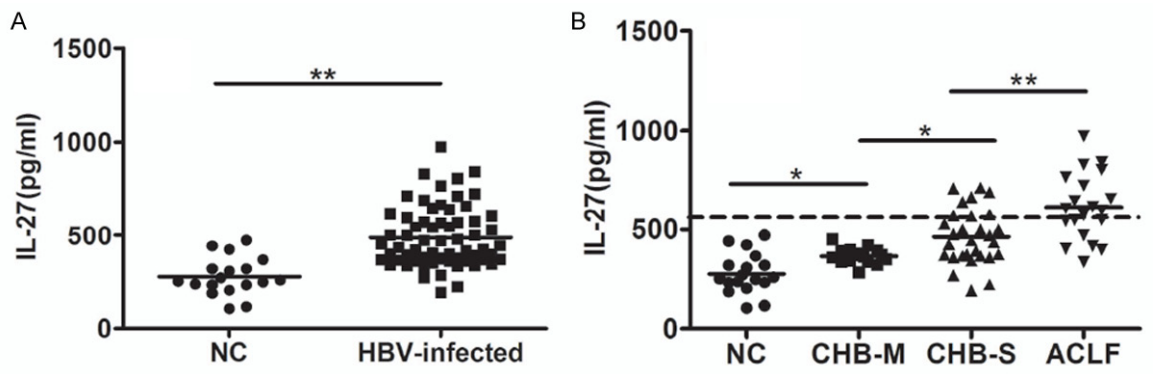


**Figure 1.** Serum levels of interleukin 27 (IL-27) in patients with HBV infected patients (ACLF, CHB-S or CHB-M) and NC group. Serum IL-27 levels were determined by a specific ELISA. A. Data are presented as dot plots, where the lines indicate the means for HBV infected patients (n=65) and NC group (n=18). B. Data are presented as dot plots, where the lines indicate the means for NC (n=18), CHB-M (n=16), CHB-S (n=29) and HBV-ACLF (n=20) group. Broken line indicates the cut-off value (mean +2.56 SD of NC samples). *P* values which were considered significant are indicated (*, $P<0.05$; **, $P<0.0001$). NC, normal control; CHB-S, severe chronic hepatitis B; CHB-M, mild chronic hepatitis B; ACLF, acute-on-chronic liver failure.

**Table 2.** Clinical features of HBV infected patients with severe liver inflammation with IL-27 overproduction

| Characteristic | Elevated IL-27 (n=23) | Normal IL-27 (n=26) | *P* value |
|---|---|---|---|
| Sex (M/F) | 21/2 | 24/2 | 0.898 |
| Age (years) | 39.96±13.14 | 36.92±10.67 | 0.377 |
| AST (U/L) | 164 (70-1496) | 182.5 (57-2646) | 0.882 |
| ALT (U/L) | 110 (27-1688) | 315 (34-2424) | 0.193 |
| TBIL (umol/L) | 422.57 (115.21-1156.96) | 312.45 (84.3-1154.40) | 0.116 |
| HbeAg (P/N) | 9/14 | 16/10 | 0.156 |
| HBV-DNA ($log_{10}$ IU/mL) | 5.34 (2.71-8.12) | 4.56 (2.70-7.44) | 0.280 |
| Antiviral therapy (Yes/No) | 14/9 | 13/13 | 0.567 |

Data are shown as means and standard error or median and range. P, positive; N, negative.

group were assessed by ELISA (**Figure 1**). Serum IL-27 levels were significantly elevated in patients infected with HBV (486.51±20.17) compared to the NC group (276.70±23.98, $P<0.0001$). For the HBV-infected subgroups, IL-27 levels in patients with CHB-M (367.46±10.14), CHB-S (463.19±25.95), and HBV-ACLF (615.57±37.90) were elevated compared with those from the NC group ($P=0.043$, $P<0.0001$, and $P<0.0001$, respectively). Furthermore, serum IL-27 levels progressively increased from CHB-M to CHB-S patients, to reach a maximum in HBV-ACLF patients (367.46±10.14 versus 463.19±25.95 versus 615.57±37.90; $P<0.0001$). Next, values higher than the mean +2.56 SD of the NC group (537.18 pg/ml) were found in 75% (15/20) of all patients with HBV-ACLF and in 28% (8/29) of patients with CHB-S. By contrast, no normal controls or CHB-M patients had elevated IL-27 levels.

*Clinical features of patients with HBV with IL-27 overproduction*

According to the cut-off value mentioned above, 49 patients with severe liver inflammation were divided into two groups: the high IL-27 group (IL-27>537.18 pg/ml, n=23) and the normal IL-27 group (IL-27<537.18 pg/ml, n=26). Next, we compared the clinical features of the patients in these two groups (**Table 2**). No significant differences were found between the two groups for the age and gender ratio. Additionally, no statistically significant differences were observed between the two groups with respect to the HBeAg and HBV-DNA loads (*P* values were 0.156 and 0.280, respectively). Antiviral

**Table 3.** Impaired liver synthetic function in patients with IL-27 overproduction

| Group | Elevated IL-27 (n=23) | Normal IL-27 (n=26) | *P* value |
|---|---|---|---|
| PTA (%) | 34 (18-126) | 66 (28-113) | 0.000 |
| ALB (g/L) | 34.04±4.66 | 37.29±4.57 | 0.018 |
| AFP (ng/mL) | 59.80 (3.40-852.00) | 135.85 (1.70-1910.00) | 0.047 |
| Cholesterol (mmol/L) | 1.50 (0.10-5.61) | 2.89 (0.25-5.24) | 0.015 |
| CHE (U/L) | 3307.43±1219.75 | 4007.54±1371.24 | 0.067 |
| Triglycerides (mmol/L) | 0.64 (0.01-7.11) | 1.18 (0.02-4.21) | 0.063 |

Data are shown as means and standard error or median and range. PTA, prothrombin time activity; ALB, albumin; AFP, alpha-fetoprotein; CHE, cholinesterase.

**Table 4.** IL-27 overproduction may indicate more complications and poor outcome

| Group | Elevated IL-27 (n=23) | Normal IL-27 (n=26) | *P* value |
|---|---|---|---|
| Complications | 17/23 | 9/26 | 0.010 |
| Asictes | 16/23 | 9/26 | 0.022 |
| Pneumonia | 4/23 | 1/26 | 0.173 |
| SBP | 7/23 | 3/26 | 0.157 |
| HE | 4/23 | 2/26 | 0.400 |
| ≥2 complications | 10/23 | 4/26 | 0.030 |
| Survived (Yes/No) | 16/7 | 24/2 | 0.040 |

SBP, Spontaneous bacterial peritonitis; HE, Hepatic encephalopathy.

therapy was strongly recommended by the updated guideline [20]. In our study, nucleoside analogues, but not interferon, was the first choice, due to the severity of the liver injury. Only 27 patients were treated with nucleoside analogues because of poor recovery of health insurance or a lack of money. However, no statistically significant differences were observed between the high IL-27 (14 patients, 60.87%) and the normal IL-27 group (13 patients, 50%) with respect to the ratio of receiving antiviral therapy (*P*=0.567).

*More accentuated liver synthetic function deterioration in patients with IL-27 overproduction*

Generally, serum levels of ALB, CHE, cholesterol (Chol), triglycerides (Trig), alpha-fetoprotein (AFP), and plasma PTA levels were common indicators to assess liver synthetic function. In our study, as shown in **Table 3**, serum levels of ALB, AFP, Chol, and plasma PTA levels were significantly decreased in the elevated group as compared to the normal group (the *P* values were 0.018, 0.047, 0.015, and <0.001, respectively). In addition, a trend for reduced CHE and Trig levels was found in patients with elevated IL-27 levels as compared to those in the group with normal IL-27 levels (**Table 3**). These findings suggest that increased IL-27 serum levels are closely correlated with impaired liver synthetic function in severe liver inflammation due to HBV infection.

*IL-27 overproduction may indicate more complications and poor outcome*

Subsequently, we assessed the complications in patients with increased IL-27 production compared with those with normal IL-27 production (**Table 4**). In our study, 17 patients in the elevated IL-27 group (63.38%) but only 9 patients in the normal IL-27 group (34.62%) had at least one of following: ascites, pneumonia, SBP, or HE, making the difference between them significant (*P*=0.010). Ascites was the most common of these complications, and showed a higher frequency in the elevated IL-27 group (69.56%, 16/23) than in the group with normal IL-27 levels (34.62%, 9/26, *P*=0.022). Although the frequency of pneumonia, SBP, and HE increased in the elevated IL-27 group as compared to the normal IL-27 group, no differences were found between them. Interestingly, 10 patients from the elevated IL-27 group (43.48%), but only 4 patients in the normal IL-27 group (15.38%) had at least two types of complications, making the difference between them significant (*P*=0.030). Finally, we calculated the survival of these patients at discharge from our liver unit, and observed a reduced survival in the elevated IL-27 group (69.56%, 16/23) as compared to the normal IL-27 group (92.31%, 24/26, *P*=0.040). Taken together, these data may indicate that IL-27 plays a role in the pathogenesis of the HBV infection.

## Discussion

In this study, our data showed that a significant increase of serum IL-27 levels was found in HBV-infected patients with severe liver inflammation. This increase was especially pro-

nounced in HBV-ACLF patients. More accentuated deterioration in the liver synthetic function was found in patients with IL-27 overproduction than in those with normal IL-27 levels. Moreover, a higher rate of complications and reduced survival were shown in patients with IL-27 overproduction than in those with normal IL-27 levels. Collectively, these data support the idea that IL-27 may play a pro-inflammatory role in the pathogenesis of liver injury in HBV-infected patients with severe liver inflammation.

Accumulating evidence indicates that IL-27, a member of the IL-12 family of cytokines, plays roles in regulating TH cell differentiation. Although IL-27 was originally characterized as a pro-inflammatory cytokine, subsequent reports have focused on its anti-inflammatory effects. However, more recent studies that have examined the influence of IL-27 on regulatory T cells (Treg) revealed that this cytokine has new pro-inflammatory properties. In a T cell transfer model of colitis, the transfer of IL-27ra-/- cells reduced the symptoms of colitis and led to an increased percentage of transferred cells with upregulated Foxp3+ in the gut and secondary lymphoid organs, compared to the transfer of wild-type (WT) cells [24]. In addition to this study, a transgenic mouse model in which the IL-27 p28 and EBI3 subunits are overexpressed (IL-27 tg mice) was used to investigate the impact of IL-27 on Treg cells *in vivo*. IL-27 tg mice almost completely lack Tregs in lymphoid organs and succumb to systemic inflammation [25]. However, when IL-27 is present during the generation of the Treg pool, Treg reconstitution is inhibited [26]. Taken together, these results indicate that IL-27 may be a potent antagonist of Treg cell differentiation. Together with our previous study in which we observed a decrease in circulating Treg cells in severe liver inflammation due to HBV infection [19], this points towards the need to undertake further studies to elucidate the relationship between IL-27 and Treg cells during HBV infection.

Recent studies on IL-27 have focused on disease-prone models and healthy populations, but the role of IL-27 in patients with HBV infection has yet to be understood. Several studies have demonstrated that IL-27 plays a key pathogenic role in liver damage in a T cell-mediated liver injury model and in CHB patients [15, 27]. In addition, two recent studies revealed that IL-27 may act on hepatocytes and hepatic stellate cells, contributing to the antiviral response [28, 29]. Consistent with the previous studies, our data indicate that serum IL-27 levels are significantly increased in HBV-infected patients with severe liver inflammation as compared to healthy controls and CHB-M subjects, and are especially higher in HBV-ACLF patients. In addition, more accentuated deterioration of the liver synthetic function was found in patients with IL-27 overproduction than in those having normal IL-27 levels. Common parameters, such as PTA, ALB, AFP, CHE, Chol, and Trig levels were significantly decreased in the elevated IL-27 group. Moreover, patients with IL-27 overproduction had higher rates of complications and reduced survival as compared to those with normal IL-27 levels. Taken together, these results indicate that IL-27 may play a pro-inflammatory role in the pathogenesis of HBV-associated immunological abnormalities.

In agreement with our study, a recent report indicated that serum IL-27 levels were significantly higher in an HBV-infected group. However, when liver cirrhosis or HCC patients were compared with CHB subjects, IL-27 expression did not show statistically significant differences for a relationship with deteriorating liver conditions [16], which seems to contradict our results. An important explanation to address this paradox could be found in the differences in participants' inflammatory profiles. Patients enrolled in our study, such as the ones with CHB-S and HBV-ACLF, had a more accentuated deterioration in their conditions, and presented more severe liver inflammation than patients with liver cirrhosis or HCC [20]. Considering these pieces of evidence together, we speculate that the impact of IL-27 is perhaps best determined by the immunological context. Thus, further studies are needed to clarify the context in which IL-27 may perform its different roles.

However, two limitations of this study should be noted. First, we aimed to explore the role of IL-27 in patients with severe liver inflammation. As we excluded many patients with a history of immunomodulatory drug treatment prior to registration, only 49 patients remained eligible for this study. Further studies with a larger sample size are definitely needed to confirm our pre-

liminary results. Second, this is only a descriptive study reporting that IL-27 may be pro-inflammatory in patients with severe liver inflammation. Future studies to explore the mechanisms of pathogenesis are therefore urgently needed.

In summary, an increase in serum IL-27 levels in patients with HBV infection suggests that this cytokine may be implicated in the pathogenesis of this disease. Additionally, more accentuated liver synthetic function deterioration, a higher frequency of complications, and reduced survival were found in patients with IL-27 overproduction, suggesting that this cytokine may play a pro-inflammatory role in the development of severe liver damage during the course of the HBV infection. Although further studies are required to clarify the immunologic context in which IL-27 works, this cytokine may be a useful serological marker for disease severity and may become a new therapeutic target.

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## Disclosure of conflict of interest

None.

## Abbreviations

ACLF, acute-on-chronic liver failure; CHB, chronic hepatitis B; NC, normal control; HBV, hepatitis B virus; IL-27, interleukin-27.

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