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# Impaired TLR3/IFN-β signaling in monocyte-derived dendritic cells from patients with acute-on-chronic hepatitis B liver failure: Relevance to the severity of liver damage

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ABSTRACT

Toll-like receptors (TLRs) are a class of proteins that play key roles in innate immunity through recognition of microbial components. TLR3 is expressed abundantly in dendritic cells, and is responsible for recognizing viral pathogens and inducing interferon beta (IFN-β) production. Although TLR3 has been reported to be involved in several diseases caused by viral infections, its role in hepatitis B virus (HBV)-induced hepatitis is still largely unknown. We found that expression of TLR3 and IFN-β was decreased significantly in monocyte-derived dendritic cells (MoDCs) from patients with chronic hepatitis B (CHB, $n = 40$) or acute-on-chronic hepatitis B liver failure (ACHBLF, $n = 60$), compared with normal controls ($n = 20$). We observed a further decrease in TLR3 and IFN-β in ACHBLF compared to CHB patients. Compared with surviving patients, TLR3 and IFN-β expression was significantly lower in non-surviving ACHBLF patients, which strongly indicated a correlation between TLR3 signaling impairment in MoDCs and disease severity in ACHBLF patients. Further linear correlation analysis demonstrated significant correlations between expression of TLR3 signaling components (TLR3 and IFN-β) and disease severity markers (prothrombin activity and total bilirubin) for individual ACHBLF patients. To the best of our knowledge, this is the first study to show that MoDC impairment is correlated with severe liver damage in ACHBLF patients, which suggests the potential of TLR3/IFN-β expression in MoDCs as a diagnostic marker.



## Introduction

Hepatitis B virus (HBV) infection has become one of the most serious public health threats in recent years. Two billion people worldwide have been infected with HBV, and more that 350 million have become victims of chronic HBV infection [1]. Chronic infection in some patients may progress rapidly towards liver failure, a condition referred to as acute-on-chronic hepatitis B liver failure (ACHBLF) [2].

Mechanisms responsible for HBV infection-associated liver damage remain incompletely understood, however, increasing evidence points to a central role for immunological rather than direct viral effects. Over the past few decades, numerous studies have established the role of the acquired immune system in triggering ACHBLF [3,4]. The recent discovery that dendritic cells (DCs) are involved in the pathogenesis of ACHBLF has led to the recognition that the innate immune system may also be important in HBV infection under some circumstances [5–7].

DCs are potent antigen-presenting cells, which display a remarkable capacity to capture antigens in peripheral tissues, process them, and present them to $CD4^+$ and $CD8^+$ T-cells in regional lymph nodes, thereby initiating priming and differentiation of pathogen-specific T-cells [8]. The antigen-presenting ability of DCs is based on their pattern recognition receptors (PRRs) that detect pathogen components [9]. Toll-like receptors (TLRs) are one of the most important categories of PRRs. They are especially abundant on DCs and play a critical role in the pathophysiology of a number of hepatic diseases [10–15]. TLR3 are able to recognize dsRNA, which may be the virus genome or replication intermediate, and play a key role in host anti-viral defenses [16]. Several studies have demonstrated the pivotal position of TLR3 in host responses against a number of viruses (e.g., West Nile virus and respiratory syncytial virus) [17]. Recent research has indicated that TLR ligands inhibits HBV replication in the liver of HBV transgenic mice [11], which suggests that TLR3 may involved in immunity against HBV infection. Besides, TLR3 also has been reported to participate in hepatitis C virus (HCV)-induced cellular activation and

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cytokine production [12]. The expression of TLR3 on peripheral DCs in patients with chronic hepatitis B (CHB) is lower than in healthy individuals [18], which suggests that expression of TLR3 on DCs is related to the ability of the host to combat HBV infection. However, direct evidence to support a correlation between ACHBLF severity and TLR3 expression remains to be discovered.

In this study, we showed that TLR3 expression and IFN-β secretion were reduced significantly in MoDCs from patients with CHB or ACHBLF, and we demonstrated that impaired TLR3 signaling in DCs was associated with liver damage in ACHBLF.

## Materials and methods

*Subjects.* Blood samples were collected from CHB and ACHBLF patients who were diagnosed according to previously described criteria [19]. In particular, the ACHBLF patients had a history of CHB, with serum total bilirubin (TBiL) more than five times the upper limit of normal (⩾85 μmol/L), prothrombin activity (PTA) <40%, recent occurrence of hepatic encephalopathy (⩾grade II), plus ascites or hepato-renal syndrome. They were also negative for other viruses, e.g., HCV, hepatitis D virus and human immunodeficiency virus. Patients had not received any anti-viral treatment or immunotherapy for 6 months prior to blood collection. Normal controls ($n = 20$) (age-, sex- and race-matched) had no evidence of prior exposure to HBV (negative for hepatitis B surface antigen). The basic demographic and clinical characteristics of these subjects are listed in Table 1. Experiments and procedures were in accordance with the Helsinki Declaration of 1975, and approved by the Human Ethics Committee of Shanghai Fudan University.

*Measurement of liver function and serological HBV markers.* Liver function tests such as serum albumin, TBiL and alanine aminotransferase (ALT), and hematological tests including PTA were performed using standard methods in a clinical setting. Hepatitis B serological markers were tested using a commercially available radioimmunoassay (Abbott). The level of HBV DNA was quantified using a DNA assay (PG BIOTECH; sensitivity >500 copies/ml).

*Generation of monocyte-derived DCs (MoDCs).* Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized whole blood via Ficoll density-gradient centrifugation. PBMCs were separated into monocytes and lymphocytes by immunomagnetic anti-CD14-beads, according to the manufacturer's instructions (Miltenyi). The purity of $CD14^+$ monocytes was always >90%. Monocytes were cultured with 1000 μ/ml recombinant human (rh) granulocyte macrophage colony-stimulating factor and 500 μ/ml rh interleukin-4 (PeproTech) in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco) for 6 days, as described previously [20]. The medium was replenished with cytokines every 2 days. DC maturation was induced by addition of 50 μg/ml poly(I:C) (Sigma). This concentration of poly(I:C) was established as optimal in preliminary experiments. Cells were spun down and supernatants assayed for IFN-β as described later.

*Flow cytometric analysis.* Expression of surface markers was analyzed by flow cytometry using conjugated monoclonal mouse anti-human antibodies: fluorescein isothiocyanate anti-human CD83, phycoerythrin (PE)-Cy5 anti-human CD80, PE-Cy5 anti-human CD86, PE-anti-human HLA-DR, and PE anti-human TLR3 (eBiosciences). Flow cytometry analysis was performed on FACSCalibur (BD Biosciences) instruments and analyzed using FlowJo software (Tree Star Inc.).

*RNA isolation and quantitative real-time PCR (qPCR).* Total RNA was isolated using Trizol reagent (Invitrogen), following the manufacturer's protocol. The RNA was then reverse transcribed to cDNA using random hexamer primers at 25 °C for 10 min, 42 °C for 1 h, and 70 °C for 10 min, according to manufacturer's protocol (Fermentas). Subsequently, cDNA was added to a PCR mixture that contained 7.5 μl SYBR Green Supermix (TOYOBO) and 1 μl of each sense and antisense primers (Invitrogen) in a total volume of 15 μl. After an initial denaturation step at 95 °C for 1 min, amplifications consisted of 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s (ABI Prism 7900HT Sequence Detection System). Primer pairs used were specific for human TLR3 (forward 5′GCTGGAAAATCTCCAAGAGC3′; reverse 5′CTTCCAATTGCGTGAAAACA3′; amplicon: 159 bp), RIG-I (forward 5′ATGGGACGAAGCAGTATTTAG3′; reverse 5′GCTTGGGATGTGGTC TACTC3′; amplicon: 92 bp) and β-actin (forward 5′GGGAAATCGT GCGTGACAT3′; reverse 5′GTCAGGCAGCTCGTAGCTCTT3′; amplicon: 106 bp). β-actin was amplified as an internal control in each PCR. Both melting-curve and gel-migration analyses were used to ensure the absence of non-specific primer–dimer products. The relative amounts of PCR product were determined using the comparative Ct method ($2^{-\Delta\Delta C_T}$).

*ELISA.* MoDCs ($2 \times 10^5$ cells/well) were stimulated for 16 h with poly(I:C), and supernatants were collected and used to assess the secretion of IFN-β using a commercial ELISA kit (USCN) according to the manufacturer's instructions. The absorbance was read at 450 nm using an ELISA plate reader.

*T-cell proliferation assay.* $CD4^+$ T lymphocytes were purified from PBMCs with magnetic beads (Miltenyi). Mixed lymphocyte reactions were performed by co-incubation of mature MoDCs isolated from eight ACHBLF patients, 10 CHB patients, and six normal controls with $CD4^+$ T lymphocytes isolated from four allogeneic healthy buffy coat donors. Cell proliferation was determined using CCK-8 solution (Beyotime) according to the manufacture's instructions.

*Statistical analysis.* All data were analyzed using SPSS 11.0 software. Values were expressed as means ± SEM. Student's *t* test was

**Table 1**
Baseline characteristics of the study subjects.

| Patients | Health control | CHB | ACHBLF |
|---|---|---|---|
| Cases | 20 | 40 | 60 |
| Ages (years) | 32 (21–57) | 34 (21–67) | 40.9 (14–80) |
| Sex (m/f) | 13/7 | 27/13 | 51/9 |
| eAg/eAb | NA | 22/18 | 18/42 |
| ALT (U/L) | 25.3 (11–37) | 108.5 (46–410) | 642.3 (37–4120) |
| ALB (g/L) | NA | 42.6 (32–53) | 35.3 (25.8–41.6) |
| TBiL (μmol/L) | NA | 11.8 (2.4–33.9) | 450.1 (103.3–845.1) |
| PTA (%) | NA | 93 (64–125) | 31.9 (14–64.5) |
| HBV DNA ($10^4$ copies/ml) | NA | 468 (0.3–8620) | 101.5 (<0.05–500) |
| Encephalopathy grade III/IV (%) | NA | 0 | 31.67 |
| Ascites (%) | NA | 0 | 86.67 |
| Mortality (%) | NA | 0 | 58.33 |

NA, not applicable. Data are presented as median and range. ALT, alanine transaminase; ALB, albumin; TBiL, total bilirubin; PT, prothrombin time; PTA, prothrombin activity.

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used for the comparison of continuous variables between two groups. The Wilcoxon matched-pairs *t* test was used to compare data from the same individuals. Correlations were determined using Pearson's correlation test. Differences were considered statistically significant when $P$ was <0.05.

## Results

### *Decreased expression of cell surface molecules and impaired T-cell stimulation in MoDCs from CHB and ACHBLF patients*

MoDCs are a widely-used *in vitro* model for studying DC maturation and function [20]. Therefore, MoDCs were differentiated with monocytes isolated from human blood samples (Table 1). Successful differentiation of MoDCs was confirmed by detecting the expression of HLA-DR, CD83, CD86 and CD80, the surface markers of DCs [21]. In premature MoDCs, only HLA-DR was highly expressed (data not shown), while in mature MoDCs, a marked induction of HLA-DR, CD83, CD86 and CD80 was observed as expected (Fig. 1A–D). Expression levels of these markers were significantly lower in MoDCs from CHB and particularly ACHBLF patients, as compared with normal controls (Fig. 1E).

The decreased expression of cell surface markers may have been associated with impaired function of MoDCs from CHB and ACHBLF patients. To validate this hypothesis, we tested MoDC function by measuring their ability to induce proliferation of $CD4^+$ T-cells. As expected, MoDCs from CHB and particularly ACHBLF patients induced only weak proliferation of $CD4^+$ T-cells (Fig. 1F). These results demonstrated the impaired function of MoDCs from patients with HBV infection, and the severe phenotype was associated with an aggravated disease state.

### *Impaired TLR3 signaling in MoDCs from CHB and ACHBLF patients*

We explored the underlying mechanism that leads to the functional impairment of MoDCs. Mature MoDCs are induced by poly(I:C), a synthetic analog of dsRNA [21]. The response to dsRNA involves two distinct pathways, through binding to TLR3 or retinoic acid inducible gene-I (RIG-I) [22]. We analyzed expression of TLR3 and RIG-I in MoDCs from patients and controls. The protein level of TLR3 in MoDCs was analyzed by flow cytometry using the characterized antibody against human TLR3. TLR3 was significantly lower in MoDCs from CHB and ACHBLF patients, as indicated by mean fluorescence intensity (MFI). We also observed a further decrease in TLR3 expression in MoDCs from ACHBLF patients compared to that from CHB patients (Fig. 2A and B). The significant downregulation of TLR3 in patient-derived MoDCs was further confirmed by qPCR analysis (Fig. 2C and D). However, the expression level of RIG-I showed no obvious change among the three groups (Fig. 2E).

In MoDCs, poly(I:C) stimulation of TLR3 activates interferon regulatory factor three and leads to the production of IFN-β [23]. Therefore, decreased TLR3 level is expected to impair TLR3 signaling and further influence downstream IFN-β production. We ana-



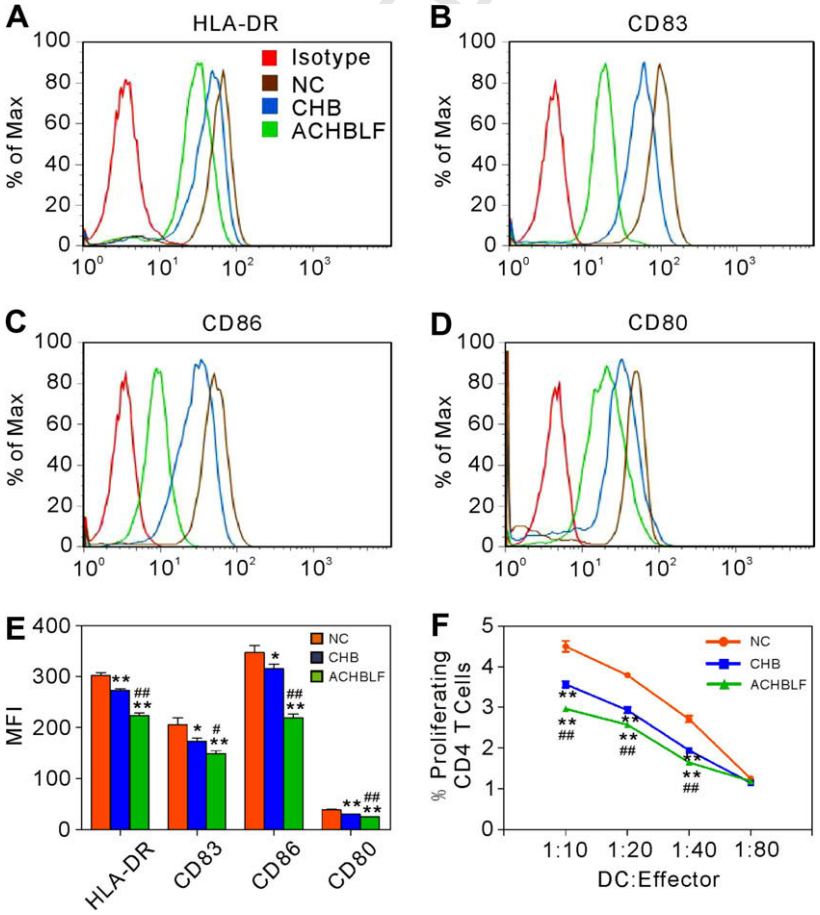


**Fig. 1.** The phenotypic profiles and function of MoDCs from normal controls and patients with CHB or ACHBLF. (A–D) Flow cytometric analysis of CD83, HLA-DR, CD86 and CD80 expression in mature MoDCs from CHB and ACHBLF patients. Representative histograms (A–D) and MFI (E) are shown. (F) Induction of $CD4^+$ T-cell proliferation by MoDCs. Data are mean ± SEM from three independent experiments. $^*P < 0.05$, $^{**}P < 0.005$, versus normal control; $^{\#}P < 0.05$, $^{\#\#}P < 0.005$ versus CHB group.

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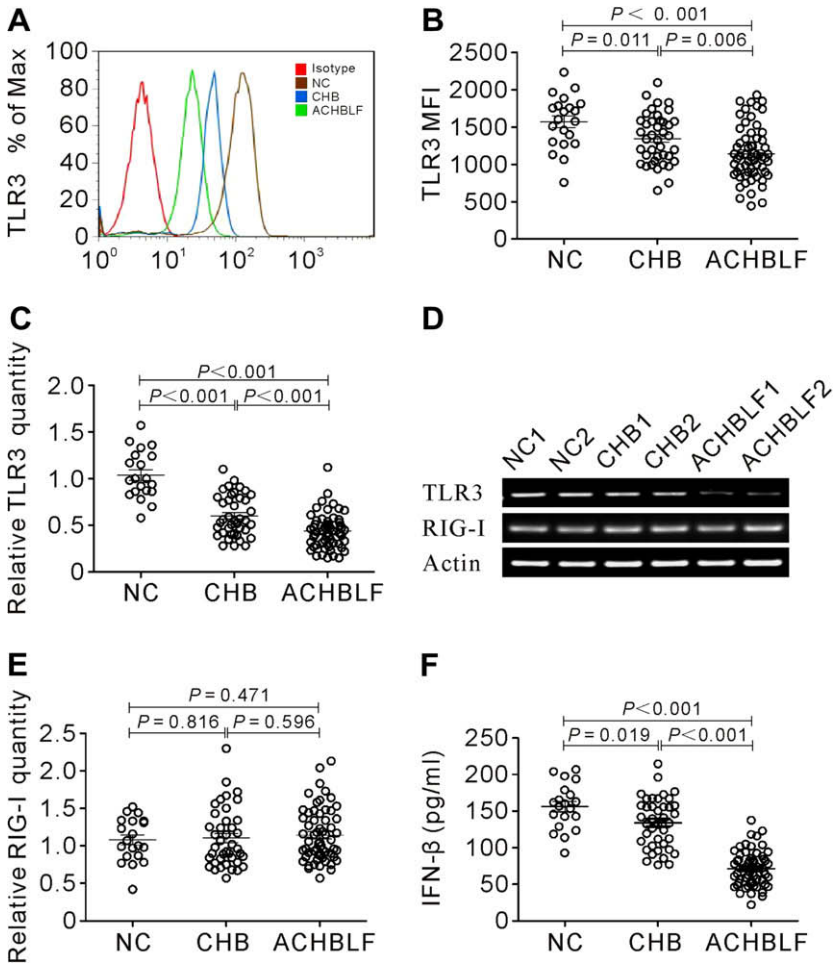


**Fig. 2.** Expression levels of TLR3, RIG-I and IFN-β in MoDCs from normal controls and patients with CHB or ACHBLF. (A,B) Flow cytometric analysis of TLR3 protein level. Representative histogram (A) and MFI (B) are presented. (C) qPCR analysis of TLR3 mRNA level. (D) Agarose gel electrophoresis analysis of the amplification products of TLR3, RIG-I and the house keeping gene β-actin. (E) qPCR analysis of RIG-I mRNA level. (F) ELISA of IFN-β secretion in the supernatants from cultured MoDCs. Data are mean ± SEM from three independent experiments.

lyzed IFN-β production in cultured MoDCs, and ELISA indicated decreased IFN-β production in MoDCs from patients, especially those with ACHBLF (Fig. 2F). These results demonstrated that the TLR3/IFN-β signaling was impaired in MoDCs from CHB and ACHBLF patients, which probably led to the functional impairment of MoDCs in these patients.

*Decreased expression of TLR3, and IFN-β in MoDCs from non-surviving patients*

Since a more severe impairment of function and TLR3/IFN-β signaling was detected in MoDCs from patients with ACHBLF, which is an aggravated disease state, we speculated that impaired TLR3/IFN-β signaling in MoDCs may be associated with severity of ACHBLF. We grouped the ACHBLF patients into survivors and non-survivors, which represented different degrees of severity, and analyzed the expression of TLR3 and IFN-β in MoDCs. The expression of both proteins was significantly downregulated in non-survivors versus survivors (Fig. 3A–C). However, no obvious differences were observed when the patients were divided into hepatitis B e antigen $(HBeAg)^+$ and $HBeAg^-$ groups (Fig. 3A–C). Additionally, we found no changes in RIG-I expression between survivors and non-survivors, or between $HBeAg^+$ and $HBeAg^-$ groups (Fig. 3A). These data strongly suggested that the progressive impairment of TLR3/IFN-β signaling may have been correlated with the deterioration of ACHBLF.

*Linear correlation between TLR3/IFN-β expression in MoDCs and disease severity markers in ACHBLF patients*

Among the clinical parameters for ACHBLF evaluation, HBV load is correlated positively with virus replication, and ALT represents hepatic function. In addition, jaundice and PTA are considered essential criteria for the diagnosis of acute-on-chronic liver failure (ACLF) [19]. These conditions may be responsible partly for the high morbidity and mortality recorded in ACHBLF patients. To confirm the correlation between TLR3 signaling and disease severity in ACHBLF, linear correlation analysis was performed. The results showed that TLR3 expression and IFN-β induction in cultured MoDCs was correlated positively with PTA but negatively with TBiL (Fig. 4A–D). However, TLR3/IFN-β expression was not correlated with serum ALT level or HBV load (Fig. 4E–H). These results demonstrated that an impaired TLR3/IFN-β signaling pathway in MoDCs was correlated negatively with disease severity in ACHBLF patients.

## Discussion

To the best of our knowledge, this is the first study to analyze the involvement of TLR3 in HBV infection, by using MoDCs from CHB and ACHBLF patients, and to demonstrate that TLR3 was downregulated markedly in MoDCs from these patients. The decrease in TLR3 correlated significantly with aggravated ACHBLF. Furthermore, production of IFN-β, the downstream cytokine of

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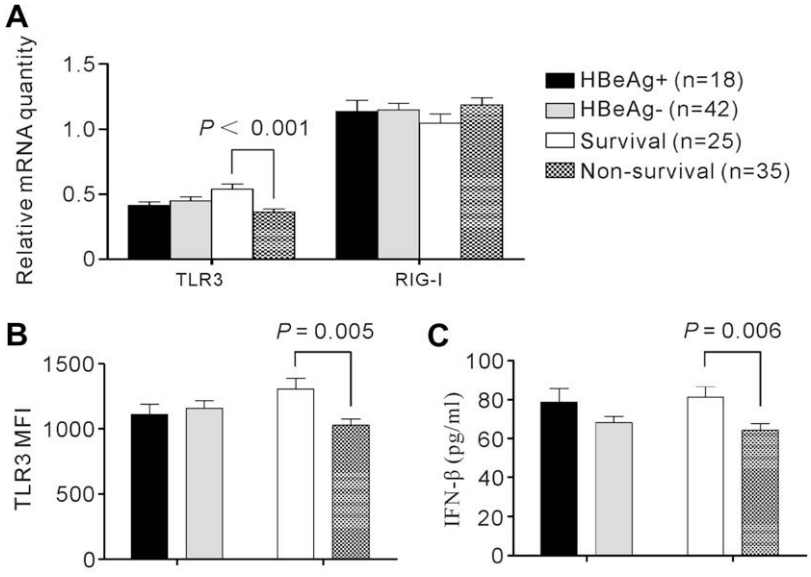


**Fig. 3.** Expression levels of TLR3, RIG-I and IFN-β in MoDCs from different groups of ACHBLF patients. (A) qPCR analysis of TLR3 and RIG-I mRNA level in MoDCs from different groups of ACHBLF patients. (B) Flow cytometric analysis of TLR3 protein level in MoDCs from different groups of ACHBLF patients. (C) Induction of IFN-β in MoDCs from different groups of ACHBLF patients. Data are mean ± SEM from three independent experiments.

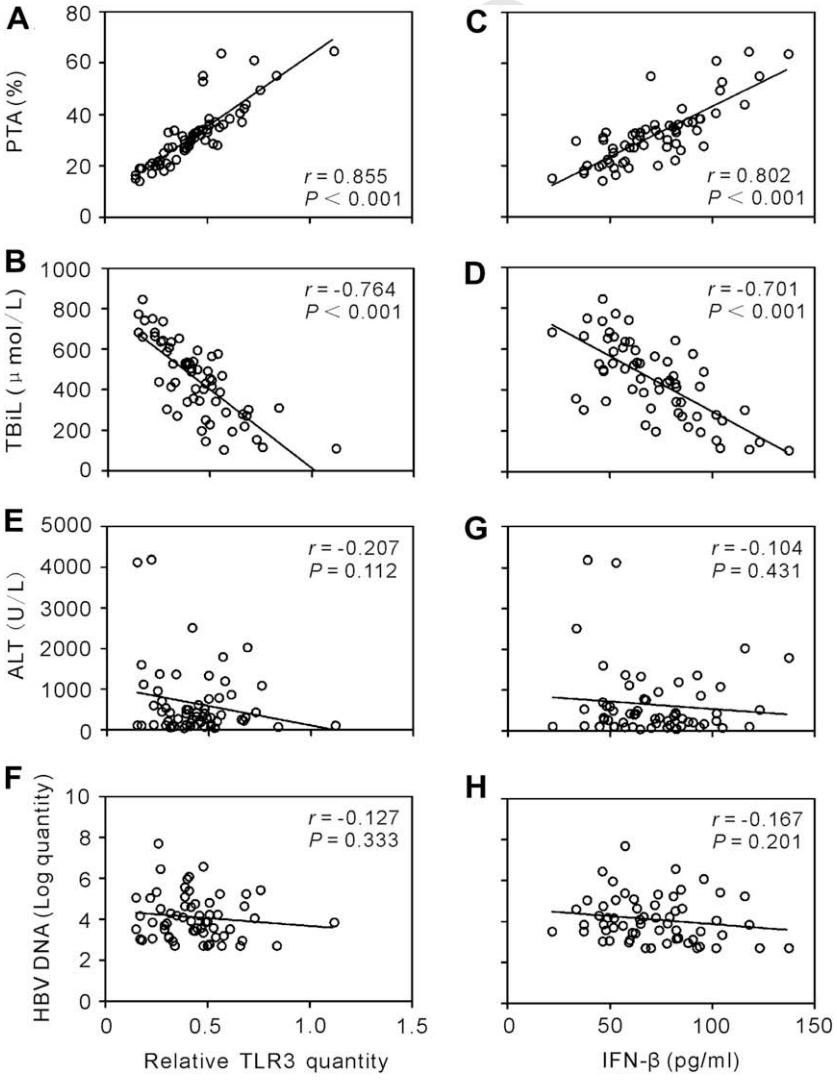


**Fig. 4.** Linear correlation analysis between TLR3/IFN-β levels in MoDCs and clinical parameters in individual ACHBLF patients. (A, B, E and F) Linear correlation between TLR3 and different clinical parameters. (C, D, G and H) Linear correlation between IFN-β and different clinical parameters.

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TLR3 signaling, was also reduced in MoDCs and negatively correlated with disease severity in ACHBLF patients.

ACLF is a severe life-threatening condition, and liver transplantation is the only available therapeutic option. Reactivation of HBV infection is one of the major causes of ACLF in the Asian region [24]. Recently, innate immune recognition at the initial stage of virus infection has shown its predominant role in priming and regulating the inflammatory response through PRRs [9,25]. Functionally impaired DCs play important roles in suppressing host immune responses and facilitating viral persistence in CHB. However, little is known regarding the status of MoDCs in HBV infection. Here, we observed that the expression of cell surface molecules was decreased significantly and was accompanied by marked impairment of the T-cell stimulation of MoDCs from CHB and ACHBLF patients. These data confirm the important function of DCs in host immunity against HBV infection.

TLRs capture signals derived from viral particles and subsequently initiate signaling for inflammatory cytokine responses, such as IFN-α/β and tumor necrosis factor-α production [26]. TLR2 and TLR3 have been reported to participate in HBV- and HCV-induced cellular activation and cytokine production [12,27,28]. In addition, TLR3 signaling has been highlighted during West Nile virus infection, not only for eliciting cellular anti-viral activity, but also for shaping a detrimental innate and adaptive immune response [29]. We confirmed that TLR3 was decreased significantly in HBV-infected human MoDCs, especially from ACHBLF patients. As a downstream target of TLR3, IFN-β was also found to be decreased significantly in the same samples. These results suggest that HBV infection can alter the innate immune response by downregulation of the TLR3/INF-β signaling pathway, which may contribute to aggravation of chronic infections.

Population based prospective studies are required to focus on the contribution of host (innate immune status) and virus (viral load or genotype) factors to treatment outcome, which may help to design possible therapeutic targets and identify diagnostic markers for ACHBLF. In this study, we demonstrated by linear correlation analysis that TLR3 expression and IFN-β induction in cultured MoDCs were correlated positively with PTA, but negatively with TBiL. However, the direct evidence support TLR3 functionally involved in HBV infection need further verification.

In conclusion, we demonstrated a correlation between impaired TLR3 signaling in MoDCs and disease severity in ACHBLF patients. This indicates that TLR3 expression and IFN-β production in MoDCs may serve as diagnostic markers for ACHBLF.

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