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Upregulated Expression of A20 on Monocytes is Associated With Increased Severity of Acute-on-Chronic Hepatitis B Liver Failure

A Case-Control Study

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Abstract: A20 expression is increased in various inflammatory diseases. However, the role of A20 in acute-on-chronic liver failure is unknown. This study was to evaluate A20 expression on monocytes and its associations with the severity of acute-on-chronic hepatitis B liver failure (ACHBLF).

Thirty-seven patients with ACHBLF, 20 patients with chronic hepatitis B (CHB), and 15 healthy controls (HC) were enrolled in this case-control study. A20-positive monocytes were identified using flow cytometry. Serum levels of interleukin (IL)-10, IL-12p70, and TNF-α were determined using bead cytometry. A20 and IL-10 expressions were examined in THP-1 cells stimulated by lipopolysaccharide (LPS).

The frequency of $A20^+$ monocytes was significantly increased in patients with ACHBLF compared with HC (median [interquartile range, IQR]: 15.7 [22.8]% vs 2.5 [4.7]%, $P < 0.001$). Increased monocyte A20 expression was detected during the progression phase (including the mild/moderate and severe grades of ACHBLF) compared with patients in the recovery phase (both $P < 0.05$), and in the ACHBLF worsening group compared with patients in the improvement group ($P < 0.001$). LPS treatment upregulated A20 and IL-10 expressions in THP-1 cells. A20 expression on monocytes from patients with ACHBLF was positively correlated with total bilirubin ($r = 0.60$, $P = 0.0001$), direct bilirubin ($r = 0.63$, $P < 0.0001$), and MELD score ($r = 0.43$, $P = 0.008$), and inversely with prothrombin activity ($r = -0.33$, $P = 0.046$). IL-10 and TLR4 expression levels in monocytes, and serum levels of IL-10, IL-12p70, and TNF-α were increased in patients with ACHBLF compared with patients with CHB and HC.

Increased A20 expression on monocytes was associated with the severity of ACHBLF.

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Abbreviations: A20 or TNFAIP3 = tumor necrosis factor (TNF)-α-induced protein, ACHBLF = acute-on-chronic hepatitis B liver failure, AFP = alpha-fetoprotein, AIH = autoimmune hepatitis, ALB = albumin, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate transaminase, CHB = chronic hepatitis B, DBIL = direct bilirubin, ELISA = enzyme-linked immunosorbent assay, GGT = gamma glutamyl transpeptidase, GLB = globulin, HBeAg = Hepatitis B e Antigen, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, HC = healthy controls, HIV = human immune deficiency virus, IBIL = indirect bilirubin, IL-10 = interleukin-10, IL-12 = interleukin-12, IL-6 = interleukin-6, INR = international normalized ratio, M cells = monocytes, MELD = model for end-stage liver disease, PBMC = peripheral blood mononuclear cell, PT = pro-thrombin time, PTA = prothrombin activity, TBIL = total bilirubin, TNF-α = transforming growth factor-α, TP = total protein.

INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, with a seroprevalence varying from 1.7% (Latin America) to 11.3% (East Asia and Pacific).[1] HBV infection is associated with an increased risk of cirrhosis, hepatic decompensation, and hepatocellular carcinoma.[2] Hepatic decompensation may be gradual or acute, which is termed acute-on-chronic liver failure (ACLF).[3] Patients with chronic hepatitis B (CHB) may experience a severe acute exacerbation of the disease that progresses into liver failure, which is defined as acute-on-chronic hepatitis B liver failure (ACHBLF), manifesting as jaundice and coagulopathy (international normalized ratio [INR] > 1.5) complicated within 4 weeks by ascites and/or encephalopathy in a patient with chronic liver disease.[3,4] Liver transplantation is the only curative therapeutic option for ACLF, with a 5-year survival rate of 85%. However, infectious complications often preclude transplant in patients with ACLF, and many patients die while being on the waiting list.[5] In addition, many patients with ACHBLF die despite significant reduction of HBV DNA, although antiviral treatment might provide some short-term survival benefits.[6] Some risk factors for mortality were identified, including pre-existing cirrhosis, prolonged prothrombin time, elevated bilirubin, low albumin levels, low platelet counts, and the presence of encephalopathy/ascites.[3] Different models have also been proposed,[3] including the model for end-stage liver disease (MELD).[7]

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The pathogenesis of ACLF is strikingly similar to the characteristics of septic shock, which is characterized by progressive vasodilatory shock and multiple organ failure.[8] Liver dysfunction leads to several immunological abnormalities because both humoral immunity and cell-mediated immunity are depressed. The inappropriate inflammatory response and immune dysfunction in ACLF increase the susceptibility to infections.[9] Some evidence suggests that the inflammatory mechanisms observed in ACLF are not specific to HBV infection.[3] In ACLF, HLA-DR is depressed in monocytes, as well as monocyte activation, leading to an overproduction of anti-inflammatory cytokines (such as interleukin [IL]-10), and to the activation and upregulation of toll-like receptors (TLR, such as TLR4).[3,6,10] Therefore, monocytes play an important role in the pathogenesis of ACLF, and deactivation of monocytes directly influences the outcomes of these patients.[3,11]

A20, also known as the tumor necrosis factor (TNF)-α-induced protein (TNFAIP3), was identified in endothelial cells as a primary response gene induced upon treatment with TNF-α.[12,13] The ubiquitin-modifying enzyme A20 is widely accepted as a key regulator of inflammation and immunity.[14–16] Previous studies demonstrated that A20 expression is increased in various inflammatory diseases.[17,18] A20-deficient mice are hypersensitive to TNF-α and die prematurely because of severe multiorgan inflammation and cachexia.[17] A20 expression is strongly induced by multiple stimuli including the proinflammatory cytokines TNF-α and lipopolysaccharide (LPS) and microbial products that trigger pathogen recognition receptors such as TLRs[19–21] and may play a role in CD14 cell immunoparalysis, which leads to multiple organ failure.[22] During endotoxemia, previous studies have shown that TNF-α first peaks, followed by peaks of A20 and IL-10 levels.[23,24]

However, the exact expression of A20 in patients with ACHBLF has not been fully elucidated. The hypothesis of the present study was that increased endotoxemia, LPS, TNF-α, and inflammatory cytokines let to increased A20 levels, which in turn induced CD14 cell immunoparalysis, increased IL-10 levels, and then multiple organ failure. Therefore, the present study aimed to evaluate the expression of A20 and cytokines in monocytes and to investigate A20 levels at different stages of ACHBLF and the association of A20 with ACHBLF severity. The present study might improve our understanding of functions of A20 and might provide useful clues for future treatments for ACHBLF.

METHODS

Participants

This was a case-control study carried out in 37 patients with ACHBLF; 20 patients with CHB and 15 healthy controls who visited the Tangdu Hospital of the Fourth Military Medical University (China) between July 2012 and December 2013 were enrolled in this study.

Patients with ACHBLF had a medical history of CHB, serum total bilirubin (TBIL) levels > 5 times the upper limit of normal (ULN), and prothrombin activity (PTA) $< 40\%$.[4] Among the 37 patients with ACHBLF, 10 were in the recovery phase. All patients with ACHBLF received conventional inpatient treatment including antiviral treatments and vital sign support according to the Asian Pacific Association for the Study of the Liver (APASL)[4] and the 2012 liver failure diagnosis and treatment guidelines.[25] Exclusion criteria were: (1) history of alcohol abuse; (2) intravenous drug abuse; (3) pregnancy; (4) concomitant chronic hepatitis C virus or human immune deficiency virus (HIV) infection; (5) inflammatory disease; (6) autoimmune hepatitis; (7) metabolic liver diseases; (8) hepatocellular carcinoma; or (9) use of drugs affecting immune parameters such as corticosteroids or immunosuppressive medications within the last 3 months before the study entry.

Patients with CHB were positive for hepatitis B surface antigen and presented serum ALT levels 2 to 10 times the ULN, according to the Chinese Guidelines for the prevention and treatment for CHB (2010 version).[26]

Healthy controls had to be negative for hepatitis B surface antigen.

Patients with ACHBLF were divided into the mild/moderate subgroup (TBIL ≥ 171 μmol/L or TBIL increased ≥ 17.1 μmol/L/d; $20\% < \text{PTA} \leq 40\%$ or $1.5 < \text{INR} \leq 2.6$; no complication or hepatic encephalopathy lower than the second degree) or the severe subgroup (PTA $\leq 20\%$ or INR ≥ 2.6, severe complications).[25] Patients with ACHBLF were further divided into improvement subgroup (including recovery phase patients) and worsening subgroup, according to the 2012 Liver failure diagnosis and treatment guidelines.[25]

The present study was approved by the Research and Ethical Committee of the Tangdu Hospital of the Fourth Military Medical University. All participants provided a written informed consent before the collection of blood samples, in accordance with the Declaration of Helsinki.

Outcomes

The primary outcome was monocyte A20 expression. Secondary outcomes were the effect of LPS on A20 upregulation in monocytes, correlations between A20 expression and other cytokines, and the association between A20 expression and ACHBLF.

Liver Function

TBIL and hematological tests were performed using standard methods in a clinical setting. Hepatitis B markers were tested using a commercial radioimmunoassay (Kechuang, Shenzhen, China). HBV DNA levels were quantified using a real-time polymerase chain reaction (PCR) assay (Qiagen, Shenzhen, China), with a lower limit of detection of 100 copies/mL. The MELD score was used to assess the participants.[7]

Flow Cytometry Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll–Hypaque gradient (Sigma, St Louis, MO), according to the manufacturer's protocol. The PBMCs were stained with the appropriate antibody or isotype-matched control antibody for 30 min at room temperature (RT) in the dark. Anti-CD14-PE, anti-CD14-PerCP Cy5.5, anti-IL-10-PerCP Cy5.5, anti-TLR4-PE and isotype-matched control antibodies were purchased from BD Biosciences (San Jose, CA). Intracellular cytokine staining was performed using the Cytofix/Cytoperm™ fixation/permeabilization kit (Cat. No. 554714; BD Biosciences, San Jose, CA).

PBMCs were stimulated with 1 μg/mL of LPS (Sigma, St Louis, MO) as TLR4 ligand and 2.5 ng/mL of the TLR7/8 ligand R848 (Sigma, St Louis, MO) for 6 h. Brefeldin A (BD Biosciences, San Jose, CA) and ionomycin (1.71 g/mL; BD Biosciences, San Jose, CA) were added 5 h before harvesting the cells to prevent cytokine secretion. Stained cells were analyzed on an Arial II flow cytometer with the FlowJo 7.6 software (BD Biosciences, San Jose, CA).

After being stained with anti-CD14-PerCP Cy5.5 antibody, PBMCs were fixed with 4% paraformaldehyde (10 min), permeabilized with 0.1% phosphate buffered serum (PBS)-Tween-20 for 20 min, and incubated in 1× PBS, 10% normal goat serum, 0.3 M glycine, and 5% bovine serum albumin to block nonspecific protein–protein interactions. Cells were then incubated with the rabbit antihuman A20 antibody (ab92324, 1/50 dilution; Abcam, Cambridge, UK) for 30 min at RT. A DyLight 488 goat antirabbit IgG secondary antibody (1/500; Abcam, Cambridge, UK) was applied for 30 min at RT. The isotype control antibody was rabbit IgG (monoclonal) (1 μg/1 × 10^6 cells), which was applied under the same conditions. More than 50,000 events were acquired. Stained cells were analyzed on a multicolor Arial II flow cytometer with the FACSDiva version 6.1.3 (BD Biosciences) and FlowJo 7.6 software.

Cytometric Bead Assay

Serum levels of IL-10, IL-12p70, and TNF-α were determined using a human cytometric bead array kit (CBA, BD Biosciences, San Jose, CA), according to the manufacturer's instructions.

A20 and IL-10 Expression Levels in THP-1 Cells Stimulated with LPS

The monocyte cell line THP-1 was purchased from the Shanghai Institute of Life Science Cell Resource Center (China) and was cultured in RPMI 1640 (Hyclone, Utah, Logan City) containing 10% fetal calf serum (Gibco, Carlsbad, CA) and antibiotics. All cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C. THP-1 cells (0.5×10^6 cells/mL) were plated in a 12-well plate (Corning Inc, Corning, NY), were cultured to confluence, and were stimulated with LPS (100 ng/mL) for 12 h.[27] THP-1 cells were used to detect the expression of A20 and IL-10 before or after LPS stimulation by flow cytometry.

STATISTICAL ANALYSIS

All experiments were carried out by technicians blinded to the clinical data of the patients. All statistical analyses were conducted using SPSS 19.0 (IBM, Armonk, NY). Normally distributed data are expressed as means ± standard deviation (SD). Comparisons between groups were performed using independent samples *t*-tests, and intragroup comparisons were performed using 1-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Nonnormally distributed date is expressed as median (interquartile range, IQR). Comparisons between groups were performed using the Mann–Whitney *U* test. Intragroup comparisons were performed using the Kruskal–Wallis *H* test. The Pearson correlation coefficient analysis was used for the correlations between A20 expression and clinical parameters of patients with ACHBLF. P values < 0.05 0.05 were considered statistically significant.

RESULTS

Characteristics of the Participants

The 3 groups were comparable for sex distribution and age. All biochemical parameters were significantly worst in the ACHBLF group compared with the CHB group (all $P < 0.05$) (Table 1). In the ACHBLF group, the mean MELD score was 23.6 ± 0.74.

TABLE 1. Characteristics of the Participants

	ACHBLF (n = 37)	CHB (n = 20)	HC (n = 15)	*P* ACHBLF vs CHB
Sex, male/female	31/6	15/5	11/4	0.49
Age (year)	43.8 ± 8.9	40.4 ± 5.0	38.2 ± 5.5	0.11
HBsAg/HBeAg (+)	+	+	-	–
HBV-DNA log10 cps/ml	4.61 ± 0.20	6.65 ± 0.31	NA	< 0.0001
TBIL (μmol/L)	281.08 ± 34.11	43.97 ± 9.63	14.58 ± 2.11	<0.0001
DBIL (μmol/L)	190.65 ± 23.62	24.29 ± 6.26	8.65 ± 1.23	<0.0001
IBIL (μmol/L)	85.76 ± 12.34	19.68 ± 3.62	6.05 ± 0.89	<0.0001
ALT (IU/mL)	445.74 ± 120.40	197.70 ± 47.02	21.04 ± 2.40	<0.0001
AST (IU/mL)	289.36 ± 60.02	157.9 ± 41.53	18.96 ± 1.02	<0.0001
TP (g/L)	63.79 ± 1.45	65.00 ± 1.61	NA	0.02
ALB (g/L)	35.32 ± 0.89	40.15 ± 1.11	NA	<0.0001
GLB (g/L)	30.31 ± 0.76	24.48 ± 1.39	NA	<0.0001
PT (s)	22.17 ± 2.16	12.06 ± 0.50	NA	<0.0001
PTA (%)	51.17 ± 4.95	88.85 ± 4.79	NA	<0.0001
INR	1.97 ± 0.20	1.12 ± 0.05	NA	<0.0001
GGT (U/L)	95.42 ± 10.91	77.04 ± 15.54	NA	<0.0001
ALP (IU/L)	148.76 ± 13.86	117.3 ± 22.14	NA	<0.0001
AFP (ng/mL)	178.08 ± 21.06	71.86 ± 8.47	NA	<0.0001
Blood glucose (mmol/L)	5.33 ± 0.44	5.65 ± 0.48	NA	0.01
Serum creatinine	5.65 ± 0.87	NA	NA	–
MELD score	23.6 ± 0.74	NA	NA	–

Note. The data are shown as mean ± standard deviation (SD). ACHBLF = acute-on-chronic hepatitis B liver failure, AFP = alpha-fetoprotein, ALB = albumin, ALP = alkaline phosphatase, ALT = alanine transaminase, AST = aspartate transaminase, CHB = chronic hepatitis B, DBIL = direct bilirubin, GGT = gamma glutamyl transpeptidase, GLB = globulin, HBeAg = Hepatitis B e Antigen, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, HC = healthy controls, IBIL = indirect bilirubin, INR = international normalized ratio, MELD = model for end-stage liver disease, NA = not available, PT = pro-thrombin time, PTA = prothrombin activity, TBIL = total bilirubin, TP = total protein, – = Not available.

Increased Frequency of A20 Expression on Monocytes in Patients with ACHBLF

The frequency of A20 expression on monocytes was significantly higher in the ACHBLF group compared with healthy controls (median (IQR), 15.7 (22.8)% vs 2.5 (4.7)%, $P < 0.001$). The frequency in patients with CHB was not significantly different compared to healthy controls ($P > 0.05$) (Figure 1).

The Frequency of A20 Expression on Monocytes Correlates with Different Disease Phases and Disease Progression

Increased A20 expression was detected during the progression phase, including the mild/moderate (n = 15, 17.4 [18.8]%) and severe grades of ACHBLF (n = 12, 31.2 [23.5]%), compared with patients in the recovery phase of ACHBLF (n = 10, 4.0 [7.1]%, all $P < 0.05$) (Figure 2A). The A20 expression levels in the ACHBLF worsening group (n = 15, 30.1 [26.1]%) were higher than in the improvement group (n = 22, 6.8 [17.5]%, $P < 0.001$) (Figure 2B).

Correlations Between A20 Expression and Clinical Parameters of ACHBLF Patients

A20 expression in CD14 cells from patients with ACHBLF was positively correlated with total bilirubin ($r = 0.60$, $P = 0.0001$), direct bilirubin ($r = 0.63$, $P < 0.0001$) levels, and MELD score ($r = 0.43$, $P = 0.008$), and inversely correlated

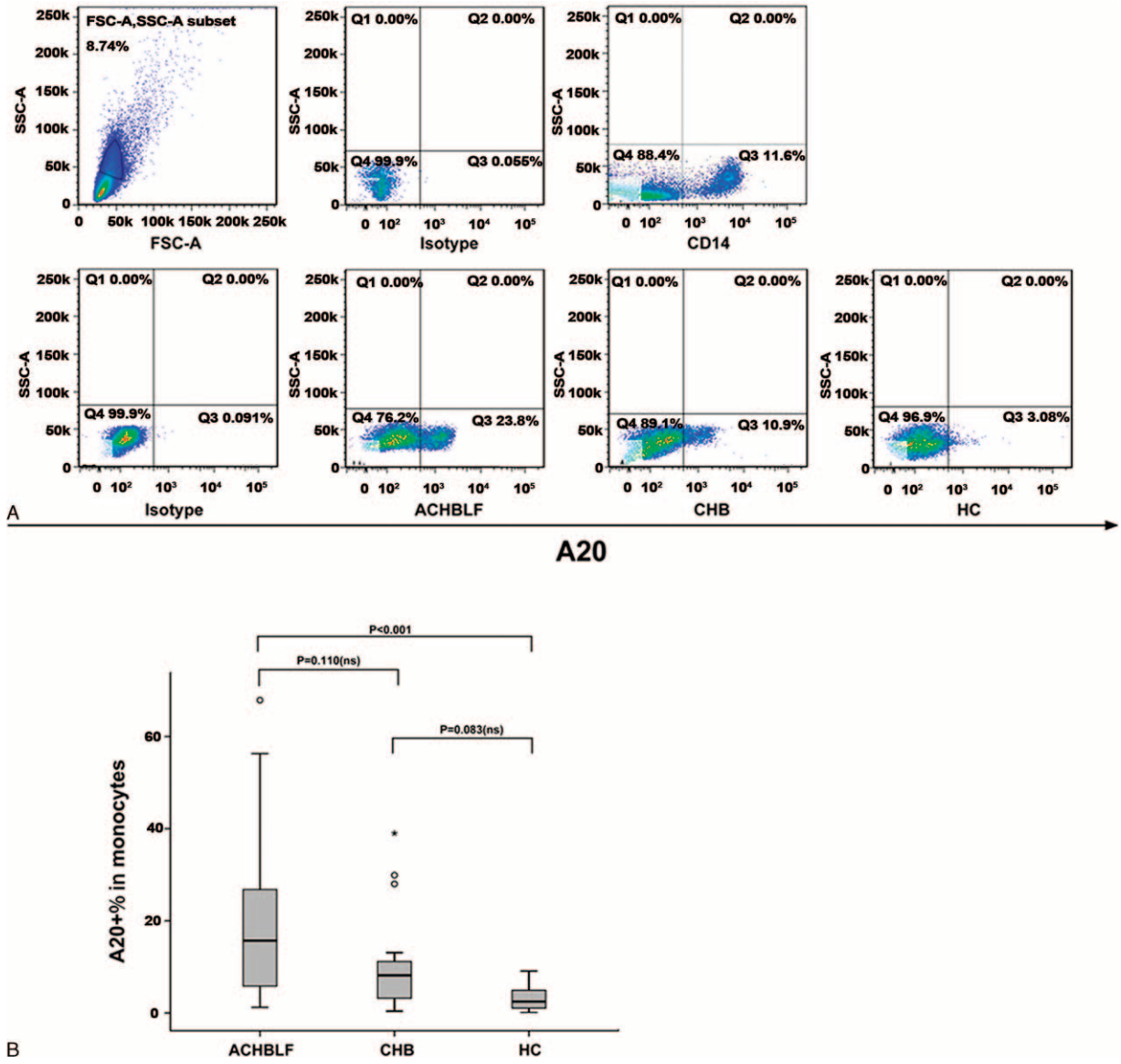

FIGURE 1. Frequency of A20 expression on monocytes from ACHBLF patients, chronic hepatitis B (CHB) patients, and healthy controls (HC). $CD14^+$ cells were determined as monocytes. A20 expression on monocytes was determined by flow cytometry. (A) Representative A20 staining for 1 ACHBLF patient, 1 CHB patient, 1 HC, and isotype control. (B) Flow cytometry data for $A20^+$ monocytes from 37 ACHBLF, 20 CHB, and 15HC. Data are shown as median (IQR). ns: no significant difference. ACHBLF = acute-on-chronic hepatitis B liver failure, CHB = chronic hepatitis B, HC = healthy controls, IQR = interquartile range.

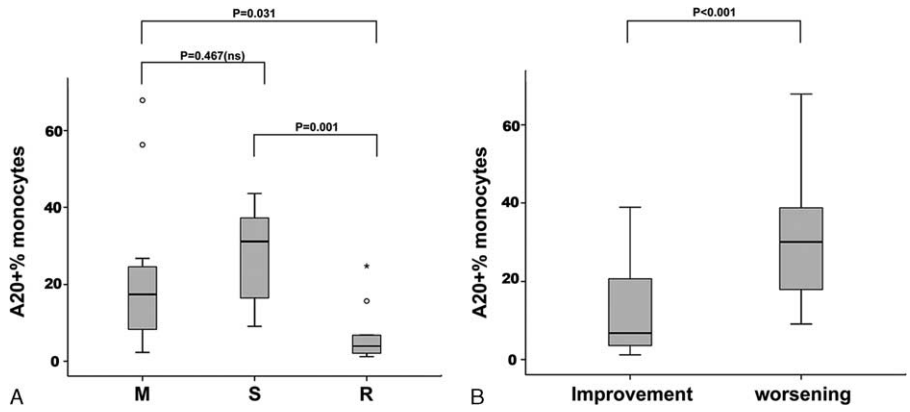

FIGURE 2. Frequency of A20$^+$ monocytes from patients with different severities of disease and prognoses. A20 expression on monocytes was determined by flow cytometry. (A) Frequency of A20$^+$ monocytes from patients in different severities of ACHBLF. M: mild/moderate ACHBLF (n = 15), S: severe ACHBLF (n = 12), R: recovery phase (n = 10). (B) Frequency of A20$^+$ monocytes among patients with different prognoses. Improvement group (including recovery phase patients) (n = 22) and worsening group (n = 15). Data are shown as median (IQR). ns: no significant difference. ACHBLF = acute-on-chronic hepatitis B liver failure, IQR = interquartile range.

with the prothrombin activity (PTA) ($r = -0.33$, $P = 0.046$) (Figure 3Figures 3A–D). However, no correlation was observed between A20 expression and TP, ALB, ALT/AST, HBV-DNA, PT, or INR (Figures 3E–J).

Serum Levels of TNF-α, IL-10, and IL-12

Levels of IL-10, IL-12, and TNF-α were significantly higher in the ACHBLF group compared with the CHB and control groups (IL-10: 5.90 [8.80] vs 3.45 [2.35] and 2.80 [1.40] pg/mL, all $P < 0.001$) (IL-12: 4.00 [2.15] vs 2.20 [1.77] and 2.10 [1.60] pg/mL, all $P < 0.001$) (TNF-α: 22.00 [17.50] vs 12.00 [13.75] and 12.00 [13.00] pg/mL, $P < 0.05$) (Figure 4).

IL-10 Expression in Monocytes from Patients with ACHBLF

The frequency of IL-10 expression on monocytes using flow cytometry was higher in the ACHBLF group compared with CHB and healthy controls (5.6 [9.9]% vs 2.7 [1.9]% and 2.5 [1.8]%, both $P < 0.001$) (Figure 5Figures 5A and B). Furthermore, the frequency of IL-10 expression on monocytes was significantly

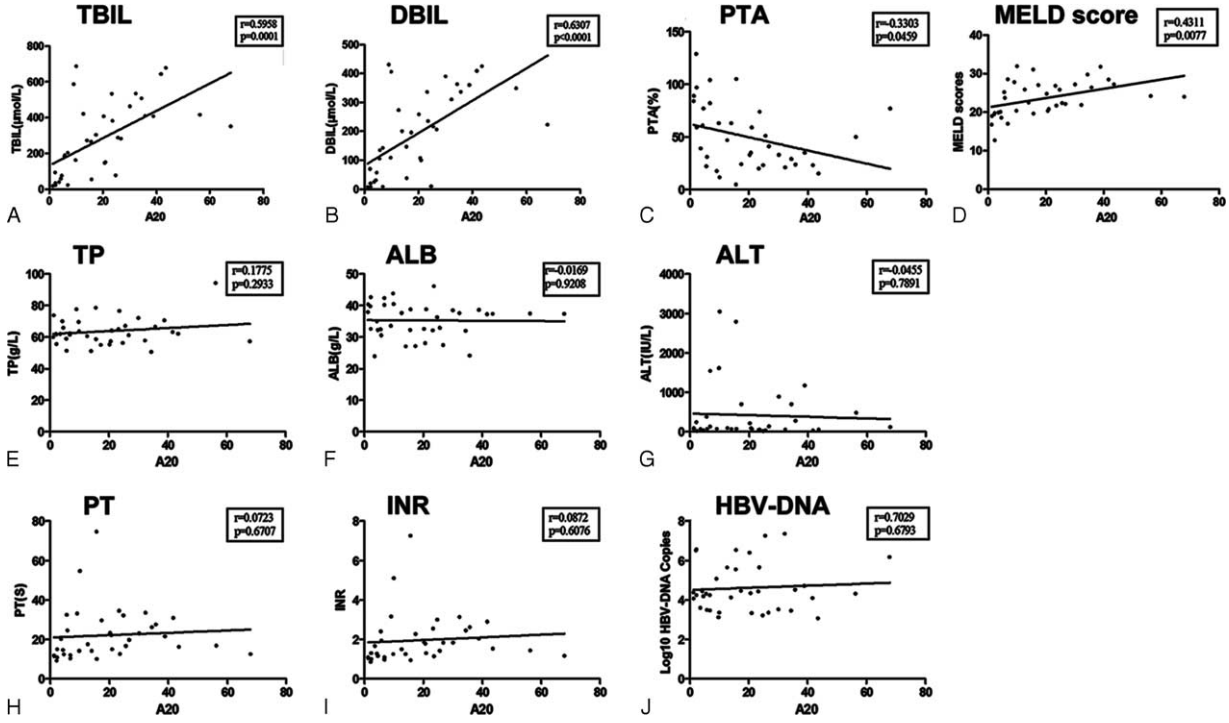

FIGURE 3. Linear correlations between A20 expression on monocytes and markers of disease severity in patients with ACHBLF: (A) TBIL, (B) DBIL, (C) PTA, (D) MELD score, (E) TP, (F) ALB, (G) ALT, (H) PT, (I) INR, and (J) HBV-DNA. ACHBLF = acute-on-chronic hepatitis B liver failure, ALB = albumin, ALT = alanine transaminase, DBIL = direct bilirubin, GLB = globulin, HBV-DNA = hepatitis B virus-DNA, INR = international normalized ratio, MELD = model for end-stage liver disease, PT = pro-thrombin time, PTA = prothrombin activity, TBIL = total bilirubin, TP = total protein, .

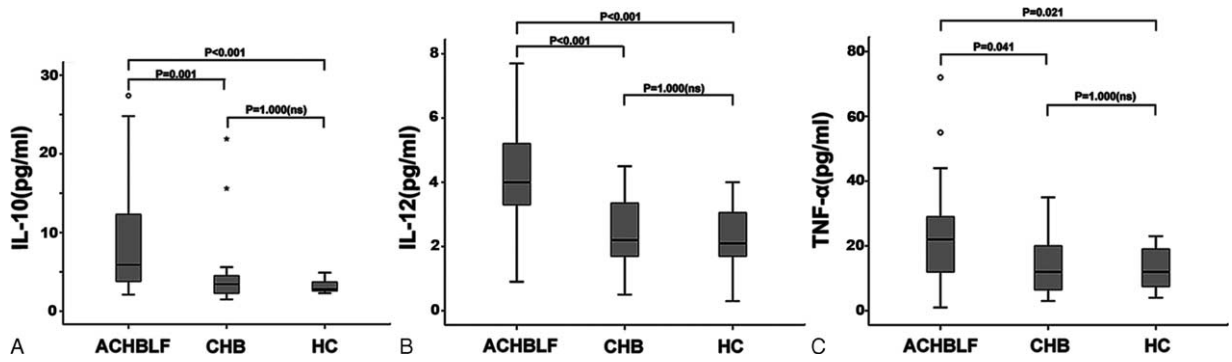

FIGURE 4. Serum levels of IL-10, IL-12p70, and TNF-α among the 3 groups (ACHBLF vs CHB vs HC). Cytokine levels were determined by cytometric bead assay. Data are shown as median (IQR). ns: no significant difference. ACHBLF = acute-on-chronic hepatitis B liver failure, CHB = chronic hepatitis B, HC = healthy controls, IQR = interquartile range.

higher in the 27 patients with ACHBLF in the progression phase (the 10 recovery patients were excluded) compared with the CHB group (5.90 [10.3]% vs 3.6 [2.1]%, $P = 0.008$) (Figures 5A and C). The frequency of IL-10 expression on monocytes in patients with CHB was not significantly different compared with healthy controls ($P > 0.05$) (Figures 5B and C).

Frequency of TLR4 Expression in Monocytes from Patients with ACHBLF

The frequency of TLR4 expression in monocytes was higher in the ACHBLF group compared with the CHB group (7.1 [5.6] % vs 3.6 [2.1] %, $P < 0.001$) (Figure 6Figures 6A and B).

A20 and IL-10 Expressions in THP-1 Cells After Stimulation with LPS

The frequency of A20 and IL-10 expressions in THP-1 cells was increased after stimulation with LPS for 12 h compared with baseline (A20: $5.03 \pm 0.47\%$ vs $0.36 \pm 0.08\%$, $P < 0.01$; IL-10: $11.09 \pm 0.35\%$ vs $5.03 \pm 0.42\%$, $P < 0.01$) (Figure 7Figures 7A and B).

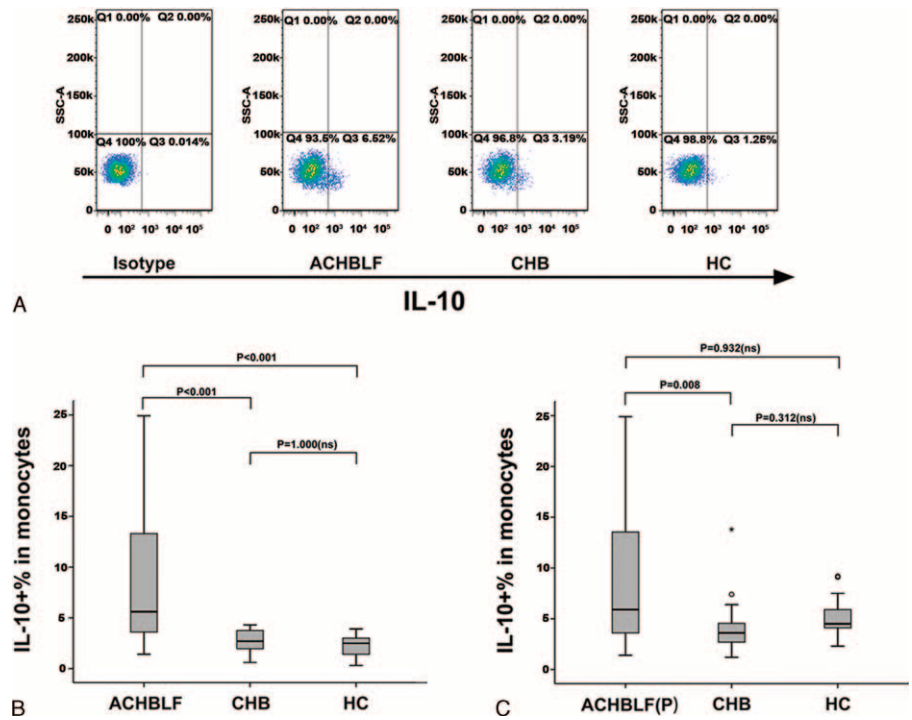

FIGURE 5. Levels of IL-10 in monocytes from patients with ACHBLF, CHB, and HC. Levels of IL-10 in monocytes were determined by flow cytometry. (A) Representative staining for IL-10 expression in monocytes from 1 ACHBLF patient, 1 CHB patient, 1 HC, and 1 isotype control. (B) Frequency of IL-10$^+$ monocytes from 37 ACHBLF, 20 CHB, and 15 HC. (C) Frequency of IL-10$^+$ monocytes from 27 patients with ACHBLF in the progression phase (the 10 recovery patients were excluded), 20 patients with CHB, and 15 HC. Data are shown as median (IQR). ns: no significant difference. ACHBLF = acute-on-chronic hepatitis B liver failure, CHB = chronic hepatitis B, HC = healthy controls, IQR = interquartile range.

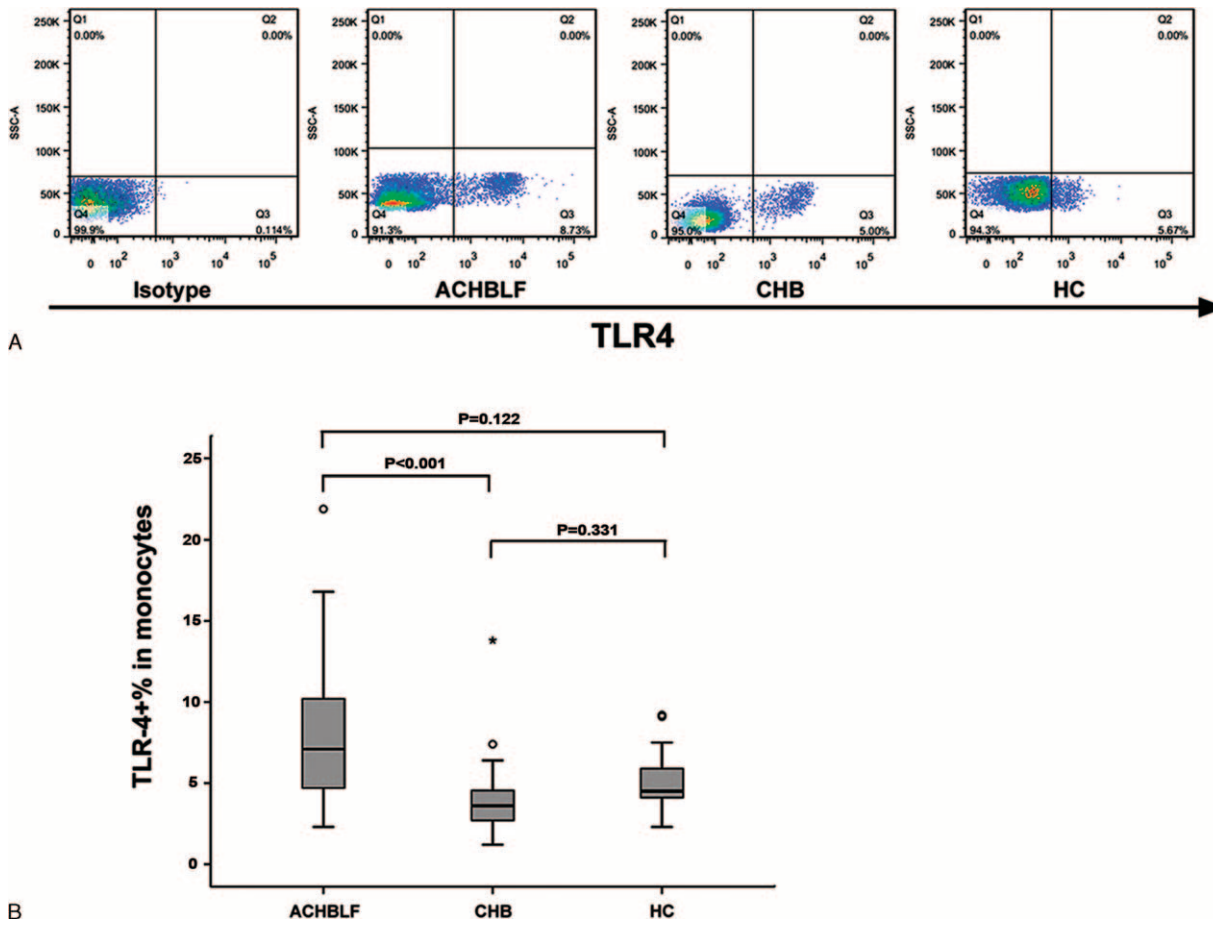

FIGURE 6. Frequency of TLR4$^+$ monocytes from patients with ACHBLF and CHB and HC. TLR4$^+$ monocytes were determined by flow cytometry. (A) Representative staining for TLR expression in monocytes from 1 ACHBLF patient, 1 CHB patient, 1 HC, and 1 isotype control. (B) Frequency of TLR4$^+$ monocytes from 37 ACHBLF, 20 CHB, and 15 HC. Data are shown as median (IQR). ns: no significant difference. ACHBLF = acute-on-chronic hepatitis B liver failure, CHB = chronic hepatitis B, HC = healthy controls, IQR = interquartile range, TLR = toll-like receptors.

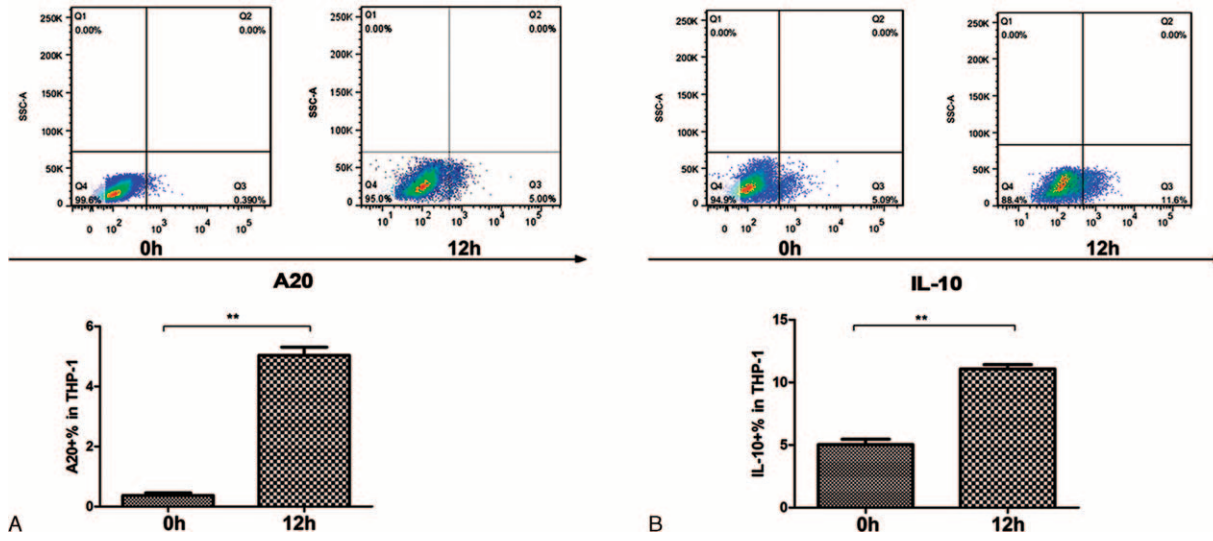

FIGURE 7. A20 and IL-10 expressions in THP-1 cells stimulated with LPS. THP-1 cells were treated with LPS (100 ng/mL) for 12 h. A20 (A) and IL-10 (B) expressions in THP-1 cells were determined by flow cytometry. Data are shown as mean ± SD. $**P < 0.01$. LPS = lipopolysaccharide, SD = standard deviation.

DISCUSSION

A homeostatic response following hepatic injury includes a balance between pro- and anti-inflammatory components to limit the extent of parenchymal damage and to promote tissue recovery. However, this balance might be lost in patients with ACHBLF because of uncontrolled, persistent expression of A20. In the present study, results showed that the frequency of $A20^+$ CD14 cells was significantly increased in patients with ACHBLF compared with HC. LPS treatment upregulated A20 and IL-10 expressions in THP-1 cells. A20 expression in CD14 cells from patients with ACHBLF was positively correlated with TBIL, DBIL, and MELD scores, and inversely with PTA. A20 expression was higher in patients with ACHBLF under progression compared with patients in recovery. IL-10 and TLR4 expression levels in monocytes were increased, and serum levels of IL-10, IL-12p70, and TNF-α were increased in patients with ACHBLF compared with patients with CHB and healthy controls.

Even if the patients were excluded if they were suffering from an inflammatory disease, the elevated A20 expression that was observed in the present study might have been induced by many factors. Indeed, TNF-α has been shown to be increased in patients with ACHBLF of the present study and in a previous study.[28] In addition, LPS, which is an endotoxin derived from Gram-negative bacteria in the intestinal microflora, was found to play an important role in ACHBLF.[29,30] A monocyte stimulated by LPS will vigorously produce cytokines to activate intrahepatic monocytes/macrophages and Kupffer cells, and further induce a burst of cytokines to cause liver tissue injury.[11] Meanwhile, liver dysfunction most likely further induces bacterial translocation from the gut, leading to higher levels of endotoxemia. Chronic liver diseases are typically accompanied by portal hypertension and increased gut permeability, both of which contribute to the increased accumulation of endotoxin in the peripheral blood and liver.[29–31] An *in vitro* culture assay was performed to evaluate the effects of LPS on monocytes to clarify the mechanism underlying the immunopathogenesis of ACHBLF in terms of increased A20 expression, and the results indicated that the frequency of A20 and IL-10 expressions in THP-1 cells was increased following stimulation with LPS compared with the control group. Therefore, these results suggest that A20 expression might be used as a prognostic marker for disease progression in patients with ACHBLF.

The present study showed that changes in A20 expression on monocytes were correlated with changes in TBIL, DBIL, MELD score, and PTA, and it is the first study to identify a significant correlation of A20 expression levels with ACHBLF severity, indicating a potential role for A20 in predicting disease progression. It is possible that the elevation of A20 levels was mainly induced by bacterial translocation from the gut to the portal circulation in patients with ACHBLF and CHB in an attempt to control inflammation. Indeed, increased A20 expression induces the development and persistence of an anti-inflammatory response, whereas the persistent suppression of A20 might be an attempt to prevent overstimulation in response to an extensive inflammatory process. A20 expression on monocytes may be the result of ACHBLF development representing homeostatic response to suppress excessive activation of monocytes. However, the increased A20 levels might be inextricably linked with the attendant immunoparesis of monocytes, termed the compensatory anti-inflammatory response syndrome (CARS). This syndrome enhances the susceptibility to sepsis, recurrent infections, and ultimately refractory multiple organ dysfunction syndrome (MODS) in patients with ACHBLF.[32] Previous studies in patients presenting acute deterioration due to alcoholic cirrhosis and ACLF revealed that the presence of a systemic inflammatory response (SIRS) was associated with an increased risk of subsequent infection.[33] Anti-TNF-α therapies in patients with alcoholic hepatitis showed that an inflammatory response was associated with an increased risk of infection and mortality.[34] Consequently, upregulated A20 expression in monocytes might be associated with acute-on-chronic severity of hepatitis B liver failure, as suggested by the correlation between the MELD score and A20 expression. The MELD score is one of many different models that have been proposed to evaluate the severity of end-stage liver disease[3] and have been shown to be reliable in predicting prognosis.[7] Therefore, assessing A20 expression could help predicting prognosis in patients with ACHBLF.

Recognition of LPS by monocytes is predominantly mediated by TLR4.[35,36] LPS interacts with TLR4 and induces a signal transduction pathway in which activated kinases further activate transcription factors, mainly nuclear factor κB (NF-κB),[37] which results in increased production of pro-inflammatory cytokines and leads to hepatic necrosis.[38] A20 can restrict NF-kB activity triggered by TLR4 and nucleotide-binding oligomerization domain-containing protein 2 (NOD2) by deubiquitinating TNF receptor-associated factor (TRAF) 6 and RIP2, respectively.[39,40] In the present study, patients with ACHBLF showed a significantly higher percentage of $TLR4^+$ monocytes compared with healthy controls. ACHBLF is characterized by immune disorder in which an uncontrolled TLR response plays a major pathogenic role.

IL-10 is known to be an anti-inflammatory cytokine that plays an important role in downregulating the immune response. Elevated IL-10 expression in critically ill patients has been correlated with a poor outcome due to sepsis.[41] Previous studies by our group have indicated that blockade of the A20 pathway significantly decreases IL-10 production in the mDCs of HCV-infected subjects, suggesting that A20 is associated with IL-10.[42] Furthermore, immunomodulators of A20 play an integrated role in suppressing cell functioning. A significantly higher level of IL-10 secretion from monocytes was observed in patients with ACHBLF during the progression phase (the 10 recovery patients were excluded) compared with the patients with CHB and healthy controls. The serum levels of IL-10 and TNF-α were also significantly elevated in patients with ACHBLF compared with healthy controls. Monocytes secrete large amounts of anti-inflammatory cytokines (IL-10) and are a source of the high circulating levels of IL-10 and of the development of immune dysfunction in patients with ACHBLF. Accordingly, the *in vitro* results indicated that the expression of IL-10 in THP-1 cells changed after stimulation with LPS compared with the control group. However, the results suggest that A20 and IL-10 levels were not correlated (correlation obtained from 37 ACHBLF ($r = 0.24$; $P = 0.15$); correlation obtained from 27 patients with ACHBLF in the progression phase (the 10 recovery patients were excluded) ($r = 0.22$; $P = 0.27$)), but that they do have a similar trend. Maybe the sample size of the present study was not large enough. In addition, other inflammatory markers that were not tested in the present study might be involved in this relationship. Further study is necessary to address this issue.

Several limitations of this study are worth noting: (1) a number of patients were not analyzed due to interventions performed during the period such as artificial liver support; (2) there was an absence of dynamically detected A20 expression during the disease progression; (3) the present study was

not designed to identify the mechanisms leading to A20 expression, nor to assess the mechanisms of A20-impaired immune state; (4) circulating LPS levels were not measured, but a previous study has shown that abnormal levels of LPS were observed in ACHBLF, and that the changes in LPS levels correlated with disease severity [29]; (5) no assessment of infections was prospectively done, and the retrospective data would be unreliable, preventing the correlation of A20 levels with infections; and (6) only ACHBLF was studied, and it would be interesting to observe patients with ACLF of other etiologies. A future trial with a larger sample size is warranted to confirm our findings.

In conclusion, A20 expression was higher in monocytes from patients with ACHBLF compared with HC. A20 expression was associated with the severity of ACHBLF.

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