Skewed CD39/CD73/adenosine pathway contributes to B-cell hyperactivation and disease progression in patients with chronic hepatitis B

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Abstract

Background: The mechanisms underlying B-cell hyperactivation in patients with chronic hepatitis B virus (HBV) infection remain largely undefined. The present study assessed the clinical characteristics of the CD39/CD73/adenosine pathway in patients with chronic hepatitis B (CHB).

Methods: We examined CD39 and CD73 expression and adenosine production by B-cells from 202 HBV-infected patients. B-cell-activation phenotypes were assessed by flow cytometry after CpG+CD40 ligand stimulation with or without blockade and activation of the adenosine pathway.

Results: CD39 and CD73 expression on circulating B-cells was decreased in CHB patients with high HBV DNA, HBeAg positivity, high HBsAg levels, and active liver inflammation, and was hierarchically restored in complete responders according to HBeAg seroconversion or HBsAg reduction. However, CD39 and CD73 expression on activated memory and tissue-like memory B-cell subsets in complete responders was not increased despite effective antiviral treatments. Furthermore, CD39 and CD73 expression on intra-hepatic B-cells was decreased in inflammatory livers.

In vitro, B-cells from CHB patients showed a markedly reduced capacity to generate CD39/CD73-dependent extracellular adenosine and expressed increased levels of activation markers after adenosine-production blockade. Contrastingly, metformin significantly reduced activation-marker expression via regulating AMP-activated protein kinase.

Conclusions: The skewed CD39 and CD73 expression on B-cells was associated with a high viral burden, liver inflammation, and antiviral efficacy in CHB patients, and the skewed CD39/CD73/adenosine pathway contributed to B-cell hyperactivation.
Regulation of the CD39/CD73/adenosine pathway using metformin may represent a therapeutic option to reverse HBV-induced immune pathogenesis.

**Key words:** hepatitis B virus; B-lymphocyte; CD39; 5’-nucleotidase; lymphocyte activation

**Introduction**

Chronic hepatitis B virus (HBV) infection is characterized by persistent viral replication, fluctuant liver damage, and functional impairment of host immunity to HBV [1, 2]. It is widely accepted that host adaptive immunity plays a key role in liver pathogenesis and disease progression [3, 4]. In recent years, the characteristics of HBV-specific T-cells have been extensively investigated in patients with chronic hepatitis B (CHB) [5–7], while only partial characteristics of B-cells have been disclosed. Previous studies have indicated that functional defects in B-cell responses exist in patients with CHB [8–12]. These dysfunctional B-cells were identified as atypical memory B-cells with a CD21−(also known as complement C3d receptor 2) CD27− phenotype [13, 14]. In addition, interleukin-10 (IL-10)-producing B-cells have been proposed as the regulatory B-cells that modulate HBV-specific T-cell responses [15]. Finally, our previous study, together with those of another group, showed that B-cells in patients with CHB displayed enhanced activation markers and functional impairment [13, 16, 17]. The B-cell hyperactivation and functional impairment were associated with HBV persistence and HBsAg seroconversion in CHB patients, thus B-cells played the active role in the immunopathogenesis of chronic HBV infection [17]. In the present study, we further investigated the mechanisms underlying B-cell hyperactivation in patients with CHB.

The CD39 (also known as ectonucleoside triphosphate diphosphohydrolase 1, ENTPD1)/CD73 (also known as 5’-nucleotidase ecto, NTSE)/adenosine pathway plays important roles in orchestrating injury responses in multiple organs, including the liver. Mechanically, CD39 converts 5’-adenylate triphosphate (ATP) to adenosine diphosphate/5’-adenosine monophosphate (5’AMP) and CD73 hydrolyses 5’AMP to adenosine, in a stepwise manner [18, 19]. This signaling pathway promotes tissue architecture in acute-injury settings, whereas it becomes detrimental by promoting tissue injury and fibrosis during the chronic-injury phase [20]. Several studies have investigated the role of this pathway in liver diseases, including hepatic steatosis and fibrosis [21, 22], acute liver failure [23], and ischemia/reperfusion injury [24–27]. Their findings indicated that the CD39/CD73/adenosine pathway plays a pathogenic role in ethanol-induced fibrosis and fatty liver; however, by contrast, this pathway plays a protective role in acute or ischemia/reperfusion injury in an adenosine-dependent manner [21–27]. Despite different in vivo cell types (including endothelial cells) expressing molecules with exonuclease activities, B-cells express higher levels of CD39 and CD73 [28, 29]. In addition, blockade of CD73 activity led to impaired IgG class switching for B-cells [30]. More recently, a skewed CD39/CD73/adenosine pathway in B-cells was demonstrated to correlate with B-cell activation and disease progression in patients with human immunodeficiency virus (HIV) infection [29, 31]. Little information is available regarding the characteristics of the CD39/CD73/adenosine pathway in B-cells or its clinical significance in chronic HBV infection.

In patients with CHB, we observed skewing of CD39 and CD73 expression on total B-cells, regardless of their specificity. The decreased CD39 and CD73 expression on B-cells was closely associated with viral burden and liver inflammation, and restoration of CD39 and CD73 expression on B-cells was closely associated with antiviral efficacy. Our findings suggested that the CD39/CD73/adenosine pathway has an important role underlying B-cell hyperactivation. Metformin, a clinically available drug, has the potential to regulate B-cell activation, suggesting that intervention in the CD39/CD73/adenosine pathway in B-cells using metformin might represent a therapeutic option for HBV-induced immune pathogenesis in CHB.

**Materials and methods**

**Patients**

Two hundred and two patients infected with HBV were enrolled, including 95 treatment-naïve patients, who were further categorized into 15 immune tolerance (IT) patients, 45 immune activation (IA) patients, 15 inactive carriers (IC), and 20 immune reactivation (RA) patients, and 107 complete responders (CR), who had received at least 1 year of entecavir treatment and had serum HBV DNA below a detectable level (20 IU/mL), together with alanine aminotransferase (ALT) normalization. In addition, antiviral efficacy was further determined by their HBeAg and HBsAg status [32, 33]. Twenty-five age-matched healthy controls (HCs) were simultaneously enrolled who tested serologically negative for HBV, Hepatitis C virus (HCV), and HIV. The baseline clinical characteristics of these patients and HCs are listed in Table 1.

**Liver biopsy specimens were collected from 13 IA patients.** The degree of hepatic inflammation was graded according to the modified histological activity index (HAI) [34]. Briefly, grading (G) was used to describe the intensity of necro-inflammatory activity and staging (S) was a measure of fibrosis and architectural alteration in chronic hepatitis. Increased numerical values indicated more severe disease. Intra-hepatic lymphocytes were isolated as described previously [35]. The study protocol was approved by the ethical committee of the fifth medical center of Chinese PLA general hospital and written informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

**Flow cytometry**

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly heparinized blood. To characterize the expression profiles of CD39 and CD73 on B-cells, PBMCs or intra-hepatic lymphocytes were stained with a cocktail of monoclonal antibodies (mAbs): anti-CD45-Allophycocyanin-H7 (clone 2D1, BD Biosciences, Franklin Lakes, NJ, USA), anti-CD19-Phosphoerytin-Cy7 (clone SJ25C1, BD Biosciences), anti-CD39-Fluorescein isothiocyanate (clone TU66, Biolegend, San Diego, CA, USA), anti-CD73-Brilliant violet 421 (clone AD2, Biolegend), anti-CD10-Phosphoerytin (clone H10a, BD Biosciences), anti-CD27-Brilliant violet 510 (clone L128, Biolegend), and anti-CD21- Allophycocyanin (clone B-ly4, eBioscience, San Diego, CA, USA).
To detect the B-cell-activation phenotypes, cells were stained with the following surface mAbs: anti-CD19-PerCP-Cy5.5 (clone HB19, BD Biosciences), anti-CD69-Fluorescein isothiocyanate (clone FN50, BD Biosciences), anti-CD71-Phycoerythrin-Cy7 (clone CY1G4, Biolegend), anti-CD80-Allophycocyanin (clone 2D10, Biolegend), and anti-CD73-PerCP-Cy5.5 (clone IT2.2, Biolegend). Flow-cytometer acquisition was performed on an LSR II instrument (BD Biosciences) and data were analysed using FlowJo (Version 7.6.1; TreeStar) software.

### Cell separation

CD19+ B-cells were separated from PBMCs using anti-CD19 Ab-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers’ instructions. The purity of separated cells was monitored by flow cytometry, which ranged from 95% to 99%.

### Mass spectrometry

Purified B-cells (1.25 x 10^6/mL) were resuspended in 200 µL of phosphate-buffered saline in 96-well plates in the presence of 20 µmol/L ATP (Sigma-Aldrich, St, Louis, MO, USA) for 5, 30, 45, and 60 minutes. Supernatants were collected, centrifuged, and boiled for 2 minutes to inactivate adenosine-degrading enzymes and stored at −80 °C for subsequent analysis. Purine levels in the supernatants were analysed using an LTQ XL Linear Ion Trap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) and selected reaction monitoring with 13C10-adenosine (Sigma-Aldrich) as the internal standard. The positive-ion mode was operated in the mass spectrometer and the following mass-to-charge transitions were monitored: 348→136 for 5′-AMP and 268→136 for adenosine.

### Cell culture and stimulation

Purified B-cells were resuspended at a concentration of 10^6/mL in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% L-glutamine and 10% fetal calf serum. Cells were stimulated with CpG (oligodeoxynucleotide 2006, 10 µg/mL; Invivogen, San Diego, CA, USA) and CD40 ligand (CD40L; 1 µg/mL; R&D Systems) for 12 or 24 hours in the presence of anti-CD73 Abs (1 µg/mL, clone 4G4, Hycult Biotechnology, Uden, The Netherlands) or metformin (10 and 20 mmol/L, Sigma-Aldrich). Cells were subsequently collected to detect B-cell-activation markers by flow cytometry.

HepG2.2.15 and HepAD38 cells containing the complete HBV genome and that were capable of stable HBV expression and replication in culture were seeded in 12-well plates at a density 2.5 x 10^4 cells per well. Metformin was added at the indicated concentrations (5, 10, 20, and 40 mmol/L) 24 hours later. The supernatants were collected 24 hours later and detected for HBsAg levels using a chemiluminescent immunoassay.

### Biochemical, virological, and immunological assessments

The biochemical indexes, including the levels of serum ALT, were measured using a Beckman-coulter LX-20 biochemistry automatic analyser. HBsAg, HBsAb, HBeAg, HBeAb, and HBV core Ab were measured using commercial kits (Abbott Architect, Abbott Diagnostics, Lake Forest, IL, USA). Serum HBV DNA levels and HBsAg quantifications were evaluated using commercially available real-time polymerase chain reaction kits (Amplicor, Roche Diagnostics, Branchburg, NJ, USA) and chemiluminescent immunoassay kits (COBAS HBsAg Q, Roche Diagnostics, respectively). The threshold of detection for HBV DNA was 20 IU/mL.

### Statistical analysis

All data were analysed using the SPSS software version 17.0 (IBM Corp., Armonk, NY, USA). The Kruskal-Wallis H nonparametric test or analysis of variance test was performed between multiple groups. Significant differences between two groups were determined using the Mann-Whitney nonparametric U test or Student’s t-test. Data from the same individuals were compared using the Wilcoxon’s matched-pairs signed-ranks test. For all tests, a P-value <0.05 was considered statistically significant.

### Results

CD39- and CD73-expression levels on circulating B-cells decrease with higher HBV viral burden and liver inflammation in CHB patients

To investigate the expression profiles of CD39 and CD73 on B-cells in the peripheral blood of CHB patients, we performed a
cross-sectional study comparing patients with different HBV-disease statuses. Representative flow plots from HCs, IA, and IC patients are shown in Figure 1A and the data are summarized in Figure 1B. In general, B-cells from patients with CHB showed significantly decreased expression levels of CD39 and CD73, except for the B-cells from IC patients, which expressed a comparable proportion of CD39 and CD73 to the HCs. Even in IT patients, the CD39 expression on B-cells was decreased compared with that in the HCs. Notably, B-cells from IA patients showed more profound decreases in the proportion of CD39 and CD73, and a significant decrease in the frequency of CD39⁺/CD73⁻ B-cells was observed between IA patients and IT or IC or RA patients. Further analysis showed the CD39 expression on B-cells was significantly reduced in patients with CHB with high HBV DNA, a positive HBeAg state, high HBsAg levels, and active hepatic inflammation (indicated by raised ALT and G scores), whereas decreased CD73 expression on B-cells was seen only in patients with high levels of serum HBeAg and HBsAg. The lower frequencies of CD39⁺/CD73⁻ B-cells also observed in patients with high HBV load, high HBeAg and HBsAg levels, and high ALT levels (Figure 1C). These data clearly indicated that the CD39 and CD73 expression on B-cells was lower in patients with CHB and was associated with higher viral burden and inflammation.

CD39- and CD73-expression levels on intra-hepatic B-cells decrease with liver inflammation

We further investigated the expression profiles of CD39 and CD73 on B-cells in the livers of CHB patients. As shown in Figure 2, the frequencies of intra-hepatic CD39⁺, CD73⁺, and CD39⁺/CD73⁻ B-cells were decreased further compared with those in peripheral blood, regardless of G or S score grouping. Interestingly, there was a larger reduction in CD39 expression on intra-hepatic B-cells in patients with a higher G score than those with lower G scores. These data indicated that the CD39 and CD73 expression on intra-hepatic B-cells was markedly reduced in the livers of CHB patients and may be associated with severe liver inflammation.

CD39- and CD73-expression levels on B-cells increase with antiviral efficacy

Subsequently, to investigate the expression profiles of CD39 and CD73 on B-cells during antiviral therapy, we performed a cross-sectional study comparing CR patients with different responses to antiviral therapy. As shown in Figure 3A, the frequencies of CD39⁺, CD73⁺, and CD39⁺/CD73⁻ B-cells were increased in HBeAg-negative patients compared with those in HBeAg-positive patients. Further analysis showed that the frequencies of CD39⁺ and CD39⁺/CD73⁻ B-cells were increased in patients with lower HBsAg levels, whereas there was no difference in the frequencies of CD73⁻ B-cells when the patients were grouped by HBsAg levels (Figure 3B). These data indicated that restoration of CD39 and CD73 expression on B-cells is closely associated with antiviral efficacy.

CD39 and CD73 expression on activated memory and tissue-like memory B-cell subsets is not restored despite effective antiviral treatment

We further investigated the expression profiles of CD39 and CD73 on B-cell subsets in CHB patients. The gating strategy for B-cell subsets is shown in Figure 4A and the frequencies of B-cell subsets are shown in Figure 4B. Briefly, the frequencies of immature, activated memory, and tissue-like memory B-cell subsets were increased in IA patients. In contrast, the composition of B-cell subsets in CR patients was similar to that in the HCs, with the exception of decreased frequencies of resting memory B-cells in CR patients. Further analysis showed the CD39 and CD73 expression on B-cell subsets from IA patients were profoundly decreased compared with those in the HCs, with the exception of CD39 on tissue-like memory B-cell subsets and CD73 on naive B-cells, which were expressed at comparable levels to those in the HCs (Figure 4C). By contrast, decreased CD39 and CD73 expression on B-cell subsets in CRs was seen only in activated memory and tissue-like memory B-cell subsets when compared with those in the HCs. These data indicated that CD39 and CD73 expression on B-cell subsets was decreased in IA patients. CD39 and CD73 expression could be restored in immature, naive, and resting memory B-cell subsets, but not in activated memory and tissue-like memory B-cell subsets in patients with CR.

B-cells from IA patients exhibit a defect in adenosine production in vitro

To address whether the decrease in CD39 and CD73 expression on B-cells is associated with reduced ATP consumption, we analysed the catalytic capacity of B-cells to hydrolyse ATP. As shown in Figure 5A, B-cells from IA patients degraded similar levels of ATP to 5'AMP compared with those from HCs. By contrast, B-cells from IA patients produced less adenosine than their counterparts from HCs (Figure 5B). These data suggested that B-cells from IA patients might have an impaired capacity to hydrolyse ATP.

Enhancement of B-cell activation after blockade of adenosine production

To investigate the association of the CD39/CD73/adenosine pathway with B-cell activation, B-cells from HCs and IA patients were stimulated with CpG+CD40L in the absence or presence of anti-CD39/CD73-blocking antibody, respectively. There were no statistically significant differences in the CD69⁺, CD71⁺, CD80⁺, and CD86-expression levels on B-cells from IA patients at baseline compared with those in the HCs. Interestingly, B-cells from HCs tended to express higher levels of activation markers upon CpG+CD40L stimulation, despite not reaching statistical differences, with the exception of the CD69 mean fluorescence intensity on B-cell and CD71 expression, which increased significantly compared with those from IA patients (Figure 6). Importantly, increased frequencies of CD69⁺, CD71⁺, and CD80⁺ cells were observed among B-cells from IA patients after blockade of CD73 activity. Notably, the mean fluorescence intensities of CD69, CD71, CD80, and CD86 expression on B-cells were significantly higher after blockade of CD73 activity, indicating that these markers were also increased on a per-cell basis. Thus, blocking the CD39/CD73/adenosine pathway could enhance B-cell activation in IA patients.

Metformin activates the adenosine pathway, leading to a significant reduction in B-cell activation and HBsAg production

Metformin, a clinically available drug, has been reported to activate AMP-activated protein kinase (AMPK), a key downstream molecule in the adenosine pathway [36]. Can metformin affect...
Figure 1. The expression profiles of CD39 and CD73 on B-cells in the peripheral blood of patients with chronic hepatitis B. (A) Representative flow plots showing B-cell expression of CD39 and CD73 in healthy controls (HCs), immune activation patients (IA), and inactive carriers (IC). (B) Frequencies of CD39$^+$, CD73$^+$, and CD39$^+$CD73$^+$ B-cells were determined by flow cytometry in 25 HCs and 95 patients in different stages of chronic HBV infection: immune tolerance (IT, $n = 15$), IA ($n = 45$), IC ($n = 15$), and immune reactivation (RA, $n = 20$). (C) Subjects were categorized into groups determined by their viral load, HBeAg status, HBsAg status, ALT levels, and hepatic necro-inflammation, respectively.
B-cell activation through the adenosine pathway? To address this question, we added metformin to cultures of B-cells in the presence of CpG and CD40L and found that metformin could significantly reduce the expression of activation markers, CD69, CD71, CD80, and CD86 both in terms of frequency and on a per-cell basis (Figure 7A and B). These data suggested B-cell activation could be regulated by metformin. Interestingly, metformin was also found to directly suppress the production of HBsAg in vitro [37, 38]; therefore, we further confirmed that metformin could significantly inhibit the production of HBsAg, not only in the HepG2.2.15 cell line, but also in the HepAD38 cell line, and increasing metformin doses significantly inhibited HBsAg production (Figure 7C). Taken together, the results showed that metformin could not only reduce B-cell activation, but also reduce HBsAg production, suggesting that metformin could be helpful to treat CHB patients.

Discussion

Although B-cell hyperactivation has been demonstrated in CHB patients, the regulatory mechanisms underlying B-cell hyperactivation remain largely undefined. The present study characterized the expression profiles of CD39 and CD73 on B-cells in patients with chronic hepatitis B. Thirteen patients were classified by (A) hepatic necro-inflammation grade (G) and (B) fibrotic stage (S), respectively. Frequencies of CD39⁺, CD73⁺, and CD39⁺CD73⁺ B-cells in peripheral blood mononuclear cells (PBMCs) and liver tissue were determined using flow cytometry. *P < 0.05.

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Disease status. Our data provided a possible explanation for why B-cell hyperactivation was maintained in patients with CHB and suggested that regulation of the CD39/CD73/adenosine pathway in B-cells using metformin might be a therapeutic option to reverse HBV-induced immune pathogenesis in CHB.

We thoroughly analysed the characteristics of CD39- and CD73-expression profiles on B-cells during chronic HBV infection. Our results were similar to those reported in HIV patients, in whom the CD39 and CD73 expression on B-cells decreased significantly compared with that in healthy subjects [29, 31]. The results of the present study further indicated that the decreased CD39 and CD73 expression on B-cells is associated not only with active liver inflammation, but also with high HBV viral burden in CHB patients. On the one hand, markedly reduced CD39 and CD73 expression on B-cells was observed in IA patients who often presented clinically fluctuant liver damage. According to the liver-biopsy diagnosis, the expression levels of CD39 and CD73 on B-cells in the liver, the site of inflammation, were lower than in the peripheral blood of IA patients. In addition, IC patients exhibited a comparable proportion of CD39 and CD73 on B-cells compared with that in the HCs. This cohort of patients often presented normal ALT levels. Taken together, the results showed higher reductions in CD39- and CD73-expression levels on B-cells from patients with CHB with high inflammation. From this perspective, the observed decrease in CD39 and CD73 expression on B-cells in our patients with CHB could be explained as a non-HBV-specific phenomenon because inflammatory cytokines, such as IL-4, IL-12, IL-21, and interferon gamma (IFN-γ), could prevent CD39 and CD73 expression. In contrast, CD39 and CD73 expression could be upregulated under hypoxic conditions, as well by the presence of transforming growth factor beta (TGF-β), IL-6, type I IFNs, and Wnt-signaling-pathway agonists [39]. Future studies should investigate which
factors are responsible for CD39 and CD73 downregulation in patients with CHB. On the other hand, decreased CD39 expression on B-cells was found in IT patients. A gradual increase in CD39 and CD73 expression on B-cells was observed in CR patients following effective antiviral treatment and was closely associated with HBeAg and HBsAg levels. Notably, CD39 and CD73 expression on AM and TLM B-cell subsets was not fully recovered in CR patients in this setting. These patients were commonly characterized by minimal liver damage, normal ALT levels, and persistent viral burden. It will be interesting to determine whether viral factors can selectively regulate the expression of CD39 and CD73 on B-cells as well as B-cell subsets in CHB patients. These observations indicated that the decreased CD39 and CD73 expression levels on B-cells are closely associated with high liver inflammation and viral burden in CHB patients.

B-cell activation is a necessary component of an effective immune response against HBV infection; however, continuous B-cell activation in CHB patients can be maintained in a B-cell receptor-independent manner and this type of B-cell activation is also seen in HCV and HIV infections [16, 40]. Our previous study found that CD40L and IFN-γ preferentially activated B-cells and contributed to the functional hyperactivation of B-cells in patients with CHB, which induces chronic liver inflammation [17]. Adenosine mediates anti-inflammatory effects by inhibiting activated immune cells. It was rational to propose that the decreased CD39 and CD73 expression on B-cells in CHB patients might lead to a decrease in adenosine production, ultimately contributing to B-cell activation. This hypothesis was supported by our findings that B-cells from patients with CHB displayed a reduced ability to convert ATP to adenosine and expressed increased levels of activation markers upon CpG + CD40 ligand stimulation after blockade of CD73 activity, suggesting that autocrine adenosine signaling is necessary to inhibit the activation levels of B-cells.

Another relevant finding in our study was the identification of the clinically available drug, metformin, as a potential treatment to reduce the hyperactivation of B-cells in vitro. Although adenosine can also effectively inhibit B-cell activation [28], the great advantage of metformin over adenosine is its defined pharmacokinetics and safety. Furthermore, in agreement with our previous studies [37, 38], we further confirmed that metformin could directly inhibit HBsAg production in HepG2.2.15 and HepAD38 cell lines, suggesting that metformin could be clinically useful as a B-cell-hyperactivation and HBsAg-production inhibitor.

Taken together, our data demonstrated the characteristics and clinical significance of CD39 and CD73 expression on B-cells in patients with CHB, suggesting that chronic exposure of B-cells to HBV and an inflammatory environment could skew the CD39/CD73/adenosine pathway, thus contributing to B-cell hyperactivation. Manipulation of the skewed CD39/CD73/adenosine pathway in B-cells using metformin might represent a promising strategy for immune therapy of this disease.
Figure 6. Blockade of the CD39/CD73/adenosine pathway enhances B-cell activation. Purified B-cells from immune activation patients (IA, \( n = 6 \)) and healthy controls (HC, \( n = 5 \)) were stimulated with CpG (10 µg/mL) plus CD40L (1 µg/mL) in the presence or absence of anti-CD73 antibody (1 µg/mL) for 0, 12, and 24 hours. (A) The frequencies and (B) mean fluorescence intensities (MFI) of CD69, CD71, CD80, and CD86 on B-cells were determined using flow cytometry. * \( P < 0.05 \); ** \( P < 0.01 \).

Figure 7. Metformin effectively reduces B-cell activation and HBsAg production. B-cells from healthy controls (HC, \( n = 3 \)) and immune activation patients (IA, \( n = 5 \)) were stimulated with CpG (10 µg/mL) plus CD40L (1 µg/mL) for 24 hours in the presence or absence of metformin (0, 10, and 20 mmol/L). (A) The frequencies and (B) mean fluorescence intensities (MFI) of CD69, CD71, CD80, and CD86 on B-cells were determined using flow cytometry. (C) Metformin reduced HBsAg production from HepG2.2.15 and HepAD38 cell lines. HBsAg levels in culture supernatants were determined using chemiluminescent immunoassay kits. * \( P < 0.05 \); ** \( P < 0.01 \).
Authors’ contributions
F.S.W. and J.Y.Z. conceived the project and obtained the funding. S.N.Z. and P.X. designed and performed the experiments and generated the data. S.N.Z. and N.Z. drafted the manuscript. N.Z. and H.H.L. provided essential patient samples and clinical data. C.Z., J.W.S., X.F., M.S., and L.J. provided help. All authors read and approved the final manuscript.

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Conflicts of interest
None declared.

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