

HCV inhibits M2a, M2b and M2c macrophage polarization via HCV core protein engagement with Toll-like receptor 2

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Abstract. Hepatitis C virus (HCV) establishes a persistent infection in most patients, eventually leading to chronic hepatitis C (CHC), cirrhosis and hepatocellular carcinoma. Our previous study revealed that HCV core protein (HCVc) inhibited the differentiation of monocytes into M1 and M2 macrophages. However, it remains unclear as to whether HCVc affects the polarization of M2 macrophages, and if this effect promotes the progression of chronic disease. In the present study, peripheral blood mononuclear cells (PBMCs) from patients with CHC and healthy controls (HCs) were isolated, purified and polarized to M2a, M2b and M2c macrophages. Phenotypic expression, cytokine secretion and gene expression were analyzed using flow cytometry, ELISA and reverse transcription-quantitative polymerase chain reaction, respectively. Monocytes from HCs were cultured with HCVc to study the effect of HCVc on macrophage polarization. Plasma alanine transaminase and HCV-RNA levels were significantly higher, and albumin levels were significantly lower in the CHC group than those in the control group ($P < 0.05$). M2a macrophages polarized from monocytes of patients with CHC expressed lower levels of CD209, IL-1 receptor antagonist (IL-1RA) and Fizz1 compared with those from HCs. M2b macrophages expressed lower levels of CD86 and TNF- α , and M2c macrophages expressed lower levels of CD163, TGF- β and sphingosine kinase 1 (SPHK1) in the CHC group compared with HCs ($P < 0.05$). HCVc inhibited the expression levels of CD209, IL-1RA and Fizz1 in M2a macrophages; CD86 and TNF- α in M2b macrophages; and CD163, TGF- β and SPHK1 in M2c macrophages ($P < 0.05$). HCVc significantly suppressed phagocytosis of

all subtypes ($P < 0.05$); however, this inhibition was restored by an anti-Toll-like receptor (TLR)2 antibody ($P < 0.05$). In conclusion, HCVc inhibited monocyte-derived M2a, M2b and M2c subtype differentiation via the TLR2 signaling pathway, resulting in macrophages exhibiting reduced phagocytosis in patients with CHC. This may contribute to persistent HCV infection, thus suggesting that the blockade of HCVc may be a new therapeutic approach for the treatment of HCV infection.

Introduction

Hepatitis C is a global public health burden and the hepatitis C virus (HCV) infected ~71 million people worldwide by 2020 (1). Acute HCV infection often leads to chronic, long-term infection that can progress to liver cirrhosis and hepatocellular carcinoma (HCC) after several decades (1). Impaired immune responses to viral antigens, as well as chronic immune cell infiltration and activation in the liver may be associated with chronic HCV infection and progression to liver disease (2). Novel direct-acting antiviral drugs (DAAs) targeting nonstructural viral proteins of HCV are better tolerated compared with interferon by patients and increase sustained virological response rates (3). However, despite the ~100% cure rate, DAA therapy does not prevent HCV reinfection (4). In addition, DAAs do not reduce the risk of HCC in patients with HCV-related cirrhosis, and some patients develop primary liver cancer or recurrent tumors shortly after DAA treatment (5). Therefore, it is clinically and economically important to study the pathogenesis of chronic HCV infection and develop new immunotherapeutic drugs.

Epidemiological studies have suggested that acute HCV infection can be resolved without treatment in up to 20% of cases, which implies that the outcome can be controlled by innate and/or adaptive immune responses (6,7). Inflammatory cells and cytokine cascades activate and recruit monocytes or macrophages in chronic HCV infection. Macrophages differentiate from peripheral blood monocytes (PBMCs) and serve important roles in regulating host inflammatory responses and tissue pathology (8-12). T helper (Th)1 cytokines, such as interferon- γ , Toll-like receptor (TLR)4 ligand and lipopolysaccharide (LPS), polarize monocytes to

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classically activated M1 macrophages, producing proinflammatory cytokines, tumor necrosis factor (TNF)- α and IL-12, which in turn promote clearance of pathogens and lead to tissue damage. However, after exposure to Th2 cytokines, such as IL-4 and IL-13, monocytes polarize towards alternately activated M2 macrophages and produce anti-inflammatory mediators, including IL-10, which have an anti-inflammatory and wound-healing role (13,14). M2 macrophages are further differentiated into M2a, M2b and M2c subtypes (15). A previous study demonstrated that monocytes that are differentiated into M1 and M2 macrophages were both suppressed by HCV (16); however, it remains unclear as to whether HCV affects the polarization of the three subtypes of M2 macrophages, and if this effect promotes the progression of chronic disease. Thus, the present study hypothesized that HCV infection could affect M2 macrophage polarization.

Materials and methods

Study subjects. The present study included 25 patients with chronic hepatitis C (CHC) who visited the Digestive Diseases Hospital of Shandong First Medical University in Jining between January 2019 and April 2022. This cohort met the diagnostic criteria of the Guideline for Prevention and Treatment of Hepatitis C (2015) (17). Inclusion criteria were: i) Age, 18-70 years; ii) HCV infection for >6 months, or epidemiological history 6 months ago prior to the enrollment; iii) anti-HCV antibody and HCV-RNA positive; iv) evidence of histopathological examination in line with the diagnostic criteria for chronic hepatitis (17). Exclusion criteria were: i) Other concomitant viral hepatitis, such as chronic hepatitis B, D and other types of hepatitis; ii) drug-induced liver disease; iii) alcoholic liver disease; iv) autoimmune liver disease; v) HIV infection; vi) cancer; vii) severe cardiovascular or cerebrovascular disease; viii) hematological or thyroid disease; ix) diabetes; or x) receiving antiviral treatment.

A total of 25 healthy controls (HCs) registered in the Physical Examination Center of Digestive Diseases Hospital of Shandong First Medical University between January 2019 and April 2022 were selected as the control group. Exclusion criteria were the same as in the CHC group except for HCV infection. The present study was approved by the Ethics Committee of Digestive Diseases Hospital of Shandong First Medical University (approval code: 2018-LC-001), and all patients provided written informed consent before participating in the study.

Sample collection. A total of 4-6 ml venous blood was collected from patients and HCs in the morning on an empty stomach. The plasma was separated from cells by centrifugation at 1,500 x g for 5 min and stored at -80°C. The plasma was used to determine alanine aminotransferase (ALT), aspartate aminotransferase, alkaline phosphatase, γ -glutamyl transferase, total bilirubin, albumin (ALB) and HCV-RNA levels, and peripheral blood mononuclear cells (PBMCs) were isolated and cultured. ALT, AST, ALP, GGT and ALB were detected on the Toshiba TBA-120FR instrument by continuous monitoring method. The HCV-RNA level was detected by real-time PCR according to manufacturer's protocol.

Reagents. HCVc (amino acids 2-192) was purchased from Meridian Bioscience, Inc. Human recombinant macrophage colony-stimulating factor (M-CSF), LPS, IL-4, IL-13, IL-10 and Pam3CSK4 (TLR2/TLR1 agonist-synthetic triacylated lipoprotein), and a TLR2 antibody (cat. no. MAB2616-SP) were obtained from R&D Systems, Inc.

Cell isolation and purification. PBMCs were isolated from the peripheral blood of HCs and patients with HCV infection by Ficoll density gradient separation (Axis-Shield Diagnostics, Ltd.). Monocytes were purified by magnetic cell sorting with CD14 microbeads (BD Biosciences, cat. no. 130-050-201) according to manufacturer's protocol. The purity of the CD14⁺ monocytes was $\geq 95\%$ as determined using flow cytometry on a flow cytometer (FACScan; BD Biosciences) and the acquired data were analyzed using FlowJo software (v7.6; FlowJo LLC).

Cell culture. Purified monocytes were cultured in RPMI 1640 medium (Corning, Inc.) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and streptomycin for 5 days at 37°C in 5% CO₂. The monocytes were induced to polarize into M2 macrophages by adding 50 ng/ml M-CSF during the culture. For the polarization of M2a, M2b and M2c macrophages, M-CSF-induced macrophages were exposed to fresh culture medium supplemented with IL-4 (25 ng/ml) + IL-13 (25 ng/ml), LPS (10 ng/ml) + immune complex (33 μ l/ml), or IL-10 (25 ng/ml) for 24 h at 37°C in 5% CO₂, respectively (18). The preparation method of the immune complex was as follows: 1 μ l 1.32 mg/ml bovine serum albumin (BSA; Santa Cruz Biotechnology, Inc.) and 50 μ l rabbit polyclonal anti-BSA (Santa Cruz Biotechnology, Inc.) were mixed and incubated for 1 h at 37°C, whereupon they were stored at 4°C until use (typically overnight).

To study the effect of HCVc on macrophage polarization, HCVc (10 μ g/ml) or Pam3CSK4 (1 μ g/ml) was added to the M2 macrophage subtypes for 5 days. They were considered as HCVc group and Pam3CSK4 group, compared with W/O group that the cells were treated with medium alone. For mechanistic experiments, monocytes were pretreated with TLR2 antibody (0.15 μ g/ml) for 1 h at 37°C, and were then polarized to M2a, M2b and M2c macrophages and treated with HCVc at 37°C in 5% CO₂ for 5 days and then considered the HCVc/anti-TLR2 group.

Flow cytometry. Polarized M2 macrophages (0.1×10^6) were collected and resuspended in staining buffer (PBS supplemented with 0.5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.)) and preincubated with FcR blocking reagent (Miltenyi Biotec, Inc.) for 15 min at 4°C. Cells were simultaneously stained for 20-40 min at 4°C with CD209-V450 (cat. no. 561275), CD86-FITC (cat. no. 555657) and CD163-PE (cat. no. 556018; all from BD Biosciences; dilution 1:50), which are markers of M2a, M2b and M2c macrophages, respectively. Cells were washed with staining buffer [PBS supplemented with 0.5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.)] by centrifugation at 1,500 x g, at 4°C for 5 min and resuspended in PBS supplemented with 1% paraformaldehyde. Finally, phenotypic analysis of M2 macrophages was performed on a flow cytometer (FACScan; BD Biosciences) and the acquired data were analyzed using FlowJo software (v7.6; FlowJo LLC).

Table I. Comparison of baseline characteristics between CHC and HC groups.

Clinical indicators	HC group (n=25)	CHC group (n=25)	t-value	P-value
Age, years	49.32±1.05	48.88±1.20	0.277	0.783
Sex, male/female,	13/12	14/11		
ALT, U/l	25.45±0.90	29.86±0.90	3.478	0.001 ^a
AST, U/l	26.91±1.13	29.00±1.10	1.329	0.190
ALP, U/l	68.72±1.89	67.29±2.17	0.496	0.622
GGT, U/l	42.43±2.62	48.86±1.97	1.962	0.056
TBIL, U/l	7.79±0.31	8.58±0.31	1.817	0.075
ALB, g/l	49.43±0.53	46.64±0.67	3.268	0.002 ^a
HCV-RNA, IU/ml	<50	2.58x10 ⁶ ±0.18x10 ⁵	14.440	<0.0001 ^a

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ -glutamyl transpeptidase; TBIL, total bilirubin; ALB, albumin; CHC, chronic hepatitis C; HC, healthy control; HCV-RNA, hepatitis C virus-RNA. Data are shown as mean \pm standard deviation; ^aP<0.05 was considered to indicate a statistically significant difference.

ELISA. The cell culture supernatants were collected following macrophage polarization in each experimental condition. Concentrations of IL-1 receptor antagonist (IL-1RA) (cat. no. CHC1183), TNF- α (cat. no. 88734677) and TGF- β (cat. no. 885039088) in cell culture supernatants were measured by ELISA kits (eBioscience; Thermo Fisher Scientific, Inc.) according to manufacturer's protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was isolated using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed to cDNA using the reverse transcription kit (TransGen Biotech Co., Ltd.) at 4°C according to manufacturer's protocols. qPCR was performed using the Faststart Universal SYBR Green Master kit (Rox; Roche Diagnostics). The primer sequence pairs were designed using NCBI online primer BLAST software (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The following primers were used for qPCR: Fizz1, forward 5'-GTCAAAGCCAAGGCAGACC-3' and reverse 5'-TGAACATCCCAC-GAACCACA-3'; TNF- α , forward 5'-TGGGGAGTGTGAGGGGTATC-3' and reverse 5'-TGCACCTTCTGTCTCGGTTT-3'; sphingosine kinase-1 (SPHK1), forward 5'-CTGGCAGCTTCCTTG AACCAT-3' and reverse 5'-TGTGCAGAGACAGCAGGT TCA-3'; and GAPDH, forward 5'-GACTTCAACAGCAAC TCCCACTC-3' and reverse 5'-TAGCCGTATTCATTGTCA TACCAG-3'. Reactions were performed using 3 μ l cDNA in a 20- μ l reaction volume under the following thermocycling conditions: Pre-denaturation at 94°C for 10 sec, followed by denaturation at 94°C for 5 sec and final extension at 60°C for 30 sec, for 40 cycles. Relative gene expression was determined by normalizing the expression of each target gene to GAPDH. All the experiments were performed in triplicate and the 2^{- $\Delta\Delta$ C_q} method (19) was used to quantify the relative fold change in the mRNA expression levels of Fizz1, TNF- α and SPHK1.

Phagocytosis assay. Phagocytosis assay was performed as described previously (20,21). Polarized M2 macrophages in the presence or absence of HCVc were incubated with FITC-latex beads (cat. no. L4655; MilliporeSigma; 0.05% of the stock

concentration) in refreshed culture medium for 1 h at 37°C with 5% CO₂. Cells were then collected. Phagocytic activity of the cells was detected using a flow cytometer (FACScan; BD Biosciences) and the acquired data were analyzed using FlowJo software (v7.6; FlowJo LLC).

Statistical analysis. All experiments were repeated three times. The data were collated into an excel table. All continuous variables presented as mean values \pm standard deviation were normally distributed and were analyzed and found to be significant using the D'Agostino and Pearson omnibus normality test. Mean values were compared using an unpaired Student's t-test (two groups) or one-way ANOVA (more than two groups) followed by Bonferroni correction for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference. The statistical analysis was performed using GraphPad Prism Version 5 (GraphPad Software, Inc.).

Results

Baseline characteristics of study subjects. To test the hypothesis that HCV affects the polarization of monocytes to macrophages, 25 patients with CHC and 25 HCs were enrolled in the present study (Table I). The CHC group included 14 men and 11 women, aged 48.88±1.20 years (range, 37-60 years), and the HC group included 13 men and 12 women, aged 49.32±1.05 years (range, 39-65 years). Plasma ALT and HCV-RNA levels were significantly higher, and ALB levels were significantly lower in the CHC group compared with those in the HC group (P<0.05; Table I). There was no significant difference in the sex ratio, age distribution and other biochemical indexes between the two groups.

HCV infection inhibits monocyte polarization to M2 macrophages. Purified monocytes from the HC and CHC groups were polarized to M2a, M2b and M2c macrophages. The expression of cell surface markers, CD209 (M2a), CD86 (M2b) and CD163 (M2c), of the three types of M2 macrophages were detected using flow cytometry. The secretion of the cytokines IL-1RA, TNF- α and TGF- β , which are markers for

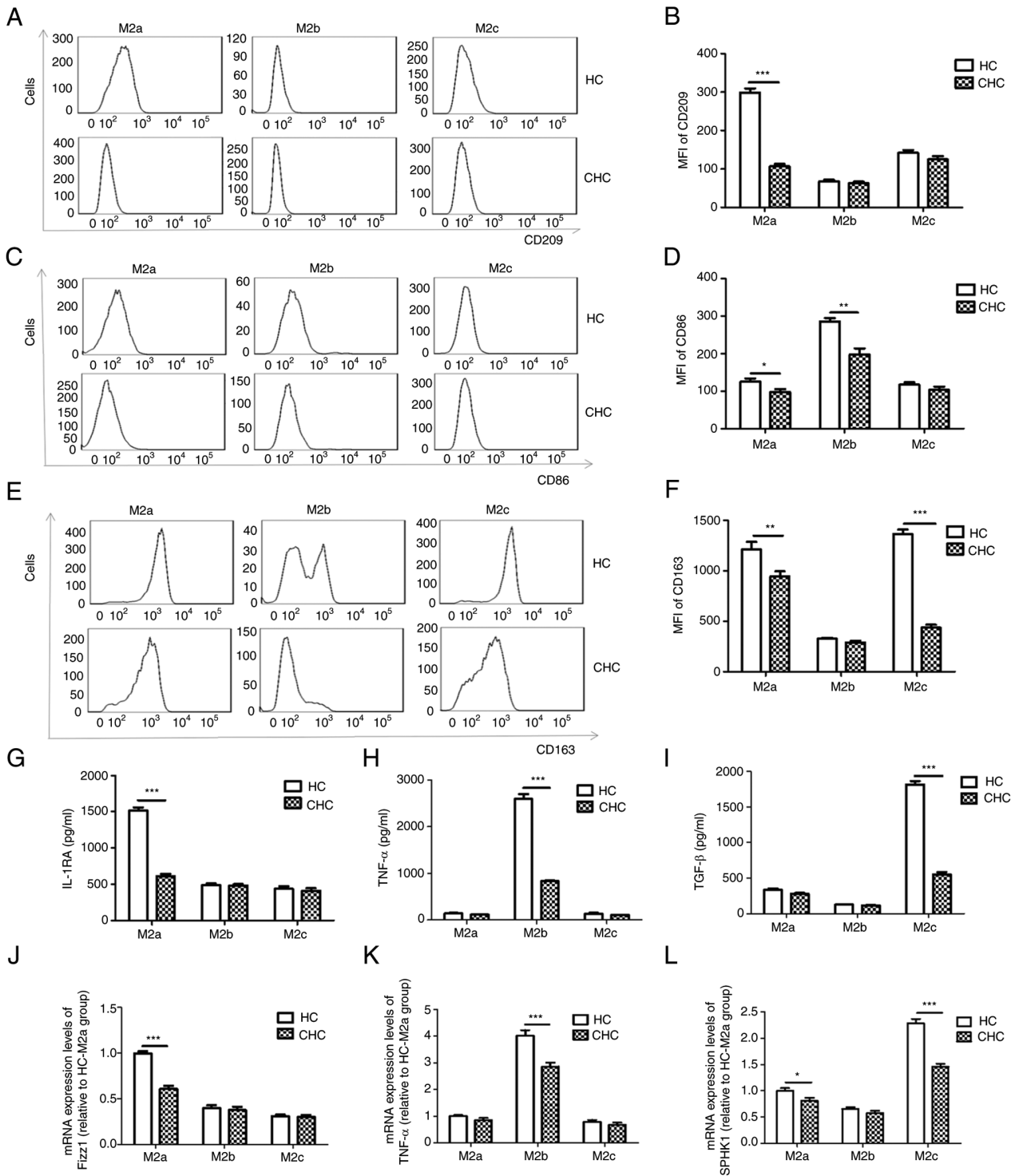


Figure 1. Differentiation of peripheral blood monocytes into M2a, M2b and M2c macrophages is impaired in patients with CHC. (A and B) M2a macrophages, which were differentiated from peripheral blood monocytes derived from patients with CHC, expressed lower levels of CD209 compared with those in the HC group. (C and D) M2b macrophages, which were differentiated from peripheral blood monocytes derived from patients with CHC, expressed lower levels of CD86 compared with those in the HC group. (E and F) M2c macrophages, which were differentiated from peripheral blood monocytes derived from patients with CHC, expressed lower levels of CD63 compared with those in the HC group. Secreted levels of (G) IL-1RA, (H) TNF- α and (I) TGF- β were decreased in M2a, M2b and M2c macrophages differentiated from peripheral blood monocytes of patients with CHC compared with those in the HC group, respectively. mRNA expression levels of (J) Fizz1, (K) TNF- α and (L) SPHK1 were decreased in the M2a, M2b and M2c macrophages differentiated from peripheral blood monocytes of patients with CHC compared with those in the HC group, respectively. To simplify the results, mRNA expression levels were calculated relative to HC-M2a group'. n=25. *P<0.05, **P<0.01, ***P<0.001. CHC, chronic hepatitis C; HC, healthy control; MFI, mean fluorescence intensity; IL-1RA, IL-1 receptor antagonist; SPHK1, sphingosine kinase 1.

M2a, M2b and M2c, respectively, were analyzed using ELISA. The mRNA expression levels of Fizz1, TNF- α and SPHK1, which are markers for M2a, M2b and M2c, respectively, were

detected by RT-qPCR. The expression levels of CD209, CD86 and CD163 on M2a macrophages (P<0.05; Fig. 1A-F), CD86 on M2b macrophages (P<0.05; Fig. 1C and D) and CD163 on

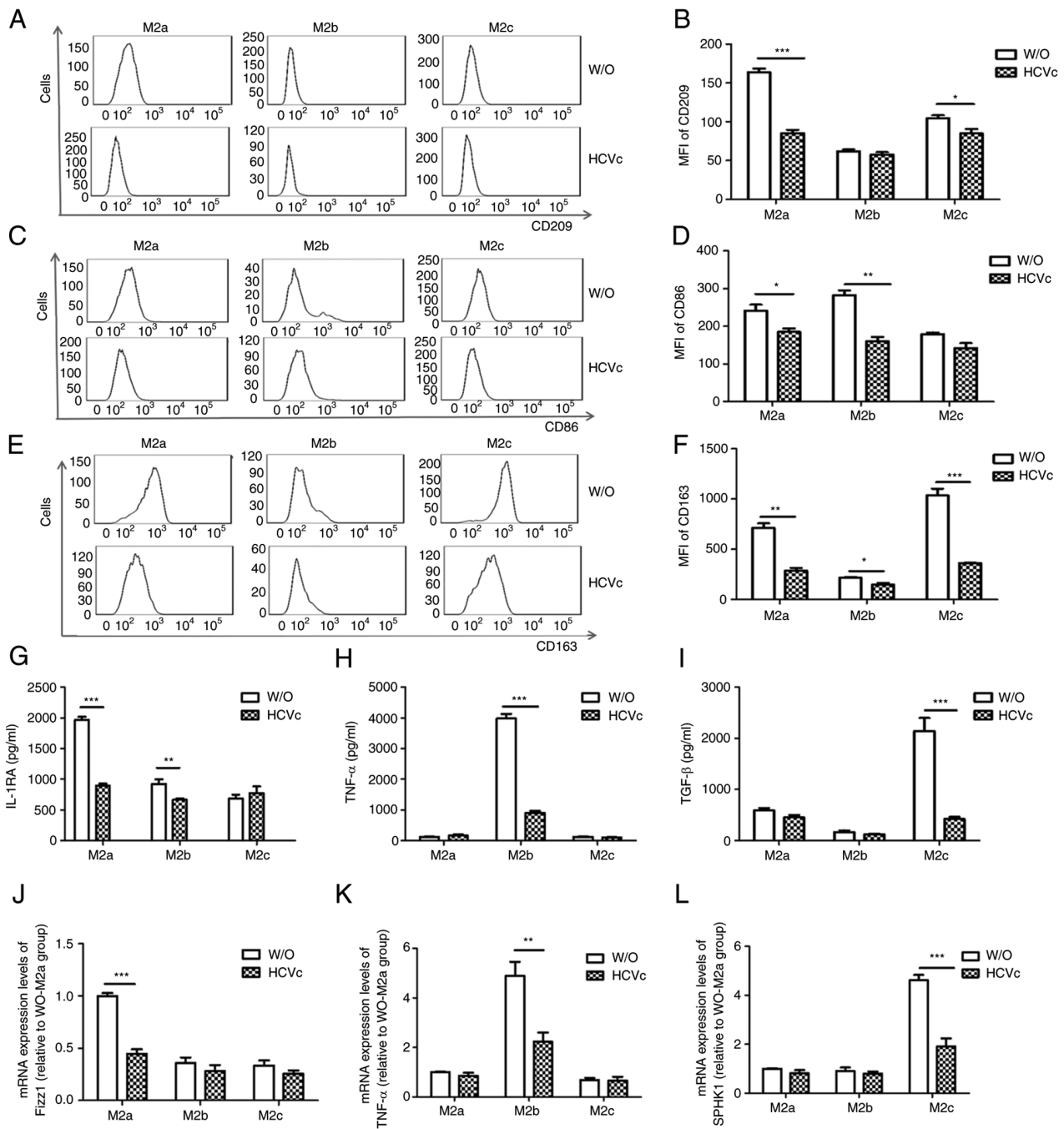


Figure 2. Differentiation of peripheral blood monocytes derived from the HC group to the three subtypes of M2 macrophages is inhibited by exposure to HCVc. (A and B) Expression of CD209 on M2a macrophages was inhibited by HCVc. (C and D) Expression of CD86 on M2b macrophages was inhibited by HCVc. (E and F) Expression of CD163 on M2c macrophages was inhibited by HCVc. Secretion levels of (G) IL-1RA, (H) TNF- α and (I) TGF- β were decreased in the M2a, M2b and M2c macrophages that were treated with HCVc, respectively. mRNA expression levels of (J) Fizz1, (K) TNF- α and (L) SPHK1 were decreased in the M2a, M2b and M2c macrophages treated with HCVc, respectively. Representative histograms of three independent experiments are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. MFI, mean fluorescence intensity; HCVc, hepatitis C virus core protein; W/O, control group (W/O, without stimulant; other groups received HCVc); IL-1RA, IL-1 receptor antagonist; SPHK1, sphingosine kinase 1.

M2c macrophages ($P < 0.05$; Fig. 1E and F) were significantly lower in patients with CHC than those in the HC group. The release levels of IL-1RA ($P < 0.05$; Fig. 1G) and the mRNA expression levels of Fizz1 and SPHK1 ($P < 0.05$; Fig. 1J and L) in M2a macrophages; the release levels of TNF- α ($P < 0.05$; Fig. 1H) and the mRNA expression levels of TNF- α ($P < 0.05$; Fig. 1K) in M2b macrophages; and the release levels of TGF- β ($P < 0.05$; Fig. 1I) and the mRNA expression levels of SPHK1 ($P < 0.05$; Fig. 1L) in M2c macrophages, were all significantly

decreased in patients with CHC compared with those in the HC group. These results suggested that peripheral monocytes derived from patients with CHC were less polarizable towards the three types of M2 macrophages compared with the HC group. Therefore, the ability of human PBMCs from patients with CHC to polarize to M2 macrophages could be impaired.

HCVc suppresses M2 macrophage polarization. According to our previous study, HCVc can inhibit the polarization

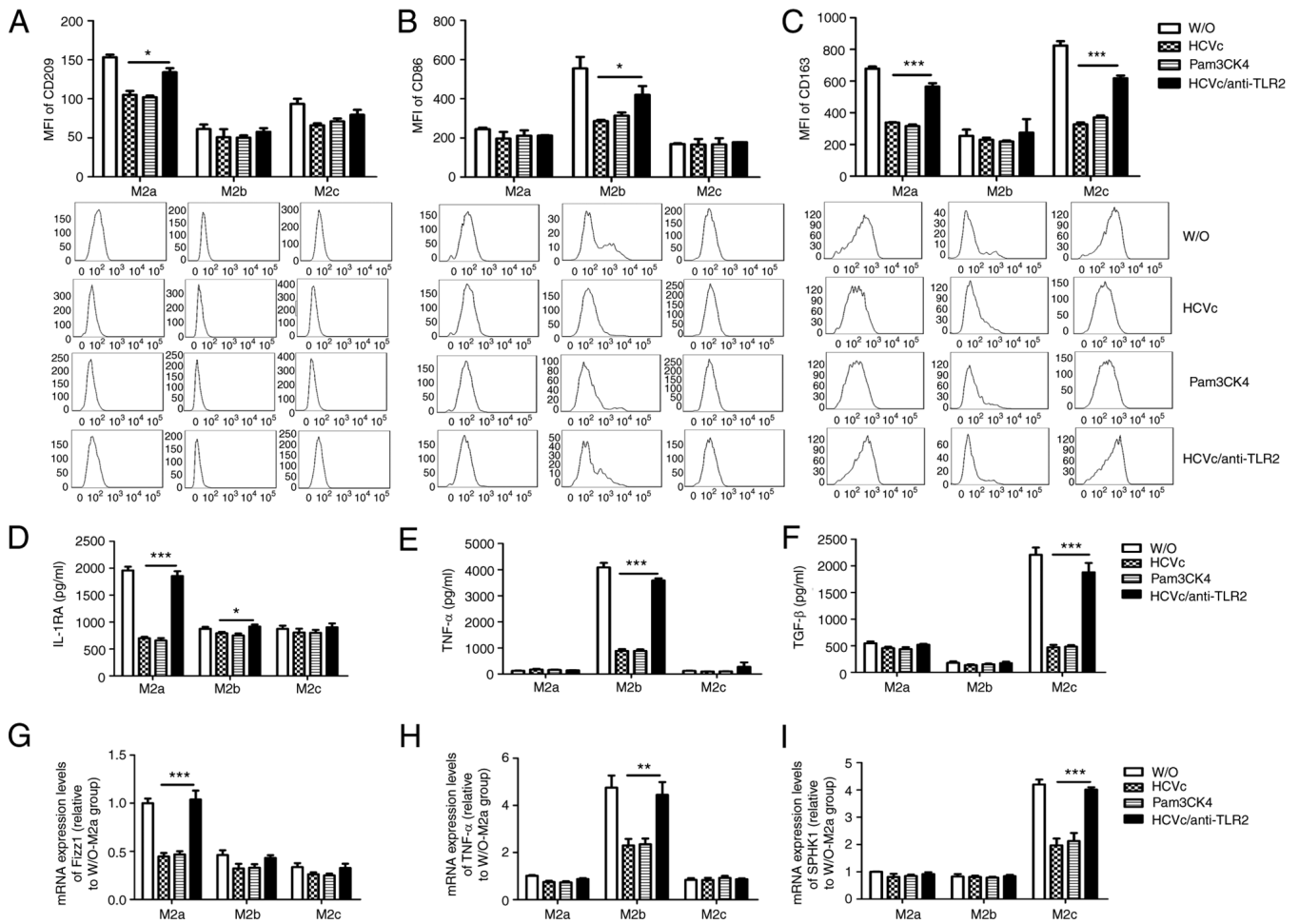


Figure 3. Macrophage polarization is inhibited by HCVc via TLR2 signaling. Expression levels of (A) CD209 in M2a macrophages, (B) CD86 in M2b macrophages and (C) CD163 in M2c macrophages were decreased by exposure to HCVc or Pam3CSK4. Secretion levels of (D) IL-1RA in M2a macrophages, (E) TNF- α in M2b macrophages and (F) TGF- β in M2c macrophages were decreased by exposure to HCVc or Pam3CSK4. mRNA expression levels of (G) Fizz1 in M2a macrophages, (H) TNF- α in M2b macrophages and (I) SPHK1 in M2c macrophages were decreased by exposure to HCVc or Pam3CSK4. (A-I) Blockade of the TLR2 signaling pathway using a TLR2 antibody partially restored macrophage polarization. Representative histograms of three independent experiments are shown. *P<0.05, **P<0.01, ***P<0.001. MFI, mean fluorescence intensity; HCVc, hepatitis C virus core protein; W/O, control group (W/O, without stimulant); other groups received HCVc, Pam3CSK4 and TLR2 antibody); IL-1RA, IL-1 receptor antagonist; SPHK1, sphingosine kinase 1; TLR2, Toll-like receptor 2.

of monocytes towards M1 and M2 macrophages (16). The present study investigated whether the impaired polarization of the three types of M2 macrophages in patients with CHC was also affected by HCVc. Therefore, purified monocytes derived from the healthy people were polarized to M2a, M2b and M2c macrophages in the presence or absence of HCVc to study the effect of HCVc on M2 macrophages polarization *in vitro*. The optimal HCVc concentration was chosen according to the results of previous studies (16,22); 10 μ g/ml was used as the standard concentration in all experiments. The results of the present study demonstrated that HCVc downregulated the expression levels of CD209, CD86 and CD163 (P<0.05; Fig. 2A-F) on M2a macrophages, as well as the release of IL-1RA (P<0.05; Fig. 2G) and the mRNA expression levels of Fizz1 (P<0.05; Fig. 2J), compared with W/O group. HCVc also downregulated the expression levels of CD86 (P<0.05; Fig. 2C and D) on M2b macrophages, as well as the release of IL-1RA and TNF- α (P<0.05; Fig. 2G and H) and the mRNA expression levels of TNF- α (P<0.05; Fig. 2K), compared with the medium controls.

In addition, HCVc downregulated the expression levels of CD209 and CD163 (P<0.05; Fig. 2A, B, E and F) on M2c macrophages, as well as the release of TGF- β (P<0.05; Fig. 2I) and the mRNA expression levels of SPHK1 (P<0.05; Fig. 2L), compared with the medium controls. The present results demonstrated that HCVc inhibited all the three types of M2 macrophage polarization, which is in accordance with chronic HCV infection.

HCVc suppresses M2 macrophage polarization through TLR2 signaling. Based on our previous research, HCVc may inhibit TLR2-mediated polarization of M1 and M2 macrophages (16). In the present study, the involvement of the TLR2 signaling pathway was further investigated in the three types of M2 macrophage polarization. Purified monocytes were pretreated with a TLR2 antibody, and in turn polarized into M2a, M2b and M2c macrophages and treated with HCVc. Pam3CSK4, a canonical TLR2 ligand, was used as a control for the HCVc binding to TLR2. Blockade of the HCVc/TLR2 interaction using the TLR2 antibody

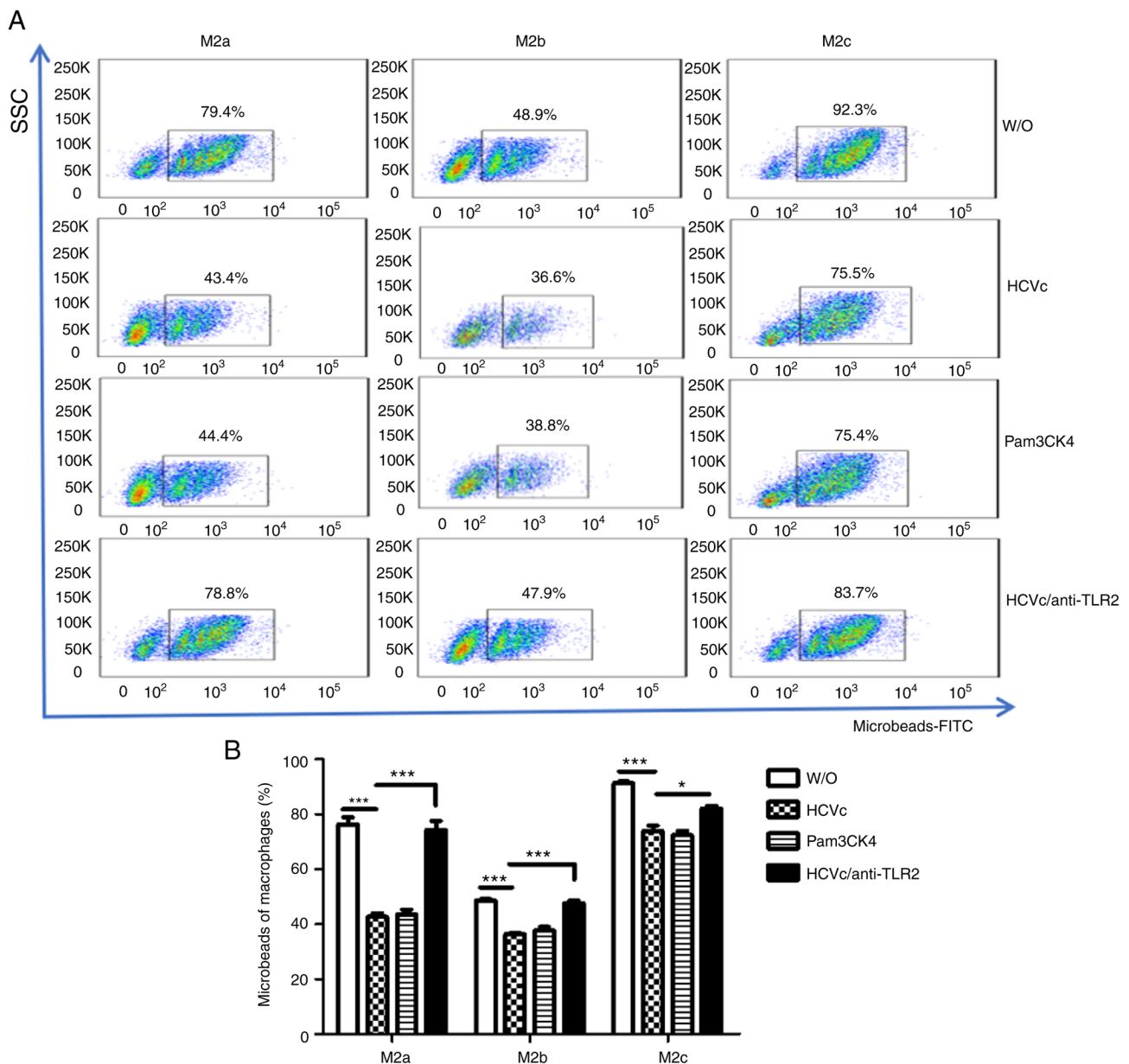


Figure 4. Phagocytic activity of M2a, M2b and M2c macrophages is inhibited by exposure to HCvC. (A) HCvC and Pam3CSK4 inhibited phagocytic activity of all three subtypes of M2 macrophages. Pre-treatment with a TLR2 antibody blocked the inhibitory effect of HCvC. (B) Statistical analysis was performed on three replicates. Flow cytometry charts are representative of one of the three replicates used for statistical analysis. * $P < 0.05$, *** $P < 0.001$. HCvC, hepatitis C virus core protein; W/O, control group (W/O, without stimulant; other groups received HCvC, Pam3CSK4 and TLR2 antibody); TLR2, Toll-like receptor 2.

upregulated the expression levels of CD209 and CD163 ($P < 0.05$; Fig. 3A and C) on M2a macrophages, as well as the release of IL-1RA ($P < 0.05$; Fig. 3D) and the mRNA expression levels of Fizz1 ($P < 0.05$; Fig. 3G), compared with HCvC group. Blockade of HCvC/TLR2 engagement using the TLR2 antibody also upregulated the expression levels of CD86 ($P < 0.05$; Fig. 3B) on M2b macrophages, as well as the release of IL-1RA and TNF- α ($P < 0.05$; Fig. 3D and E) and the mRNA expression levels of TNF- α ($P < 0.05$; Fig. 3H), compared with HCvC alone. In addition, blockade of HCvC/TLR2 engagement using the TLR2 antibody upregulated the expression levels of CD163 ($P < 0.05$; Fig. 3C) on M2c macrophages, as well as the release of TGF- β ($P < 0.05$; Fig. 3F) and the mRNA expression levels of SPHK1 ($P < 0.05$; Fig. 3I), compared with HCvC alone. By contrast,

Pam3CSK4 significantly suppressed M2 macrophage polarization compared with W/O group. In summary, HCvC inhibited M2a, M2b and M2c macrophage polarization via TLR2, and there was no difference in the effect of HCvC on the polarization of the three subtypes.

HCvC suppresses the phagocytic activity of M2 macrophages via TLR2 signaling. Macrophages have a vital role in immune surveillance through phagocytic activity; therefore, the effect of HCvC on the phagocytic activity of the three M2 subtypes was investigated. Polarized M2 macrophages treated with HCvC or Pam3CSK4 were cultured in the presence of FITC-latex beads. The phagocytic activity was then analyzed by flow cytometry. HCvC and Pam3CSK4 significantly suppressed the phagocytic activity of all three subtypes ($P < 0.05$; Fig. 4). To determine

if the suppression of phagocytosis caused by HCVC was regulated through its interaction with TLR2, purified monocytes were pretreated with a TLR2 antibody and polarized to M2a, M2b and M2c macrophages in the presence of HCVC; Pam3CSK4 was used as a control. Blocking the binding of HCVC to TLR2 using a TLR2 antibody partially restored the phagocytic activity of all three subtypes of M2 macrophages ($P < 0.05$; Fig. 4).

Discussion

Macrophages serve a vital role in the primary immune response to pathogenic agents, inflammation, repair, resolution of inflammation and tissue homeostasis (23). Macrophages exhibit a range of different phenotypes, in connection with macrophage polarization. They take part in host defense, immunoregulation and tissue recovery, and are defined by surface markers and the release of soluble cytokines (24). Activated macrophages can be divided into M1 and M2 subtypes under different stimulation conditions. In addition, M2 macrophages can be further subdivided into M2a, M2b and M2c in the appropriate context (25). IL-4 and IL-10 cytokines can activate M2a macrophages, expressing high levels of surface markers, such as IL-1RA, CD209, Fc ϵ receptor and Dectin-1 (26). IL-1, LPS, immune complexes such as viral antigens and TLR/IL-1R induce activation of M2b macrophages, which are involved in immunoregulation (27). Notably, M2b macrophages produce cytokines, such as IL-1, IL-6 and TNF- α . M2c macrophages are induced by anti-inflammatory cytokines IL-10 and TGF- β (28), and produce IL-10, TGF- β and α 1-antitrypsin. M2c macrophages also take part in extracellular matrix remodeling, tissue repair and phagocytosis of apoptotic cells (29).

During HCV infection, monocytes and macrophages mediate an abnormal inflammatory response that affects the natural history of infection (30). Plasticity and functional polarization are hallmarks of macrophages, leading to phenotypic diversity in macrophage populations. Dysregulation of macrophage polarization is associated with the pathogenesis of various diseases (31,32). According to our previous research, the polarization of monocytes in patients with CHC to both M1 and M2 macrophages was impaired compared with that of healthy monocytes (16). In the present study, the differences in M2a, M2b and M2c subtypes between patients with CHC and HCs were investigated. It was demonstrated that HCV inhibited the polarization of all three subtypes. Despite the relatively small sample size of 25 patients with CHC and 25 HCs, the results of all indicators were consistently significant between the two groups; therefore, the effect of chance could be excluded. In the context of HCV infection, impaired macrophage polarization may contribute to chronic infection, where the subsequent immunosuppression promotes liver disease.

HCVC is found in the cytoplasm and nuclei of infected cells, including hepatocytes and other cells in the liver, from which it can be secreted as well (33). A previous study showed that HCVC could inhibit the expression of TNF- α and transferrin receptor protein 1 expression and phagocytosis of HCVC particles in M1 macrophages, which should be enhanced in the conversion of M1 to M2 (34). The differentiation of

peripheral blood monocytes into M1/M2 macrophages in the presence of HCVC was studied in our previous study, which showed that HCVC could inhibit monocyte polarization to M1 and M2 macrophages (16). In the present study, the effect of HCVC on the differentiation of M2a, M2b and M2c subtypes was further investigated. As expected, HCVC inhibited polarization of all three subtypes.

It has been reported that HCVC can activate TLR2, which is expressed in human monocytes, macrophages, Kupffer cells and regulatory T cells, to induce the production of inflammatory cytokines by activating the MyD88-dependent TLR signaling pathway (35–37). HCV induces the production of myeloid derived suppressor cells (MDSCs) like suppressive monocytes via the TLR2/PI3K/AKT/STAT3 signaling pathway, which in turn induces CD4⁺Foxp3⁺ regulatory T cells and inhibits autologous CD4⁺ T cell activation (38). In our previous study, HCVC inhibited the polarization of M1 and M2 macrophages by binding to TLR2 (16). Therefore, it was hypothesized that HCVC engagement with TLR2 on monocytes could regulate the subtypes of M2 macrophages polarization. *In vitro*, peripheral blood monocytes derived from the HC group were differentiated into M2a, M2b and M2c macrophages in the presence of HCVC, and the results showed that HCVC could suppress the polarization of monocytes to all three subtypes of M2 macrophages. Moreover, the TLR2 ligand Pam3CSK4 inhibited the polarization of M1/M2 macrophages, whereas blocking the binding of HCVC to TLR2 partially alleviated the HCVC-induced inhibition of macrophage polarization. These results suggested that HCV may modulate the polarization of various subtypes of M2 macrophages through the interaction of HCVC with TLR2 on monocytes.

Macrophages have a large capacity for phagocytosis, which is the first step for the presentation of antigens of foreign pathogens. Macrophages engulf HCV intracellularly by phagocytosis (39). Consequently, the phagocytosis of HCV by macrophages contributes to viral clearance. In our previous research, the phagocytic function of M2 macrophages was revealed to be more prominent than that of M1 macrophages, and phagocytic activity of both M1 and M2 macrophages was downregulated by HCVC (16). The results of the present study are in line with the aforementioned observations. The phagocytic activity of all three subtypes of M2 macrophages was downregulated by HCVC; however, the blockade of TLR2 signaling restored the macrophage polarization and the phagocytic activity of all the types of M2 macrophages. These findings suggest a mechanism by which HCV can escape phagocytosis by macrophages.

In conclusion, the present study showed that monocyte polarization toward M2 macrophage subtypes (M2a, M2b and M2c) may be impaired in patients with CHC via the interaction of HCVC with TLR2, resulting in a decline in phagocytosis. The present study provided a novel perspective regarding the mechanism by which HCV develops into a chronic persistent infection due to HCVC. It may be proposed that blocking the binding of HCVC to TLR2 could be a therapeutic strategy against HCV infection.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QZ contributed to the conception and design of the study. SZ performed the experiments and drafted the article. XD contributed to the acquisition of the blood samples and operation of the experiments. MS and LK contributed to the implementation of the study and the acquisition of the data. DW contributed to data analysis. SZ and XD confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Affiliated Hospital of Gastroenterology of Shandong First Medical University. All patients enrolled in the present study provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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