The Effects of Bushen Jianpi Recipe on the Maturation of Plasmacytoid Dendritic Cell to Treat Chronic Hepatitis B

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Research

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Abstract

Background: Plasmacytoid dendritic cell (pDC) has been reported showing obvious maturation and deficient function in chronic hepatitis B (CHB) patients. One of the traditional Chinese medicine, Bushen Jianpi Recipe (BSJPR), has efficacy in suppressing HBV by improving immune system. In this study, we explained its mechanism on cellular level.

Methods: We measured the contents of pDC maturation-related extracellular cytokines and intracellular signaling pathway proteins in HBV-infected or not infected PBMCs and pDCs to explore the cellular mechanism of BSJPR. There were six groups: a normal serum control group added with fetal bovine serum, a rat serum control group added with normal serum, two HBV virus groups added with normal serum and HBV with different concentrations, and two BSJPR groups added with drug-containing serum and HBV with different concentrations.

Results: It was revealed that the effect of BSJPR on extracellular cytokines was vague, while intracellular toll-like receptor (TLR) /myeloid differentiation primary response 88 (MyD88) /TIR-domain-containing adapter-inducing interferon-β (TRIF) signaling pathway proteins were induced by BSJPR.

Conclusions: This study confirmed that BSJPR induces pDC to alleviate CHB via up-regulating intracellular TLR/MyD88/TRIF signaling pathway proteins rather than directly regulating extracellular cytokines.

1. Background

Chronic hepatitis B (CHB) is an infectious disease that endangers human health severely. There are more than 400 million people infected worldwide, especially in China where approximately 10–20% of the population are infected by hepatitis B virus (HBV) [1, 2]. One million people die each year because of HBV-related hepatic failure, liver cirrhosis and hepatocellular carcinoma [3, 4]. It has been confirmed that cellular immunodeficiency is an important cause of chronic and refractory hepatitis B [5, 6].

Dendritic cell (DC) is important in initiating immune response [7]. The mature DCs can induce the differentiation of naïve T cells, which builds a connection between innate immune and adaptive immune [8, 9]. DC also produces various proinflammatory cytokines in response to viral infection. Thus, the number of DC determines the immune response level and implies the severity of viral infection [10]. It has been reported that DC inhibits the replication of HBV efficiently [11]. Nevertheless, HBV can lead to impaired maturation and function of DC, and makes DC as a place for viral protein synthesis [12, 13]. For example, HBV is able to inhibit the expression of cluster of differentiation 4 (CD4), CD80 and CD86 on the DC surface [14]. In addition, HBV decreases interleukin 12 (IL-12), inhibits the activation of HBV-specific T cells, and leads to immune tolerance of HBV-specific cytotoxic T lymphocyte (CTL) [15]. To sum up, altering the expression of extracellular cytokines and the conduction of intracellular signals in DC is likely to be a reason for chronic and refractory HBV.
DC is classified into plasmacytoid DC (pDC), myeloid DC (mDC) and conventional DC (cDC) [16]. pDC is found in human peripheral blood and represent 10% of total DC in the liver [8, 17]. It is a dominated type I interferon (IFN-I)-producing cell, producing over 95% IFN-I in peripheral blood and innately responding to viral infection [18]. pDC also produces moderate amount of IL-6, tumor necrosis factor alpha (TNF-α) and small amount of IL-12 [19]. Furthermore, pDC promotes B cell differentiation into plasma cell, and recognizes viral nucleic acids and proteins. In HBV infection condition, pDC is one of the main effector cells in the early antiviral innate immune response [20]. Previous studies have shown that pDC is impaired in CHB patients [21]. CHB patients manifest decreased IFN-I, implying suppressed natural killer (NK) cell and macrophagocyte, which is a reason for HBV immunity escape [8].

Nowadays, treating CHB through traditional Chinese medicine (TCM) has been receiving increasing attention, especially in China. One of the recipes, Bushen Jianpi Recipe (BSJPR), has efficacy in inhibiting HBV at some degree by improving immune system. However, there are limited researches having explored its mechanism. In this study, we investigated the molecular mechanism of BSJPR for curing CHB in the aspect of pDC maturation-related extracellular cytokines and intracellular signaling pathway proteins.

2. Methods

2.1. Preparation of Drug-Containing Serum

The drug-containing serum was collected from rats. The daily dose of rats was 8.46 g/kg and was administered by gavage. Twelve normal SPF Wistar rats with 250 ± 20 g were purchased from SLAC Laboratory (Shanghai, China) and were randomly divided into two groups, blank control group (n = 6) and BSJPR group (n = 6). The BSJPR group was given BSJPR by gavage with 8.46 g/kg/day and the blank control group was given equal volume of saline by gavage. The drug and saline were administrated twice a day for three days, then the rats underwent fast for one night. In the next morning, the two groups were taken the same dosage of respective agent (BSJPR or saline) via gavage. Two hours after the last gavage, all rats were anesthetized by 2% pentobarbital sodium and their blood was collected from abdominal aorta. Rats were finally sacrificed by abdominal aorta bloodletting. The blood was centrifuged at 4 °C, 3000 rpm/min for 20 minutes, and the separated serum was inactivated in a water bath at 56 °C for 30 minutes. The serum then passed through a 0.22 um filter membrane and was frozen at -80 °C. Animals received humane care and the protocols were approved by the Local Ethics Committee for Animal Research Studies at the Shanghai University of Traditional Chinese Medicine.

2.2. Separation of Peripheral Blood Mononuclear Cell

5 ml of peripheral blood was collected from healthy volunteers. The blood was diluted equally with PBS. A 15 ml of centrifuge tube was filled with 6 ml of lymphocyte separation fluid that was twice as much as blood. The blood was added into the centrifuge tube, and was centrifuged at 800 rpm/min for 20 minutes at room temperature. Peripheral blood mononuclear cells (PBMCs) were extracted from the grayish white band that was between the upper and middle layer. The collected cells were suspended with twice as
much PBS and was washed twice by centrifuge with 1000 and 800 rpm/min for 10 and 5 minutes, respectively. The washed cells were mixed with 100 ul of DMSO and 900 ul of 1640 medium.

Separated PBMCs were evenly placed on 6-well plates with $5 \times 10^6$/well. The wells were divided into six groups: a normal serum control group containing 10% fetal bovine serum; a rat serum control group containing 15% normal rat serum; two HBV virus groups containing 15% normal rat serum with $10^5$/ml and $10^7$/ml HBV, respectively; two BSJPR groups containing 15% drug-containing serum with $10^5$/ml and $10^7$/ml HBV, respectively. Each group took up 5 wells and was placed in a 5% CO$_2$ incubator at 37°C.

2.3. Detection of Cytokine

Cytometric beads array was used to detect the contents of cytokines. Standard dry powder was transferred to a 15 ml of streaming sample tube, and 4 ml of Assay Diluent solution was used to dilute and resuspend the powder. This tube was labeled TOP Standard and was allowed to stand at room temperature for 15 minutes. Next, nine solutions were prepared via gradient dilution which were labeled 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256, respectively. Another tube was taken and was labeled 0 pg/ml that was only filled with 500 ul of Assay Diluent dilution. 50 ul of sample was added to analysis tube. 50 ul of capture microspheres were mixed and transferred into analysis tube. The tube was incubated for 1 hour at room temperature. 50 ul of PE detection reagent was added to the tube and was mixed by shaking. The solution was incubated for 2 hours at room temperature. Then, 1 ml of Wash Buffer was added and the solution was centrifuged at 1000 rpm/min for 5 minutes. After the supernatant being removed, 300 ul of Wash Buffer was added and the microspheres were resuspended by shaking.

2.4. Detection of pDC

The cultivated PBMCs were transferred into a 1.5 ml of EP tube. The tube was centrifuged at 1200 rpm/min for 5 minutes and the supernatant was discarded. The tube was washed with 1 ml of PBS and was centrifuged at 1200 rpm/min for 5 minutes, and the supernatant was discarded. Next, the tube was filled with 200 ul of PBS and was extracted 50 ul after mixing. 5 ul of FITC-labeled Lin-1 antibody, 2.5 ul of PE-labeled CD123, and 2 ul of Percp-labeled HLA-DR APC fluorescent CD11c antibody were added, and the solution was incubated for 20 minutes in dark. Flow cytometer settings: P5 for the exclusion of debris and cells; P6 for the weak positive and negative cells of Lin; P7 for HLA-DR/CD123 cells; P8 for CD11c + cells. The flow detection of pDC was Lin-, CD123 + and HLA-DR+. Each group of pDC was compared by Cellquest software analysis. The CD123, CD11c and HLA-DR were from BD biosoences (San Jose, California, USA).

2.5. The isolation of pDC

The cell suspension that was selected by negative separation of CD14 microbeads (Miltenyi Biotec) was centrifuged for 10 minutes, and the cell pellet was resuspended at $10^8$/300 ul. Every $10^8$ cells were filled with 100 ul of FcR Blooong Rgeagent and 100 ul of CD304 microbeads (Miltenyi Biotec). The solution was mixed and incubated for 15 minutes. Every $10^8$ cells were filled with 5 to 10 ml of sample buffer then were centrifuged at 1200 rpm/min for 10 minutes after being washed. The supernatant was discarded,
and every $10^8$ cells were filled with 500 ul of sample buffer to be resuspended. The cells were collected by MS/LS column (MS: 500 ul, LS: 3 ml). The effluent liquid was collected, and was washed with sample buffer 3 times. The MS/LS column was taken out and placed in a sterile centrifuge tube. A piston was installed to collect the cell suspension for pDC cultivation. pDCs were placed in complete medium RPMI 1640 (10% fetal bovine serum) and were filled with CpGODN 2216 (3 µg/ml) to be stimulated for 24 hours.

The cultured pDCs were separately divided into normal serum control group, rat serum control group, HBV virus group, and BSJPR group. The normal serum control group was added with 10% fetal bovine serum. The rat serum control group was added with 15% normal rat serum. The HBV virus group was added with 15% normal rat serum and with $10^5$/ml HBV. The BSJPR group was added with 15% drug-containing serum and with $10^5$/ml HBV.

**2.6. Real Time PCR Detection**

Every $10^6$ cells were homogenized in 1 ml of TRLZOL (No:10296010, Thermo fisher) and were mixed with 200 ul of chloroform (Sinopharm chemical reagent Co., Ltd). After spinning, the upper aqueous phase was filled with the same volume of isopropanol. After the second spinning, the precipitate was mixed with 1 ml of 75% ethanol. Isolated RNA was converted to cDNA according to the manufacturer’s instructions. QPCR was performed with gene-specific primers (Table 1).

**Table 1 The primers used in the present study**
2.7. Western Blot Detection

Every $10^6$ cultured cells were filled with 300 ul of tissue lysate (lysate: protease inhibitor = 99:1), then the solution was homogenized. The supernatant was taken out, and protein sample (50 µg) and protein marker (10 µl) were loaded into each lane. The membrane was blotted with the primary antibody (diluted at a concentration of 1:200) and the secondary antibody (diluted at a concentration of 1:1000), washed with 1 × TBST. The protein in membrane was visualized by the Electro-Chemi-Luminescence (ECL) method. The sources of antibodies were as follow: c-Jun (60A8) Rabbit mAb (#9165), interferon regulatory factor 3 (IRF3) (D83B9) Rabbit mAb (#4302), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p105/p50 Antibody (#3035), toll-like receptor 3 (TLR3) (D10F10) Rabbit mAb (#6961), TLR9 Antibody (#2254) and TIR-domain-containing adapter-inducing interferon-β (TRIF) Antibody (#4596) were from Cell Signaling Technology (Danvers, MA, USA), and anti-IRF7 antibody [EPR4718] (ab109255) was from Abcam (Cambridge, MA, USA).

2.8. Statistics Analysis
All data were shown as mean SEM. Differences between two groups were compared with the 2-tailed unpaired t test. Differences between multiple groups were compared by 1-way or 2-way analysis of variance with either Newman–Keuls or Bonferroni posttests (GraphPad Prism 4.0a; GraphPad Software, La Jolla, CA). \( P < 0.05 \) was considered to be significant.

3. Results

3.1. Impact of HBV on PBMC Cytokine Content

HBV had the most obvious effect on the content of IL-6. The contents of IL-6 (Fig. 1C) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 1H) increased dramatically, reaching peaks at the 36th hour and beginning to decrease. The contents of TNF-\( \alpha \) (Fig. 1F), IFN-\( \gamma \) (Fig. 1G), IL-4 (Fig. 1B) and IL-17A (Fig. 1E) reached peaks at the 8th hour and then began to drop. Furthermore, TNF-\( \alpha \), IFN-\( \gamma \) and IL-4 rose again after the 36th hour. HBV had slightly inducing effect on the content of IL-2 (Fig. 1A), and IL-10 (Fig. 1D) reached at the peak at the 24th hour and then began to fall. All in all, HBV can induce the levels of cytokines at the beginning, and most of the cytokines had their peaks of content. (Table A.1)

3.2. Effect of BSJPR on the cytokine secretion of PBMC infected by \( 10^5 \)IU/well HBV

Comparing with virus group, BSJPR had a relatively obvious inducing effect on the contents of IL-6 (Fig. 2B) and IL-10 (Fig. 2C). The contents of IL-6 and IL-10 in virus group were obviously less than those in rat serum group. And the levels of IL-6 and IL-10 in BSJPR group and rat serum group were similar. In this case, we found that BSJPR can hamper the inhibiting-effect of HBV on the contents of IL-6 and IL-10 in low concentration virus condition. (Table A.2)

3.3. Effect of BSJPR on the cytokine secretion of PBMC infected by \( 10^7 \)IU/well HBV

BSJPR had more obvious effect on the high virus-load group. The level of TNF-\( \alpha \) (Fig. 3D) in BSJPR group surpassed not only virus group but also rat serum group all the time. In addition, all of the other cytokines (IL-4 (Fig. 3A), IL-6 (Fig. 3B), IL-10 (Fig. 3C), IFN-\( \gamma \) (Fig. 3E) and IL-17A (Fig. 3F)) in BSJPR group reached at an obvious high point at the 24th hour immediately which was higher than other three groups. In this case, we concluded that BSJPR exerted its effect best at the 24th hour when dealing with high concentration virus. (Table A.3)

3.4. Regulation of BSJPR on pDC proliferation in HBV-infected PBMC
In $10^5$ IU/well HBV condition (Fig. 4A), there was nearly no difference of pDC content between virus group and rat serum group, while pDC content in BSJPR group increased evidently compared with virus group and rat serum group.

In $10^7$ IU/well HBV condition (Fig. 4B), pDC content in virus group and rat serum group decreased, while pDC content in BSJPR group maintained at the initial level. (Table A.4 and A.5)

### 3.5. The regulation of BSJPR on the HBV-infected pDC intracellular TLRs/MyD88/TRIF signaling pathway

#### 3.5.1 Real time PCR

BSJPR had an increasing effect on the mRNA expression of TLR3 (Fig. 5A), TLR9 (Fig. 5B), myeloid differentiation primary response 88 (MyD88) (Fig. 5C), TRIF (Fig. 5D), IRF3 (Fig. 5E), IRF7 (Fig. 5F) and activator protein 1 (AP-1) (Fig. 5G). The expression of these mRNAs was induced more in virus group than rat serum group, and BSJPR further induced their expression compared with virus group, especially TLR9 and IRF7. However, the expression of NF-κB mRNA (Fig. 5H) was augmented greatly in virus group compared with rat serum group, and BSJPR contained this augment. (Table A.6)

#### 3.5.2 Western Blot

BSJPR had an obviously increasing effect on the protein expression of TLR3 (Fig. 7A), TLR9 (Fig. 7B), MyD88 (Fig. 7C), TRIF (Fig. 7D), IRF7 (Fig. 7F), c-Jun (Fig. 7G) and NF-κB (Fig. 7H). The expression of TLR3, TLR9 and TRIF in virus group was less than that in rat serum group, while BSJPR distinctly induced their expression compared with virus group and rat serum group. The expression of MyD88, IRF7, c-Jun and NF-κB in virus group was higher than that in rat serum group, and BSJPR further induced their expression compared with virus group. Moreover, the expression of IRF3 (Fig. 7E) was augmented greatly in virus group compared with rat serum group, and BSJPR contained this augment obviously. (Table A.7)

### 4. Discussion

Our data suggested that BSJPR could induce pDCs to alleviate CHB and the mechanism was up-regulating relevant TLR/MyD88/TRIF signaling pathway proteins of pDC rather than directly regulating cytokines.

PBMC develops to matured DC with the help of cytokines. The effect of BSJPR on cytokines of PBMC was related with virus concentration. In the low virus concentration condition, HBV obviously suppressed IL-6 and IL-10 levels, that is consistent with previous researches [22]. And BSJPR hampered the inhibiting-effect of HBV on the contents of IL-6 and IL-10. In the high HBV concentration condition, HBV obviously suppressed TNF-α and IL-10 levels. The level of TNF-α in BSJPR group surpassed not only virus group but also rat serum group all the time. And all of the other cytokines (IL-4, IL-6, IL-10, IFN-γ and IL-17A) in
BSJPR group reached at an obvious high point at the 24th hour immediately which was higher than other three groups. In this case, we concluded that BSJPR exerted its effect best at the 24th hour when dealing with high concentration virus. It has been reported that IL-6 and TNF-α induce the maturation and differentiation of DC, while IL-10 inhibits its maturation \[23–25\]. IL-6 can suppress HBV via inhibiting the expression of hepatocyte nuclear factor (HNF) 1α and HNF 4α, two transcription factors essential for HBV gene expression and replication \[26\]. TNF-α has been confirmed to induce IL-12 and to inhibit HBV replication via a posttranscriptional mechanism \[27, 28\]. However, the increase of IL-10 was also observed in BSJPR group. IL-10 has been reported to inhibit the proliferation of pDC and consequently aggravate CHB. Thus, we cannot safely conclude that BSJPR can treat CHB via regulating cytokines.

Next, we explored the relationship between BSJPR and pDC-related TLR /MyD88/TRIF signaling pathway proteins. According to the results, compared with virus group, BSJPR had an increasing effect on the mRNA expression of TLR3, TLR9, MyD88, TRIF, IRF3, IRF7 and AP-1, and an augment effect on the protein expression of TLR3, TLR9, MyD88, TRIF, IRF7, c-Jun and NF-κB.

DCs mainly use pattern-recognition receptor (PRR) to identify pathogen-associated pattern (PAMP) thereby inducing innate immunity. TLRs are one of the important PRRs on DCs and play significant roles in maturing DCs \[29\]. It has been confirmed that TLR-mediated immune responses can restrict HBV replication at the different steps of viral life cycle \[27\]. TLR3 and TLR9 are closely related with HBV infection \[30\]. Our finding confirmed a current report that the expressions of TLR3 and TLR9 in PBMC are reduced in CHB patients \[27\]. Specifically, the mRNA expressions of TLR3 and TLR9 were induced in virus group, while their protein expressions were inhibited in virus group, indicating that HBV suppressed the translation of their mRNAs. And BSJPR can induce not only their mRNA expression but also protein expression. TLR3 can identify NF-κB, IRFs and AP-1 to enter into nuclei via MyD88-dependent signaling pathway and TRIF signaling pathway, therefore up-regulating costimulatory molecules of APC like CD40, CD80 and CD86 to induce DCs maturation ultimately \[31, 32\]. And our research confirmed that BSJPR greatly induced the mRNA expression of TLR9, IRF7, MyD88 and AP-1, and protein expression of TLR9, MyD88 and IRF7. Human pDC selectively expresses TLR9. It is located on ER to sense viral nucleic acids and induce MyD88. MyD88 consequently activates IRF7 which initiates most of the transcription of IFN-1 \[18\]. In addition, TLR9 can identify CpG sites by MyD88-dependent signaling pathway and is bound with TNFR-associated factor 6 (TRAF6). Activated TRAF6 can stimulate NF-κB, AP-1, IRFs, and induce cytokines including IL-1, IL-12, TNF-α, IFN-α, by which TRAF6 exerts presenting antigen and immune surveillance \[33–35\]. MyD88 also clears pathogen efficiently via inducing IFN-γ response, chemokines and specific memory CD8 + T cells \[36\]. The activation of MyD88-dependent TLR can promote the secretion of pro-inflammatory cytokines IL-6, IL-10 and TNF-α, which explains our finding about these cytokines. Additionally, MyD88 is capable of inducing NF-κB which induces the transcription induction of proinflammatory cytokines, chemokines, and costimulatory molecules. Notably, the mRNA expression of NF-κB in BSJPR group was less than that in virus group, indicating that the increasing expression of NF-κB protein in BSJPR group may result from other factors. All in all, we concluded that BSJPR increase pDC possibly via inducing the expression of key proteins in the pDC-related TLR /MyD88/TRIF signaling pathways.
4. Conclusions

In the study, we concluded that BSJPR can induce pDC to alleviate CHB via up-regulating relevant TLR/MyD88/TRIF signaling pathways proteins rather than directly regulating extracellular cytokines. Specifically, BSJPR has an increasing effect on the mRNA expression of TLR3, TLR9, MyD88, TRIF, IRF3, IRF7 and AP-1, and an augment effect on the protein expression of TLR3, TLR9, MyD88, TRIF, IRF7, c-Jun and NF-κB, which induces IFN-I and ultimately promotes the antiviral functions of mDC, NK cell, T cell, and B cell. The study revealed the mechanism of BSJPR on cellular level, which might have guiding significance for exploring mechanisms of other TCM.

List Of Abbreviations

pDC: Plasmacytoid dendritic cell; CHB: Chronic hepatitis B; BSJPR: Bushen Jianpi Recipe; TLR: Toll-like receptor; MyD88: Myeloid differentiation primary response 88; TRIF: TIR-domain-containing adapter-inducing interferon-β; HBV: Hepatitis B virus; DC: Dendritic cell; CD4: Cluster of differentiation 4; CTL: Cytotoxic T lymphocyte; mDC: Myeloid DC; cDC: Conventional DC; IFN-I: Type I interferon; TNF-α: Tumor necrosis factor alpha; NK: Natural killer; TCM: Traditional Chinese medicine; PBMCs: Peripheral blood mononuclear cells; IRF3: Interferon regulatory factor 3; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HNF: Hepatocyte nuclear factor; PRR: Pattern-recognition receptor; PAMP: Pathogen-associated pattern; TRAF6: TNFR-associated factor 6

Declarations

Ethics approval and consent to participate

The protocols of all animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Local Ethics Committee for Animal Research Studies at the Shanghai University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

LW, TW and PYZ concept and designed the research; LW, ZXY, CLZ, XBS and YFL performed the experiments; LW, ZXY and YFL prepared the figures; LW, ZXY, CLZ and XBS analyzed and interpreted the data; LW, ZXY and TW drafted the manuscript; TW and PYZ revised the manuscript. All authors read and approved the final manuscript.

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