

Original Research Article

Correlation between toll-like receptor 9 expression in peripheral blood dendritic cells and interferon- α antiviral sustained virological response in patients with chronic hepatitis B

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Abstract

Purpose: To investigate the link between dendritic cells (DC cells), toll-like receptor 9 (TLR9) and tumor necrosis factor- α (TNF- α) expressions in peripheral blood of patients with chronic hepatitis B (CHB).

Methods: Peripheral blood mononuclear cells (PBMC) and CD cells were prepared from CHB patients and healthy volunteers, and the cell proliferation was assessed. The surface expressions of CD83, CD86, CD40 and human leukocyte antigen DR (HLA-DR) on DC cells were assayed by flow cytometry, while the expressions of mRNAs of TLR9, MyD88 and NF- κ B in each cell-group were determined by fluorescent quantitation PCR. Protein expressions of TLR9, MyD88 and NF- κ B were analyzed by Western blot.

Results: The rate of proliferation of DC cells in the CHB patients was slower than the rate in healthy volunteers ($p < 0.05$), and the expression of co-stimulatory molecules was significantly lower in CHB patients than in healthy volunteers ($p < 0.05$). The ability to stimulate T-lymphocyte proliferation of the CHB-DC group was much weaker than that of the D-NC group ($p < 0.05$). In fluorescent quantitation assays, the relative expressions of mRNAs of TLR9, MyD88 and NF- κ B in cells of CpG-Stimulate ODN (CpG-S ODN) group were higher than those of the control group ($p < 0.05$), but the relative expressions of mRNAs of TLR9, MyD88 and NF- κ B in the cells of the CpG-S ODN group were significantly lower ($p < 0.05$). Moreover, IFN- α level in the CpG-S ODN group was much higher than that in the control group ($t = 6.633, p = 0.014 < 0.05$). However, results from Western blot showed that the relative expressions of TLR9, MyD88 and NF- κ B in the CpG-S ODN group were lower than in the control group ($p < 0.05$).

Conclusion: These results indicate that TLR9 on the surface of DC cells of CHB patients can eliminate HBV by generating IFN- α via regulation of MyD88 and NF- κ B.

Keywords: Chronic hepatitis B, Dendritic cells, TLR9, IFN- α

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INTRODUCTION

Chronic hepatitis B (CHB) is a liver injury due to hepatitis B virus (HBV). According to World

Health Organization (WHO), HBV infection is one of the top ten causes of mortality in the world, and about 350 million people are in the world are chronic HBV carriers [1]. In terms of statistics,

about 1 million people die from liver failure, liver cirrhosis and primary hepatocellular carcinoma (HCC) induced by HBV infection each year, while 70 - 90 % liver cancers are caused by CHB [2-4]. The onset of hepatitis B is complicated, and is linked to the immune response of its host [5]. Dendritic cells (DC cells), the most potent special antigen-presenting cells (APC) in mammals, are able to activate APC of resting T-cells and induce specific immune responses [6]. The cytokines, interleukin (IL) and interferon (IFN) can mediate humoral immune or allergic disorders and thus promote the synthesis of antibodies [7]. The natural immune molecule, toll-like receptor (TLR) family is a pathogen pattern-recognition receptor (PRR). In recent years, it has been found that the TLR9 signal transduction pathway plays an essential role in the occurrence and development of CHB [8-10]. It has been reported that HBC or HCV infection can significantly restrain the expression of TLR9 [11]. Some studies also showed that the number and proportion of DC cells expressing TLR9 in PBMC in patients with CHB is greatly reduced, leading to decreased expression of TLR9 and its downstream genes [12,13].

Interferon- α (IFN- α) is currently one of the choice drugs for the clinical management of CHB, and it functions as an antiviral, antitumor and immunoregulating agent [14]. In addition, IFN can enhance the biological functions of T and B lymphocytes, NK cells and macrophages, and compel the host to kill virus-infected cells and malignant tumor cells or completely eliminate them, thereby playing a role in immune surveillance and immune regulation [15-16]. It is thought that IFN- α -induced MyD88 can significantly inhibit the replication of HBV DNA and protein synthesis [17]. Moreover, the activation of NF- κ B signal transduction pathway can boost the expression of MyD88 [17]. There are very few reports about the molecular mechanism of IFN- α antiviral effect through DC cells. The present study was aimed at investigating the possibility of a correlation between IFN- α and TLR9 expressions in DC cells.

EXPERIMENTAL

In vitro culture of DC cells and characterization

A total of 90 out- and in-patients with CHB from the Department of Infectious Diseases of the First Affiliated Hospital of Xinxiang Medical University and 20 healthy volunteers from Medical Examination Center of the same hospital (from March 2014 to September 2015) were

enrolled in the study. Anti-coagulated blood was drawn from the subjects and used to prepare peripheral blood mononuclear cells (PBMC) with hydroxypropyl methylcellulose and DC cells with GM-CSF, IL-4 and Flt3-L *in vitro* directed induction and separation culture method. The morphology of DC cells acquired was examined under an inverted optical microscope and transmission electron microscope. The proliferation of DC cells of the patients with CHB and healthy volunteers was measured, and the expressions of surface molecules of DC cells, such as CD83, CD86, CD40 and HLA-DR, were determined by flow cytometry. DC cell-stimulated MLR reaction and the stimulation of T-lymphocytes by DC cells were assessed. This research was approved by the Ethical Committee of The First Affiliated Hospital of Zhengzhou University (approval no. 201703031) according to the Declaration of Helsinki promulgated in 1964 as amended in 1996 [18].

Determination of cytokines and INF

The expressions of IL-10 and IL-12, and the content of IFN- α were determined by enzyme linked immunosorbent assay (ELISA) kits (R & D System, USA) according to kit instructions. A standard curve was drawn from which the expressions of cytokines and the content of IFN- α in corresponding samples were calculated.

Fluorescent quantitation polymerase chain reaction (PCR)

The total RNA of cells was extracted from each group using Trizol total RNA extraction kit from Roche, and then electrophoresed. After DNA removal, the RNA samples were subjected to reverse transcription (reverse transcription kit M-MLV, provided by Invitrogen); the expressions of mRNA of TLR9, MyD88 and NF- κ B of each cell population were assayed with fluorescent quantitation PCR. The housekeeping gene was GADPH and the primer sequences are shown in Table 1. The amplification of fluorescent quantitation PCR was carried out using GeneScript kits according to the kit instructions. Thereafter, the amplification curve and the dissolution curve of each cDNA sample were analyzed, and the contents of mRNAs of TLR9, MyD88 and NF- κ B in each sample were calculated relative to expression amount of GADPH in the same sample using the formula:

Folds = $\Delta\Delta$ Ct. $\Delta\Delta$ Ct was the relative expression level of the target mRNA, $\Delta\Delta$ Ct = stimulation group (Ct (target mRNA) - Ct (GADPH)) - control group (Ct (target mRNA) - Ct(GADPH)).

Table 1: Sequences of primers used in fluorescent quantitation

Primer	Sequence 5'→3'
TLR-9	F-ACCTCTACCACGAGCACTCATTAC R-GACAAGTCCAGCCAGATCAAACC
MyD88	F-GCATGGAACCAAGTGGCTGTGAG R-GAGGAAGTGGGAATGGGCGGTGT
NF-κB	F-AGTTGAGGGGACTTTCCAGGC R-GCCTGGGAAAGTCCCCTCAACT
HBV	F-ATACTGCGGAATCCTACG R-CCGCGTAAAGAGAGGTGGC
NADPH	F-CTCCATCCTGGCCTCGCTG R-GCTGTACCTTCACCGTTCC

Western blot analysis

Total proteins of cells were extracted from each group. Then, the protein samples were electrophoresed and detected with 10 % polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) to determine the expressions of TLR9, MyD88 and NF-κB. The antibodies used in the study were rabbit anti-human monoclonal antibody of TLR9, MyD88, NF-κB and HRP anti-rabbit secondary antibody (Abcam, UK). The relative expression level of each target protein was calculated based on the ratio of the gray value of target band / the gray value of β-actin.

RESULTS

Morphological characteristics of DC cells

After PBMC were cultured for 48, 72, 144 and 288 h, the dendritic processes on the surface of DC cells were obvious, showing the typical morphology of DC cells (Figure 1 A). The DC cells cultured for 12 days were collected and processed for morphological observation using transmission electron microscope. It was revealed that the surface of the DC cells was rough with numerous folds and dendritic processes. The cells had irregular nuclei and small nucleoli, and the chromatin in the nucleus was highly condensed along the edge of the nucleus. Moreover, there were a large number of mitochondria and lysosomes in the cytoplasm (Figure 1 B). During cell culture, the proliferation of the cells was subjected to statistical analysis. Compared with the health volunteers, the proliferation rate of DC cells of the patients with

CHB was much slower ($p < 0.05$, Figure 1 C).

Expressions of CD83, CD86, CD40, HLADR on the surface of DC cells

After the DC cells in each group were cultured for 10 - 12 days, the monoclonal antibodies (CD83, CD86, CD40 and HLADR) were labeled with FITC and then analyzed by flow cytometry (Table 2). The results revealed that the expressions of co-stimulatory molecules of DC cells in CHB patients were significantly less than that in the healthy volunteers ($p < 0.05$).

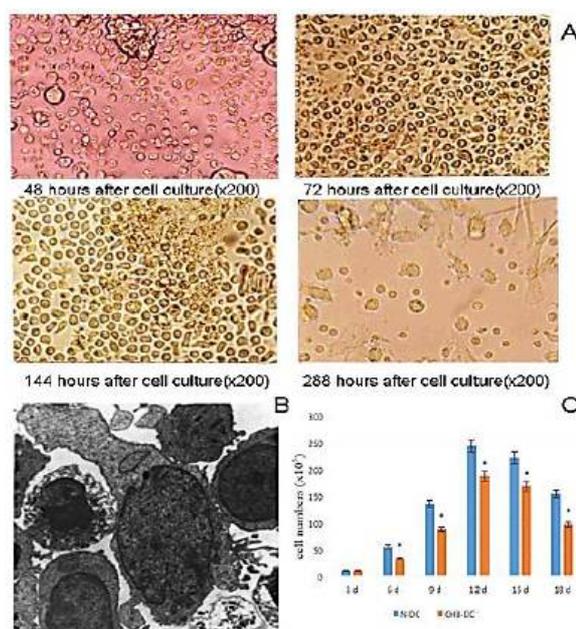


Figure 1: Morphological observation and proliferation status of DC cells cultured *in vitro*. Figure 1 A shows the morphological characteristics of DC cells cultured *in vitro* after PBMC were cultured for 48h, 72h, 144h and 288h. Figure 1 B presents the morphology of DC cells under electron microscope after 12-day culture. Figure 1 C shows the proliferation of cells in the N-DC and CHB-DC group; * $p < 0.05$ compared with N-DC group

Allogenic mixed lymphocyte reaction stimulated by DC cells (MLR)

In the three MLR groups with 5×10^3 , 1×10^4 and 5×10^4 DC cells, respectively, the OD values of stimulated T-lymphocyte proliferation of the CHB-DC group were 0.239 ± 0.108 ,

Table 2: Expression of surface molecules of DC cells in each group ($\bar{x} \pm s$)

Group	No. of cases	CD83	CD86	CD40	HLADR
N-DC	20	96.37±5.53	84.55±9.24	78.46±8.22	50.33±10.92
CHB-DC	90	35.22±6.89	46.37±8.01	43.25±6.56	37.88±7.19
T values		4.221	3.877	2.982	2.655
P values		0.016	0.035	0.041	0.048

0.534 ± 0.103 and 0.585 ± 0.091, respectively, and corresponding values for the N-DC group were 0.361 ± 0.189, 0.625 ± 0.062 and 0.728 ± 0.077, respectively. The CHB-DC group had much weaker stimulatory effect on T lymphocyte proliferation when compared to the N-DC group ($p < 0.05$, Figure 2A). On day 7 of culture, TNF- α was added, and the culturing was continued non-stop for 48 h. The mature DC cells had much larger stimulation on the T-lymphocyte proliferation when compared to the immature DC cells ($p < 0.05$, Figure 2B).

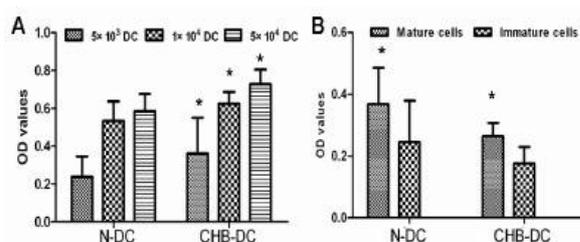


Figure 2: Effect of DC cells on the proliferative capacity of T-lymphocytes between two groups. 2A: Results of the T-lymphocyte proliferation stimulated by DC cells in each group. 2B: The effect of mature/immature DC cells in each group on the proliferation of T lymphocytes; * $p < 0.05$

DC cell-secreted cytokines

The culture supernatants of DC cells and the reaction supernatants of MLR were collected and assayed for changes in the expressions of IL-10 and IL-12 with ELISA. As shown in Table 3, the level of IL-10 in the reaction supernatants of MLR in N-DC group was 26.79 ± 7.63 pg/mL, and that in the CHB-DC group was 20.55 ± 6.52 pg/mL. There was no statistical difference between the two groups ($p > 0.05$). However, in the culture supernatants of DC cells and the reaction supernatants of MLR, the contents of IL-12 in the CHB-DC group were 26.53 ± 3.26 pg/mL and 97.21 ± 17.29 pg/mL, respectively; and in the N-DC group the corresponding values were 88.42 ± 2.38 pg/mL and 208.46 ± 21.73 pg/mL, respectively. In other words, the CHB-DC group had much more IL-12 than the N-DC group ($p < 0.05$).

Table 3: Levels of cytokines in the culture supernatants of DC cells (mean ± SD)

Group	MLR reaction supernatants		DC culture supernatants IL-12 (pg/mL)
	IL-12 (pg/mL)	IL-10 (pg/mL)	
N-DC	88.42±2.38	26.79±7.63	208.46±21.73
CHB-DC	26.53±3.26	20.55±6.52	97.21±17.29
t-value	5.218	1.107	4.503
P-value	0.019	0.535	0.022

Fluorescent quantitation PCR results

The mRNA expressions of TLR9, MyD88 and NF- κ B in the DC cells of the control, CpG S-ODN and CpG-ODN group were determined by fluorescent quantitation PCR. As shown in Figure 3A, the solubility curve of the mRNAs of TLR9, MyD88, NF- κ B and the housekeeping gene GAPDH all presented single peaks, suggesting that the amplification primers used were not primer dimers. The single peaks also confirmed the absence of non-specific amplification.

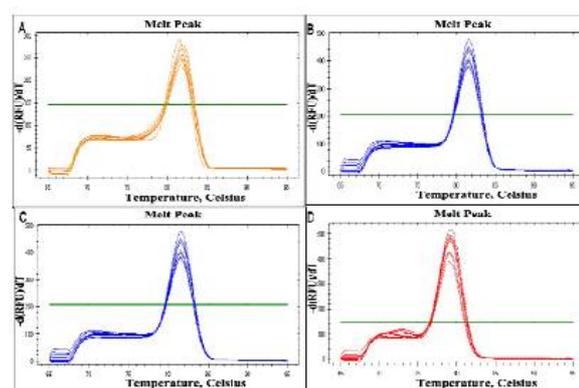


Figure 3: The solubility curves of mRNA amplification of targeted genes. A: Solubility curve of TLR9, B: Solubility curve of MyD88, C: Solubility curve of NF- κ B, D: Solubility curve of GAPDH; * $p < 0.05$

The expression levels of TLR9, MyD88 and NF- κ B mRNA in the cells of the three groups were determined separately by fluorescence quantitative PCR, and the results are shown in Figure 4. The relative expression level of TLR9 mRNA was 1 in the control group and 2.36 ± 0.59 in the CpG S-ODN group ($p < 0.05$); the relative expression level of TLR9 mRNA in the CpG N-ODN group was 0.21 ± 0.07, which was much lower than that in the control group ($p < 0.05$, Figure 4 A). The relative expression level of MyD88 mRNA was 1 in the control group, and much less than 3.17 ± 0.44 in the CpG S-ODN group ($p < 0.05$); the relative expression level of MyD88 mRNA in the CpG N-ODN group was 0.34 ± 0.15, which was much lower than that in the control group ($p < 0.05$, Figure 4 B). The relative expression level of NF- κ B mRNA was 1 in the control group, and much less than 3.29±0.56 in the CpG S-ODN group ($p < 0.05$); the relative expression level of MyD88 mRNA in the CpG N-ODN group was 0.34 ± 0.15, which was much lower than that in the control group ($p < 0.05$, Figure 4 C). The levels of IFN- α in the culture supernatants of each group were assayed, and the results revealed that the level of IFN- α in the culture supernatants of the control group was 38.59 ± 6.77 pg/mL, which was

significantly lower than the value of 58.46 ± 8.13 pg/mL for the CpG S-ODN group ($t = 6.633$, $p = 0.014 < 0.05$); the level of IFN- α in the CpG S-ODN group (11.39 ± 5.38 pg/mL) was significantly lower than that of the control group ($t = 5.279$, $p = 0.028 < 0.05$; Figure 4 D).

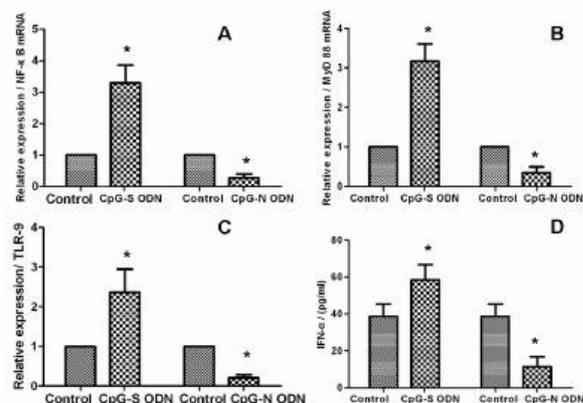


Figure 4: The mRNA expression levels of TLR9, MyD88 and NF- κ B, and the level of IFN- α in serum of each group. A: The relative expression level of TLR9 mRNA of the three groups. B: The relative expression level of MyD88 mRNA of the three groups. C: The relative expression level of NF- κ B mRNA of the three groups; * $p < 0.05$

Expression of TLR9, MyD88 and NF- κ B in N-DC cells after TLR9 inhibition

The expressions of TLR9, MyD88 and NF- κ B in N-DC cells in the control and CpG N-ODN group were measured using Western Blot, and the results are shown in Figure 5. The relative expression level of MyD88 in the control group (0.89) was much higher than that in the CpG N-ODN group (0.28) ($p < 0.05$; Figure 5 A). The relative expression level of NF- κ B was 0.93 in the control group, which was much higher than 0.44 in the CpG N-ODN group ($p < 0.05$; Figure 5 C).

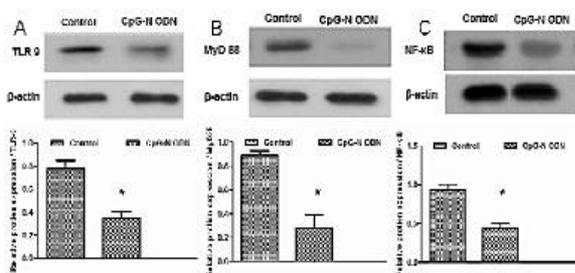


Figure 5: Results of Western blot. A: Relative expression levels of TLR9 in the control group and CpG-N; ODN group. B: Relative expression levels of MyD88 in the control and CpG-N ODN group. C: Relative expression levels of NF- κ B in the control group and CpG-N ODN group; * $p < 0.05$

Fluorescent quantitative PCR was used to determine the contents of HBV DNA in the prepared culture supernatants of N-DC in the control and CpG-N ODN groups. The results are shown in Figure 6A. The relative expression level of HBV DNA in the control group was 1, which was significantly lower than the value of 3.27 ± 1.33 for the inhibited ODN group ($p < 0.05$). The content of IFN- α in the control group culture supernatants was 57.65 ± 11.24 pg/mL while the IFN- α level in the inhibited ODN group was 17.26 ± 7.88 pg/mL ($t = 6.431$, $p = 0.017 < 0.05$).

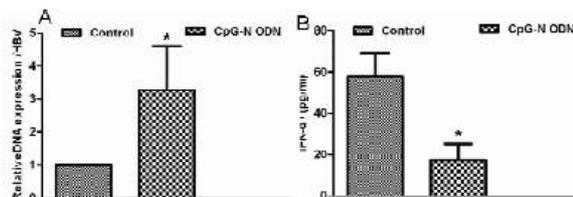


Figure 6: Levels of HBV DNA and IFN- α in the control group and CpG-N ODN group. A: level of HBV DNA in the control group and CpG-N ODN group. B: level of IFN- α in the control group and CpG-N ODN group; * $p < 0.05$

DISCUSSION

The mechanism involved in the occurrence and progression of hepatitis B (CHB) has been a topic of concern and research for majority of medial workers, but so far, no definite conclusion has been arrived at. In previous studies of immunology linked to persistent CHB infection, scholars largely focused on acquired immunity. It has been suggested that if the link between persistent CHB infection and natural immune system is deeply investigated, it is expected to set up a new direction for the study and treatment of HBV infection [19].

As a type of pathogen pattern-recognition receptor (PRR), TLR acts as a bridge between natural and acquired immunity. Studies in which the corresponding ligands of TLR3, TLR4, TLR5, TLR7 and TLR9 were injected into HBV transgenic mice showed that all the ligands were able to restrain the replication of HBV DNA in hepatocytes within 24 h [20]. This action is an IFN- α/β dependent, non-cytolytic effect, and these TLR ligands can induce the production of cytokines that inhibit the replication of HBV [12,21]. This is in accordance with the outcomes of the present study.

TLR2 shares the same MyD88 signal recognition pathway with TLR9. Recently, it was found that TLR9 is involved in the immune defense of HBV infection and its expression is associated with the chronicity of hepatitis and immune tolerance [12].

This is in line with the changes seen in MyD88 in the present study. The study carried out by Chuang *et al* showed that the expression of TLR9 was located in tissues or cells rich in immunocytes, such as spleen, lymph nodes and peripheral lymphocytes [22]. In human PBMC, TLR9 is mainly expressed in B-lymphocytes, monocytes and DC cells, and its function is to identify the double-stranded DNA from bacteria or virus and unmethylated CpG-ODN, thereby playing an essential role in the process of exogenous CpG DNA activation of innate immunity [23]. The results from morphological observations of DC cells were consistent with those from studies on activation and inhibition of TLR9 in this investigation. The TLR9 signal transduction pathway plays a crucial role in the occurrence and progression of CHB [24-26]. The findings of the present study reveal that when the expression of TLR9 mRNA was upregulated, the expressions of MyD88 and NF- κ B mRNA also increased, suggesting that the expression of TLR9 may be positively related to those of MyD88 and NF- κ B in the peripheral CD cells in patients with CHB. Besides, other studies have demonstrated that TLR9 can boost the expression of IFN- α , while IFN- α can induce the mature of DC cells. Therefore, it can be inferred that decreases in TLR9 control the expression of IFN- α , and thereby give rise to inability of immature DC cells to activate adaptive immune responses of Th1 and Th2. Thus, HBV cannot be eliminated, thereby resulting in persistent infection of HBV and chronic hepatitis. In order to verify this hypothesis, the present study adopted Western blot, ELISA and fluorescent quantitation PCR to determine the expressions of TLR9, MyD88 and NF- κ B as well as the content of HBV DNA in the specimens.

CONCLUSION

The results show that following HBV infection, the TLR9 on the surface of DC cells activated NF- κ B via regulation of the expression of MyD88, thereby generating IFN- α , and finally activating the anti-viral mechanism of the body leading to the elimination of the virus.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities

pertaining to claims relating to the content of this article will be borne by the authors, all authors read and approved the manuscript for publication. Li Jiansheng conceived and designed the study, Zhu Bin, Wang Tianbao, Wei Xiaoxia, Zhou Yancai collected and analyzed the data, Zhu Bin wrote the manuscript.

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