

siRNA-mediated knockdown of FoxP3 promotes the ratio of T-helper 1 (Th1) to Th2 in chronic hepatitis B patients

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To determine the effect of regulatory T cells on the ratio of Th1/Th2 differentiation in peripheral blood mononuclear cells of chronic hepatitis B patients, FoxP3, the essential transcription factor for Tcells differentiation and function, was knocked down by FoxP3 siRNA, which was affirmed by real-time polymerase chain reaction for decreased expression of FoxP3. Then, at different time points, comparing with control groups, we detected enhanced lymphocyte proliferation and up-regulated Th1-type cytokines, but down-regulated Th2-type cytokines by ELISA. Finally, to research the involved mechanisms, an increased ratio of T-bet/GATA-3 mRNA expression was found by real-time polymerase chain reaction. Our results suggest that siRNA-mediated knockdown of FoxP3 could regulate the immune balance of Th1/Th2 in chronic hepatitis B patients, which may be mediated partly by regulating transcription factors T-bet and GATA-3.

Key words: FoxP3, siRNA, Th1, Th2, T-bet, GATA-3

Kronik Hepatit B'li hastalarda, FoxP3'ün siRNA-aracılıklı susturulması T-helper 1 (Th1) Th2 oranını artırmaktadır

Kronik hepatit B'li hastaların periferik kan mononükleer hücrelerinde Th1/Th2 farklılaşma oranının düzenlenmesinde T hücrelerinin etkinliğinin saptanması için, T hücrelerinin farklılaşma ve fonksiyonunda esansiyel transkripsiyon faktörü olan FoxP3, FoxP3 siRNA ile susturulmuş ve FoxP3'ün azalmış ekspresyonu gerçek zamanlı polimeraz zincir reaksiyonu ile gösterilmiştir. Ardından, değişik zaman noktalarında kontrol grubuyla karşılaştırıldığında, artmış lenfosit çoğalması ve Th1 – tipi sitokinlerde artış, ancak Th2 – tipi sitokinlerde ELISA ile “down – regülasyon” saptanmıştır. Son olarak, etkin olan mekanizmaların tespiti için gerçek zamanlı polimeraz zincir reaksiyonu ile T-bet/GATA-3 mRNA ekspresyonunun arttığı bulunmuştur. Bu sonuçlara göre, kronik hepatit B'li hastalarda FoxP3'ün siRNA – aracılıklı susturulması ile kısmen T-bet ve GATA-3 gibi transkripsiyon faktörlerinin düzenlenmesiyle, Th1/Th2 immün dengesi değiştirilebilir.

Anahtar kelimeler: FoxP3, siRNA, Th1, Th2, T-bet, GATA-3

INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health problem worldwide and especially in China. Although the factors that contribute to the viral clearance in acute self-limiting HBV infection or to the viral persistence in chronic infection remain not completely clear, many studies suggest that host immune response to HBV is a critical

factor in determining the outcome of HBV infection (1). During the natural course of HBV infection, individuals with acute, self-limiting hepatitis B can develop a strong polyclonal cellular response of both CD8+ and CD4+T cells, while chronic infection is characterized by a weak and monoclonal T-cell response with an imbalance of Th1/Th2. Th1

cells have been reported to be correlated with the hepatic inflammatory activity of chronic hepatitis B (CHB), which favors clearance of virus, while Th2 cells may be associated with the persistence of HBV infection (2). Moreover, dominance of Th1-type cytokines such as interferon- γ (IFN- γ) and interleukin-2 (IL-2) exerts cellular and noncellular antiviral effects, but dominance of Th2 cytokines (IL-4, IL-10) contributes to chronicity of HBV infection.

It has been revealed that CD4+CD25+ regulatory T cells (Tregs) play an essential role in maintenance of immunologic tolerance to both self and foreign Ags suppressing aggressive T cell response (3-5). In the chronic infections caused by the human immunodeficiency virus (HIV) and hepatitis C virus (HCV), Tregs have been confirmed to suppress effective antiviral immune responses (6,7). Meanwhile, during the research on chronic HBV infection, it was found that the frequency of CD4+CD25+ Tregs significantly increased in peripheral blood of CHB patients totally when compared with healthy controls, and had a positive correlation with serum HBV DNA load in CHB patients, with $>10^7$ copies/ml serum HBV DNA. Furthermore, the depletion of CD4+CD25+Tregs leads to the increase of HBV Ag-stimulated IFN- γ production and cellular proliferation of peripheral blood mononuclear cells (PBMCs) from HBV-infected patients, and co-culture of CD4+CD25+Tregs with effector cells significantly suppressed HBV Ag-stimulated IFN- γ production and cellular proliferation (8,9). Thus, although the exact mechanism has not been well defined yet, Tregs may play an important role in HBV persistence by modulating virus-specific immune responses.

Presently, few studies have been conducted in CHB patients about the effect of Tregs on Th1 and Th2 differentiation. Thus, in this study, considering that FoxP3, a forkhead family transcription factor, is not only a critical regulator of Tregs' development, function, and homeostasis (10-12) but also relatively unique to the CD4+CD25+ cell population of PBMCs (8), we tried to knock down FoxP3 by siRNA to see whether there was an alteration in the Th1/Th2 ratio in PBMCs of CHB patients. We also discuss the involved mechanisms.

MATERIALS AND METHODS

Human Subjects

Included in this study were 30 CHB patients (11

females, 19 males; average age: 43.8 years) who had been admitted to the Sixth People's Hospital of Shanghai Jiaotong University from December 2009 to May 2010, with the presence of HBsAg for more than six months, presence of HBeAg or HBeAb, positivity of HBV DNA ($>1 \times 10^3$ copies/ml) and elevated alanine aminotransferase (ALT) levels (>40 IU/L) without any antiviral or immunomodulatory therapy within six months. Patients were excluded if they had any causes of liver disease other than HBV infection, such as HCV, HEV, hepatocellular carcinoma (HCC), HIV, alcohol, and autoimmune diseases. The baseline clinical data of patients are shown in Table 1. Among all of the subjects, samples from 20 patients (7 females, 13 males; average age: 41.3 years) were prepared for detecting the expression of target genes and the level of cytokines. Meanwhile, samples from the other 10 patients (4 females, 6 males; average age: 48.8 years) were for investigating lymphocyte proliferation.

Permission for the study was obtained from the local ethics committee, and written informed consent was provided by all enrolled subjects in this study. All investigations were conducted according to the principles expressed in the Declaration of Helsinki.

Human FoxP3 siRNA

FoxP3 siRNA reagent purchased from Santa Cruz (sc-43569, Santa Cruz, USA) was a pool of three target-specific 19-25 nt siRNAs. The sequence for each siRNA duplex is given in Table 2. Due to the secrecy policy of Santa Cruz, the sequence of the negative control siRNA (sc-37007, Santa Cruz, USA) was not allowed to be shown. Both reagents were dissolved to a final concentration of 10 μ M according to the manufacturer's instructions.

Table 1. Summary of clinical characteristics of CHB patients enrolled in the study

Characteristic	Number/ (mean $\pm$ SD)
Age (years)	43.8 $\pm$ 17.5
Female	11
Male	19
HBV DNA (copies/ml)	
1 $\times$ 10 ³ -1 $\times$ 10 ⁵ copies/ml	9
> 1 $\times$ 10 ⁵ copies/ml	21
ALT (IU/L)	203.4 $\pm$ 72.3
HBeAg positive	14
HBeAb positive	16
HBcAb positive	30

Cell Preparation

Soon after being isolated from heparinized blood samples (10 ml for each) using Ficoll-Hypaque (Sigma, USA) density separation and washed with phosphate buffered saline (PBS), PBMCs were suspended at a density of 1.0×10^7 cells/ml and cultured in 6-wells plates (Corning, NY) with RPMI-1640 (Gibco, USA) medium supplemented with 10% fetal bovine serum (Gibco, USA), 50 IU/ml penicillin, 50 µg/ml streptomycin, and 5 µg/ml phytohemagglutinin (PHA, Sigma, USA), which would be incubated for 36 hours (h) at 37°C in a humidified incubator with 5% CO₂ to prepare for the following transfection.

Transfection with siRNA

Thirty-six hours after incubation, cells were centrifuged and resuspended at a concentration of 2.0×10^7 cells/ml with electroporation buffer (Bio-Rad, USA); 8.0×10^6 cells were transfected with 80 nmol of siRNA in a 4 mm cuvette (Bio-Rad, USA) with a total volume of 400 µl of electroporation buffer, which was carried out using Gene Pulser Xcell (Bio-Rad, USA) under the condition of a unique square wave pulse of 200 V for 10 ms. Immediately after electroporation, cuvettes were incubated for 10 minutes (min) at 37°C. Cells were transfected with FoxP3 siRNAs as the test group (FoxP3 siRNA group), with control-siRNA as a negative group (Control-siRNA group), and with RNase-free water as a blank group (Blank group).

Analysis of FoxP3 Expression

Real-time PCR for FoxP3 mRNA expression

After transfection as previously described, cells were

resuspended by 0.8 ml culture medium described above and transferred into 12-well culture to be incubated for 24 h, 48 h and 72 h at 37°C in a humidified incubator with 5% CO₂. Then, cells were collected for total RNA isolation according to the protocol for Trizol Reagent (Invitrogen, CA), while the supernatants collected would be stored at -20°C for the following cytokine assay. cDNA was generated using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). Relative quantitative real-time polymerase chain reaction (PCR) was performed using SYBR[®]Premix Ex Taq[™] reagents (TaKaRa, Japan) on a LightCycler (Roche Diagnostic). The primers for FoxP3 and GAPDH are shown in Table 2 and the thermal cycle parameters were 30 seconds (s) at 95°C followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. Then, FoxP3 expression was normalized relative to GAPDH by $2^{-\Delta\Delta Ct}$. To ensure reliable results, three parallel reactions of each sample were run. (To research into the mechanism of how knocking down FoxP3 promoted the ratio of Th1/Th2, we also did real-time PCR for T-bet and GATA-3 mRNA expression, the primers of which are also shown in Table 3, as done for FoxP3 mRNA expression described above).

Lymphocyte Proliferation Assay

To determine whether knocking down FoxP3 can promote lymphocyte proliferation, we performed lymphocyte proliferation assay of each group described above using CCK-8 assay kit (Beyotime, China). Immediately after transfection, cells from each group were seeded at 5×10^3 cells/well in a 96-well culture plate. After cells were incubated for 24 h, 48 h, 72

Table 2. The sequences for three types of FoxP3 siRNA

Type	Sense (5'to 3')	Antisense (5'to 3')
siRNA-A	UCUUCGAAGACCUUCUCAC	GUGAGAAGGUCUUCGAAGA
siRNA-B	UGAACCAGUGGUAGAUCUC	GAGAUCUACCACUGGUUCA
siRNA-C	UGAAGUAAUCUGUGCGAGC	GCUCGCACAGAUUACUUCA

Table 3. The primer sequences for FoxP3, T-bet, GATA-3 and GAPDH

Gene	Orientation	Sequence (5' to 3')
FoxP3	Forward	CTGACCAAGGCTTCATCTGT
	Reverse	AACTCTGGGAATGTGCTGTT
T-bet	Forward	ATGTGACCCAGATGATTGTGCT
	Reverse	GTAAAGATATGCGTGTGGAAGC
GATA-3	Forward	CCTCATTAAGCCCAAGCGAAG
	Reverse	TTGGCATTTCCTCCAGAGT
GAPDH	Forward	CTACCTGGGCATAGGCAACG
	Reverse	TCACATTCTGGCTCTGTTGGG

h, and 96 h, 100 μ l CCK-8 was added to the culture medium followed by incubation for an additional 2 h according to the manufacturer's instruction. Three replicates were carried out for each group. At last, the absorbance was determined at 450 nm using the microplate photometer (Spectra, Australia).

Cytokine Assay by ELISA

To examine any alteration of Th1 and Th2 differentiation, the collected supernatant of the cells mentioned above was investigated for Th1-type (IFN- γ , IL-2) and Th2-type (IL-4, IL-10) cytokines with ELISA kits (R&D, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm on a microplate photometer.

Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) 13.0. Statistical significance was assessed with the Student's *t* test. Differences between experimental groups were analyzed using paired *t* test or one-way ANOVA for more than two subgroups. All values were expressed as Mean $\pm$ SD. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Efficient Knockdown of FoxP3 by FoxP3 siRNA

To confirm the effect of FoxP3 siRNAs on FoxP3 expression, we examined FoxP3 expression of PBMCs from each group at the mRNA level. Compared to the Control siRNA group and Blank group (regarded as 100% of FoxP3 expression, not shown by Figure 1), our data demonstrated that the FoxP3 siRNAs treatment significantly inhibited FoxP3 mRNA expression, while no differences were detected in FoxP3 expression between the Control siRNA and Blank groups (Figure 1).

Increased Proliferation of Lymphocytes after Knockdown of FoxP3

To determine whether FoxP3 exerts a suppressive function of lymphocytes in CHB patients, after transfection with siRNA, we performed the lymphocyte proliferation assay for each group. The data revealed that the FoxP3 siRNA group had a significantly increased proliferation compared to the Control siRNA or Blank group ($p < 0.01$ for all), and no difference existed between Control siRNA and Blank groups ($p > 0.05$, shown by Figure 2).

Knockdown of FoxP3 Up-regulates Th1 (IFN- γ , IL-2) and Down-regulates Th2 (IL-4, IL-10) Cytokines

To investigate Th1 and Th2 differentiation during

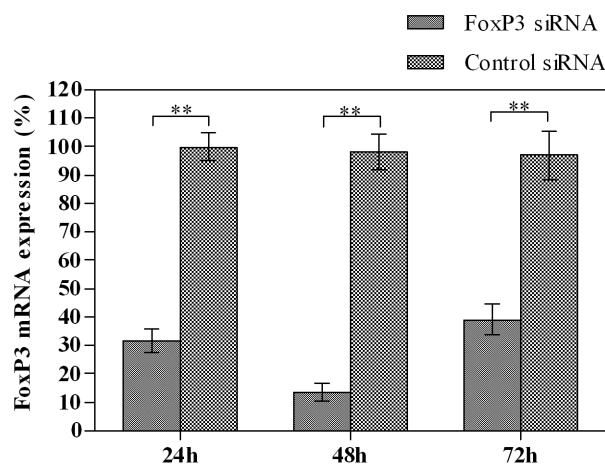


Figure 1. After the treatment, the expression of FoxP3 mRNA was measured by real-time PCR for each group. Values are mean $\pm$ SD, $n=20$, ** $P < 0.01$.

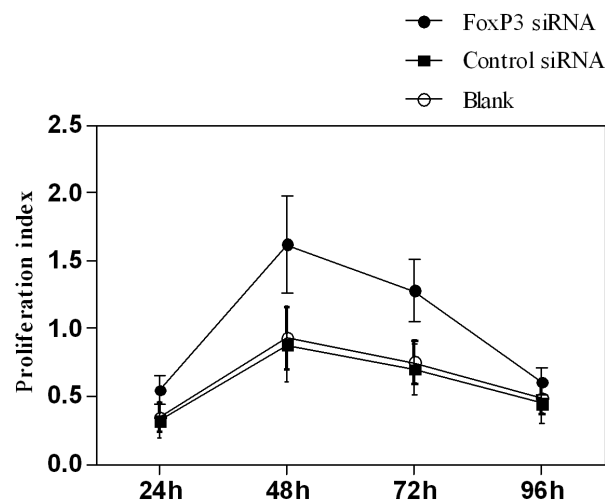


Figure 2. After knockdown of FoxP3, proliferation of lymphocytes in PBMCs from CHB patients was increased significantly. Values are mean $\pm$ SD, $n=10$.

the increased lymphocyte proliferation after knockdown of FoxP3, we measured the levels of cytokines in culture supernatants of cells of each group after incubation for 24 h, 48 h and 72 h. Th1 cytokines (IFN- γ , IL-2) detected by ELISA were significantly increased and Th2 cytokines (IL-4, IL-10) were decreased following FoxP3 siRNAs treatment compared with Control siRNA and Blank groups (Figure 3A, B, C, D). Additionally, there were no statistical differences among the levels of these cytokines between the Control siRNA and Blank groups.

These data evidently indicated that FoxP3 expression might participate in mediating Th1/Th2 differen-

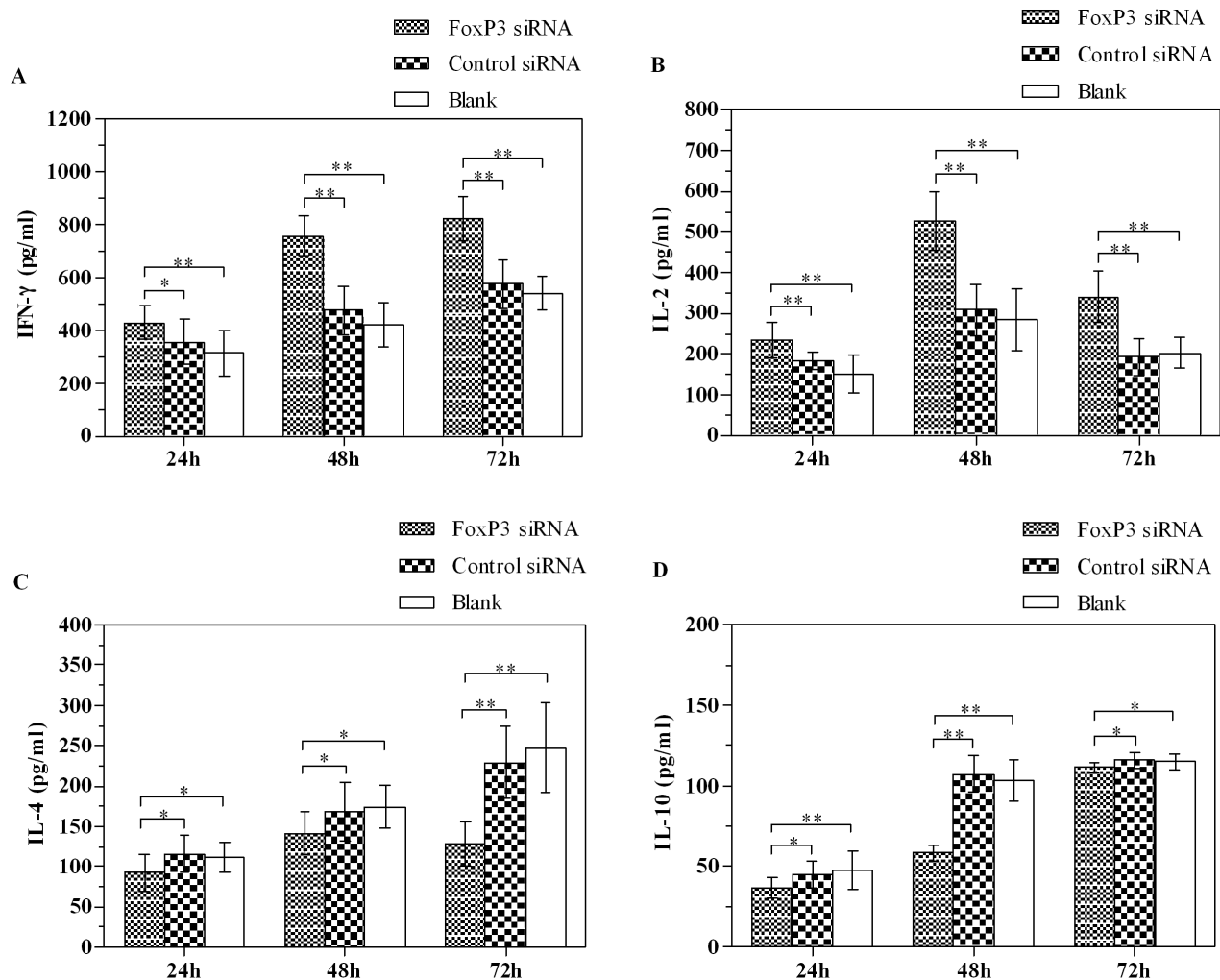


Figure 3. Cytokines assay of PBMCs from CHB patients for each group at three time points (24h, 48h and 72h). **A)** The secretion of IFN- γ . **B)** The secretion of IL-2. **C)** The secretion of IL-4. **D)** The secretion of IL-10. Values are mean $\pm$ SD, * $P < 0.01$, * $P < 0.05$, $n = 20$. PMBCS: Peripheral blood mononuclear cells

tiation and function. Thus, inhibition of FoxP3 expression could be used to reverse the decreased ratio of Th1/Th2 in patients with immune tolerance.

Knockdown of FoxP3 by FoxP3 siRNA Promotes T-bet But Reduces GATA-3 Resulting in an Increased Ratio of T-bet/GATA-3

As previous studies indicated, T-bet is the specific transcription factor for Th1 cells and GATA-3 for Th2 cells. Thus, both T-bet mRNA and GATA-3 mRNA of each group were examined after incubation for 48 h when proliferation of lymphocytes reached the climax as shown by the lymphocyte proliferation assay. Expression of T-bet mRNA was up-regulated significantly, while expression of GATA-3 mRNA was down-regulated (Figure 4A, B). Moreover, the alteration in the T-bet/GATA-3

ratio, which indexes the condition of Th1/Th2 differentiation, was assessed. Compared with Control siRNA and Blank groups (regarded as 100% of T-bet and GATA-3 expression, not shown by Figure 4), knockdown of FoxP3 tended to increase the expression ratio of T-bet/GATA-3 in CHB patients (Figure 4C). Our results suggested that knockdown of FoxP3 could result in an increased ratio of T-bet/GATA-3 through promoting T-bet but reducing GATA-3. These results indicated that we might regulate the balance of Th1 and Th2 immune response by down-regulating FoxP3 expression in CHB patients.

DISCUSSION

Tregs have attracted the attention of related researchers over the past few years for their ability to suppress CD4 and CD8 effector T-cell responses,

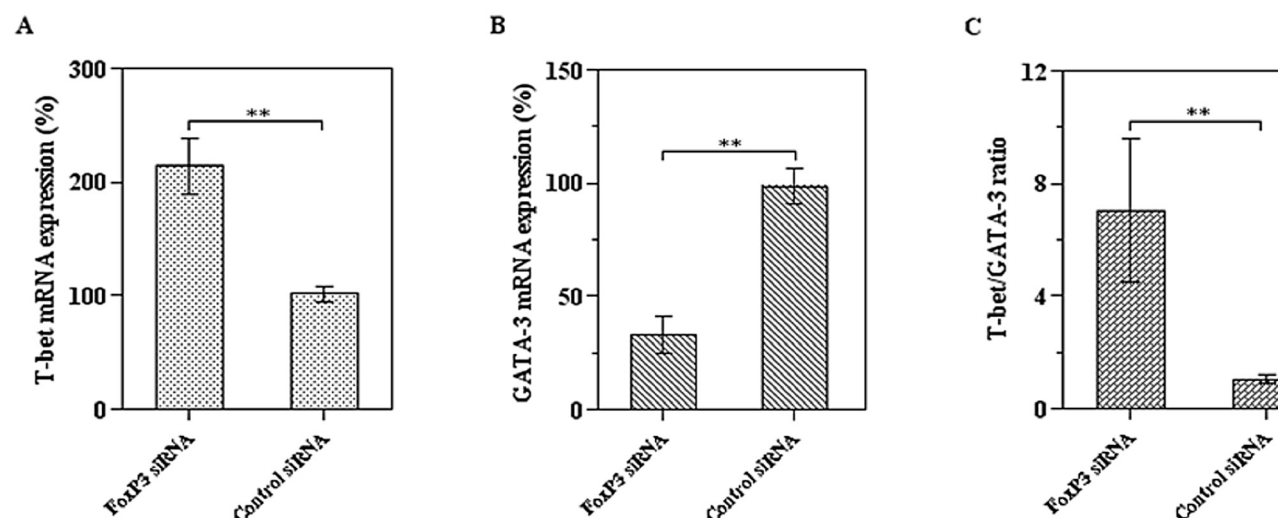


Figure 4. Knockdown of FoxP3 increased the ratio of Th1/Th2 in PBCMs from CHB patients. **A)** The expression of T-bet mRNA was measured by real-time PCR. **B)** The expression of GATA-3 mRNA was measured by real-time PCR. **C)** The ratio of T-bet/GATA-3 mRNA expression was calculated as the index of Th1/Th2 immune balance. Values represent means $\pm$ SD, * $P < 0.01$, ** $P < 0.05$, $n = 20$.

which favors chronicity of virus infection including HBV. Recently, many studies have revealed that stable FoxP3 expression restricts to Tregs and is required not only for Tregs' differentiation (10,11,13) but also for their suppressor function, proliferative potential and metabolic fitness (14,15). Furthermore, loss of FoxP3 or its diminished expression in Tregs leads to loss of the Tregs phenotype and suppressor function and even acquisition of effector T cell properties, including production of immune response-promoting cytokines such as IL-2, IL-17 and IFN- γ (13,16), which we hope could happen in CHB patients.

In this study, we tried to knock down FoxP3 of Tregs by siRNAs to see whether the treatment can result in an alteration of Th1/Th2 in CHB patients, which has not been reported to date. As Foxp3 expression is relatively unique to CD4+CD25+Tregs of PBCMs, CD4+CD25+ Tregs activity can be evaluated by measuring FoxP3 expression in total PBCMs rather than isolating CD4+CD25+Tregs. Thus, after the efficiency of knocking down FoxP3 by siRNAs was confirmed by detecting the decrease of FoxP3 expression in PBCMs of CHB patients using real-time PCR, a lymphocyte proliferation assay was performed. Our results showed that the proliferation of lymphocytes was increased significantly perhaps as knockdown of FoxP3 could inhibit Tregs differentiation and their suppressor function, which was consistent with a previous report showing that the depletion of CD4+CD25+Tregs led to an

increase of cellular proliferation of PBCMs from HBV-infected patients (8).

Subsequently, Th1-type (IFN- γ and IL-2) and Th2-type (IL-4 and IL-10) cytokines were measured as the index of Th1/Th2 immune balance. As our data showed, the levels of both IFN- γ and IL-2 were increased significantly, while the levels of IL-4 and IL-10 were decreased after knockdown of FoxP3. These data strongly indicate that knockdown of FoxP3 may reverse the imbalance of Th1/Th2 in PBCMs of CHB patients mainly by promoting Th1 polarization. In another word, Tregs may play a role in the decreased ratio of Th1/Th2 mainly by depressing Th1 differentiation during chronic HBV infection, consistent with previous reports about other diseases (17,18).

Furthermore, naive T cells differentiate toward different T cell subtypes based on the expression of certain transcription factors. T-bet, a member of the T-box family of transcription factors, has been reported to involve polarization toward Th1 cells, while GATA-3, a zinc finger protein, has been reported to be involved in the course of Th2 differentiation (19-21). Either of the two transcription factors can inhibit the polarization which the other facilitates. Our results showed knockdown of FoxP3 induced significantly increased T-bet mRNA expression and decreased GATA-3 in PBCMs of CHB patients, making an enhanced T-bet/GATA-3 ratio, in accordance with reversion of Th1/Th2 imbalance demonstrated by the secretions of Th1-type

and Th2-type cytokines. Thus, our finding suggested that Tregs, which relatively uniquely expressed FoxP3, may play a role in Th1/Th2 imbalance in CHB patients by regulating the transcription factors T-bet and GATA-3, which can influence the secretion of Th1-type and Th2-type cytokines as reported in previous studies. However, the related mechanisms are too complex to be elucidated clearly by our limited work alone, leaving many questions requiring further study to answer, for example, whether or not FoxP3 expression of Tregs influences the expression of T-bet and GATA-3 directly, and if not, what is the bridge?

In conclusion, we successfully knocked down the expression of FoxP3 by siRNAs reversing the Th1/Th2 imbalance in PBMCs of CHB patients in vitro. This provided the evidence that knockdown of FoxP3, which could inhibit Tregs' differentiation and suppressor function, may be for Th1 immune response but against Th2 immune response, which can be applied for enhancing immunity in CHB patients. A therapeutic approach to regulate the expression of FoxP3 or block the effect of FoxP3 on the transcription factors T-bet/GATA-3 may improve the prognosis of patients with chronic HBV infection.

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