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MicroRNA-146a Feedback Suppresses T Cell Immune Function by Targeting Stat1 in Patients with Chronic Hepatitis B

Saifeng Wang,* 1 Xiaojun Zhang,* 1 Ying Ju,* Bao Zhao,* Xiaoli Yan,* Jun Hu,* Lei Shi,† Lebing Yang,‡ Zhibo Ma,* Lizhao Chen,* Yali Liu,§ Zhongping Duan,¶ Xinyue Chen,§ and Songdong Meng*

More than 350 million people are chronically infected with hepatitis B virus, and dysfunctional T cell responses contribute to persistent viral infection and immunopathogenesis in chronic hepatitis B (CHB). However, the underlying mechanisms of T cell hyporesponsiveness remain largely undefined. Given the important role of microRNA-146a (miR-146a) in diverse aspects of lymphocyte function, we investigated the potential role and mechanism of miR-146a in regulating T cell immune responses in CHB. We found that miR-146a expression in T cells is significantly upregulated in CHB compared with healthy controls, and miR-146a levels were correlated with serum alanine aminotransaminase levels. Both inflammatory cytokines and viral factors led to miR-146a upregulation in T cells. Stat1 was identified as a miR-146a target that is involved in antiviral cytokine production and the cytotoxicity of CD4+ and CD8+ T cells. In vitro blockage of miR-146a in T cells in CHB greatly enhanced virus-specific T cell activity. Therefore, our work demonstrates that miR-146a upregulation in CHB causes impaired T cell function, which may contribute to immune defects and immunopathogenesis during chronic viral infection. The Journal of Immunology, 2013, 191: 293–301.

Hepatitis B virus (HBV), a strictly hepatotropic DNA virus, infects ∼350 million people worldwide. Chronic HBV infection (CHB), which is correlated with a prominently increased risk of cirrhosis, liver failure, and hepatocellular carcinoma, causes approximately 1 million deaths worldwide annually from persistent liver diseases (1, 2). As in the natural course of HBV infection, robust viral-specific CTL and CD4+ T cell responses are observed during resolution of acute and self-limited HBV infections, whereas chronic infection is characterized by only weak and impaired T cell responses; T cells are believed to play a critical role in the control of HBV replication and infection (3–5).

The natural course of CHB may have four phases, as follows: the immune-tolerant (IT) phase, defined by high viral loads but normal alanine aminotransferase (ALT) levels and no or minimal changes in liver histology; the immunoactive (IA) phase, defined by moderate-to-high viral loads with elevated ALT levels and symptomatic hepatitis; the low replicative phase; and the asymptomatic chronic carrier state. Several molecular mechanisms have been reported for the involvement of T cell tolerance and exhaustion, most likely driven by long-term intensive viral antigenic stimulation in CHB, which includes both direct and indirect mechanisms that may act synergistically. The direct effects caused by intrinsic defects include upregulation of co-inhibitory molecule programmed death-1 on T cells and its ligand programmed death-L1 on dendritic cells (DCs) (6, 7), as well as CTLA-4 and proapoptotic protein Bcl2-interacting mediator on HBV-specific CD8+ T cells (8, 9). The indirect effects caused by extrinsic defects include enhanced regulatory T cell (Treg) activities and the tolerogenic nature of the liver microenvironment (2, 10). Further understanding the molecular defects underlying T cell hyporesponsiveness will allow the development of new strategies for the establishment of immunotherapeutic regimens by targeted reversal of tolerizing mechanisms (2, 7–9).

There is a growing body of literature concerning the biological functions of microRNAs (miRNAs) in the regulation of the development and differentiation of mammalian immune cells, as well as their involvement in immune responses to infection and the development of immune diseases (11, 12). In particular, miR-146a is involved in diverse aspects of lymphocyte function, including the modulation of T cell activation (13), Treg suppressor activity (14), and macrophage and DC homeostasis and function (15, 16). Previous studies indicate that miR-146a, which expression is predominantly driven by NF-κB, acts as a negative feedback regulator of TLR or retinoic acid-inducible gene signaling pathways by targeting key upstream components, including IL-1R–associated kinase (IRAK) 1, IRAK2, and TNFR-associated factor 6 in macrophages or intestinal epithelial cells (17–20).

Consistent with these findings, deficient miR-146a expression in mice results in the hyperresponsiveness of macrophages to LPS, the development of an autoimmune disorder, and myeloid sar-
comas and some lymphomas due to an increase in NF-κB transcrip- 
tional activity (21, 22). miR-146a deregulation in T cells and 
DCs has also been observed in human autoimmune diseases, 
including systemic lupus erythematosus and rheumatoid arthritis 
through abnormal activation of type I IFN signaling pathways (23–25). However, to date, there is no report on the 
regulation of the immune response by miR-146a during clinical 
viral infections. Indeed, despite a number of reports demon- 
strating miR-146a-controlled TLR signaling in inflammatory 
settings, the role of miR-146a in the T lymphocyte–mediated 
adaptive immune response, which plays a pivotal role in viral 
infections, is just beginning to be explored.

In this study, we focused on the potential role of miR-146a in 
regulating T cell immune responses in patients with CHB. We 
found that miR-146a expression is substantially upregulated in 
CD4+ and CD8+ T cells from CHB patients. Moreover, we un-
covered a role of miR-146a in suppressing antiviral T cell function 
responses by targeting Stat1.

Materials and Methods

Subjects

Forty-two CHB patients were enrolled in this study. CHB patients are 
defined as those who are seropositive for hepatitis B surface Ag (HBsAg) for 
at least 6 mo and may have exhibited symptoms of hepatitis. The CHB 
patients were assigned to two distinct groups with respect to ALT levels and 
serum HBV DNA loads according to a previous study (6). The first group 
was defined as IT patients (n = 20) with high viral loads but normal ALT 
levels (<40 U/L) and minimal changes in liver histology. The second 
group was defined as IA patients (n = 22) with high viral loads, elevated 
ALT levels (>40 U/L) and symptomatic hepatitis. In addition, 10 healthy 
uninfected subjects with matched age and sex were enrolled as controls.

The clinical characteristics of the enrolled subjects are listed in Table I. All 
patients were hospitalized in Beijing You’an Hospital of Capital University of 
Medical Sciences from July 2010 to July 2011. All blood samples were 
collected with the approval of the Beijing You’an Hospital, Capital Uni-
versity of Medical Sciences Research Ethics Committee, and written 
informed consent was obtained from each subject.

Abs and reagents

PerCP anti-human CD3, FITC anti-human CD4, allopurinol-cyanin anti-
human CD4, PE anti-human CD8, and allopurinol-cyanin anti-human 
CD8 were purchased from eBioscience (San Diego, CA). FITC anti-
human perforin, FITC anti-human GrA, and FITC anti-human GrB were 
from BD Biosciences (San Jose, CA). In addition, the mouse anti-human 
actin Ab (sc-47778), mouse anti-human Stat1 (sc-346), and the HRP-
conjugated secondary Abs were purchased from Santa Cruz Biotechnol-
ogy (Santa Cruz, CA). Recombinant human TNF-α, IFN-γ, and IL-6 were 
purchased from PeproTech (Rocky Hill, NJ). The chemically synthesized 
mir-146a mimic and inhibitor, as well as the Stat1-specific small inter-
fering RNA (siRNA), were synthesized by Ribo Life Science (Suzhou, 
China).

Isolation of PBMCs and purification of CD8+ and CD4+ T cells

PBMCs were isolated from freshly heparinized blood by Ficoll-Hypaque 
(GE Healthcare) density gradient centrifugation. CD4+ T cells were pu-
rified from PBMCs by negative selection, and CD8+ T cells were purified 
by positive selection using the MiniMACS system (Miltenyi Biotec, 
Bergisch-Gladbach, Germany), according to the manufacturer’s instruc-
tions. Isolated populations of CD4+ and CD8+ T cells exceeded 95% purity 
by flow cytometric analysis.

Cell cultures and transfection

All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C. 
Human PBMCs were maintained in RPMI 1640 (Life Technologies, Grand 
Island, NY) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM t-
glutamine, 20 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, 
and 50 U/ml rIL-2 (Sigma-Aldrich). The Jurkat human leukemic T cell line 
was cultured in RPMI 1640 (Life Technologies) medium containing 10% 
FBS (HyClone), 2 mM t-glutamine, 20 mM HEPES, 100 U/ml penicillin, 
and 100 mg/ml streptomycin. The 293T cells were grown in DMEM (Life 
Technologies, Grand Island, NY) supplemented with 10% FBS, penicillin/ 
streptomycin, and t-glutamine. Transient transfections of PBMCs were 
performed using the Lonza Nucleofector system (Lonza, Walkersville, 
MD). Briefly, 5 x 106 cells resuspended in 100 μl Nucleofector solution 
(Nucleofector kits for human T cells) with small RNA (100 pmol/106 cells) 
were transfected using the program for unstimulated T cells. Cells were 
immunized by electric pulse in prewarmed complete RPMI 1640 medium 
in 12-well culture plates.

miR-146a target prediction

Potential targets of miR-146a were predicted by querying PicTar (http:// 
pictar.bio.nyu.edu/) and TargetScan (http://www.targetscan.org/). The se-
quencing alignment of mir-146a/Stat1 was performed by RNAhybrid.

Untranslated region luciferase reporter assays

Dual luciferase reporter assays were performed following the manufac-
turer’s protocol (Promega). The 3'-untranslated region (UTR) of Stat1 was 
cloned downstream of the Renilla luciferase gene in the pGL3 plas-
mid. MiR-146a seed region mutants were constructed using a Quick-
Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The 
293T cells plated in a 12-well plate at a concentration of 8 x 104 cells/well 
were transfected with constructs using lipofecto (Invitrogen) at a DNA: 
lipo2000 ratio of 1:3. Samples were harvested 48 h postransfection and 
subjected to the dual luciferase assay using a Femtomaster FB12 chemi-
luminometer (Zytech, Coeur d’Alene, ID). Relative units represent the ratio 
between Renilla values and the internal luciferase control.

Quantitative real-time PCR analysis

Total cellular RNA was extracted using TRizol reagent (Invitrogen) 
following the manufacturer’s instructions. miR-146a levels were quantified 
by quantitative RT-PCR using a TaqMan miRNA kit (Applied Biosystems, 
Foster City, CA). A U6 endogenous control was used for normalization.

Detection of cytokines by ELISA

ELISA tests for detection of TNF-α, IFN-α, IL-6, IFN-γ, and IL-2 in serum 
or supernatants of cell culture were performed using ELISA kits (eBioscience).

Phenotypic analysis and intracellular staining

Jurkat or PBMCs were collected and blocked with 5% BSA at 4°C for 20 
min. Then, various fluorochrome-conjugated Abs raised against the surface 
markers of interest were added to cell pellets, incubated for 30 min on ice, 
and washed three times before analysis. Perforin, GrA, and GrB intra-
cellular staining was performed using Cytofix/Cytoperm kits (eBioscience 
and BD Pharmingen). Samples were analyzed using a FACSCalibur and 
CellQuest software (BD Biosciences) after staining.

Cytotoxicity assay

CFSE-based cytotoxicity assays were performed, as previously described 
(26). Briefly, HepG2 cells were labeled with 2 μM CFSE as the target 
cells, and then the cells were seeded into a 96-well U-bottom microtiter 
plate. T cells were added at an E:T ratio of 10:1. Plates were then incu-
bated in a humidified atmosphere of 5% CO2 for 6 h. The cells were 
harvested, and apoptosis assays were performed by propidium iodide 
staining using a Vybrant apoptosis assay kit (Invitrogen).

IFN-γ ELISPOT assay

The ELISPOT assay for enumeration of Ag-specific, IFN-γ-secreting cells 
was performed according to the protocol supplied by the manufacturer. 
Briefly, 96-well polystyrene microtiter plates (BD Pharmingen, San 
Diego, CA) were precoated with coating Ab overnight at 4°C and blocked 
for 1 h at 37°C. Isolated and transfected PBMCs (2.5 x 105 cells/well) and 
HBsAg (10 mg/ml) or hepatitis B core Ag (HBcAg) (10 mg/ml) were 
added to each well and incubated at 37°C for 48 h. Each test condition 
was performed in triplicate. The spots were counted and analyzed with an 
ELISPOT reader (Biosys).

Statistical analysis

All data were analyzed using SPSS software (SPSS Science, Chicago, IL). 
Comparison between healthy controls, IT, and IA was performed using the 
Mann-Whitney U test. For the intervention assay, statistical comparisons 
were conducted using the Wilcoxon matched pairs t test. Correlations 
between variables were determined by Spearman’s nonparametric corre-
lation. For all tests, two-sided p < 0.05 was considered significant.
Results
The expression of miR-146a in CD8+ and CD4+ T cells is significantly increased in CHB patients

To determine the effect of chronic HBV infection on miR-146a expression, we measured miR-146a levels by real-time PCR in CD4+ and CD8+ T cells from HBV-infected subjects. The clinical characteristics of the enrolled subjects are listed in Table I. Compared with noninfected individuals, the miR-146a levels in CHB were increased to various degrees in CD8+ (IT patients versus healthy controls, 0.036 ± 0.019 versus 0.037 ± 0.033, p > 0.05; IA patients versus healthy controls, 0.25 ± 0.12 versus 0.037 ± 0.033, p < 0.01) and CD4+ T cells (IT patients versus healthy controls, 0.92 ± 0.61 versus 0.48 ± 0.31, p < 0.05; IA patients versus healthy controls, 1.05 ± 0.93 versus 0.48 ± 0.31, p < 0.05) (Fig. 1A). Compared with IT patients, IA patients displayed dramatically increased miR-146a expression (>7-fold) in CD8+ T cells (p < 0.01), but not in CD4+ T cells (p > 0.05), indicating that chronic inflammation may lead to upregulation of miR-146a in CD8+ T cells. To investigate whether active viral replication correlates with miR-146a upregulation, we compared the miR-146a levels between hepatitis B e Ag (HBeAg)-positive and -negative CHB patients. As shown in Fig. 1B, CD4+ T cells in HBeAg-positive IT and IA patients exhibited significantly higher miR-146a levels than in HBeAg-negative patients, but there was no difference in miR-146a levels in CD8+ T cells, indicating that the increase of miR-146a in CD4+ T cells correlated with the HBV replication level because a significantly greater HBV DNA load was found in HBeAg-positive than HBeAg-negative patients (p < 0.01).

We further evaluated the association between miR-146a levels and ALT levels or HBV DNA loads in CHB patients. Spearman analysis revealed that there was a high correlation between miR-146a levels in CD8+ T cells and serum ALT levels (r = 0.857, p < 0.01), but only a low correlation was observed in CD4+ T cells (r = 0.122, p < 0.05) (Fig. 1C). In contrast, there was only a weak positive correlation between miR-146a levels in CD4+ T cells and HBV DNA loads (r = 0.35, p < 0.05), whereas no correlation was observed in CD8+ T cells (r = 0.105, p > 0.05) (Fig. 1D). Taken together, these data indicate that an increase in miR-146a levels in CD8+ T cells correlates with chronic necroinflammation, whereas its increase in CD4+ T cells may moderately associate with both active viral replication and chronic necroinflammation.

Table I. The clinical characteristics of studied subjects

<table>
<thead>
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<th>Group</th>
<th>HCs</th>
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<td>41 (28–57)</td>
<td>45 (22–62)</td>
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<td>9/11</td>
<td>12/10</td>
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<td>11–38</td>
<td>43–164</td>
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<td>HBeAg positive (ratio)</td>
<td>ND</td>
<td>10 (50%)</td>
<td>12 (55%)</td>
</tr>
<tr>
<td>Serum HBV DNA (Lg of copies/ml)</td>
<td>ND</td>
<td>3.7–4.95/4.28</td>
<td>3.27–7.07/5.78</td>
</tr>
</tbody>
</table>

*Data are shown as range and median. HC, Healthy control; Lg, log base 10; ND, no data.
miR-146a expression in T cells is induced by inflammatory cytokines and HBV factors

To understand how inflammation in CHB may influence miR-146a expression in T cells, we determined whether miR-146a is subject to regulation by TNF-α, IFN-α, and IL-6, which are the major proinflammatory cytokines involved in hepatic inflammations (27, 28). The upregulation of miR-146a followed a dose-response curve and time course after treatment with TNF-α in Jurkat cells (Fig. 2A). Time course analysis showed a dramatic increase in miR-146a expression in CD8+ T cells, with a maximum occurring between 4 and 16 h (Fig. 2B). Compared with CD8+ T cells, TNF-α only stimulated moderate miR-146a upregulation in CD4+ T cells (Fig. 2C). As for IL-6 and IFN-α, significant upregulation of miR-146a was observed in CD4+ T cells after treatment, but only moderate miR-146a upregulation was observed in CD8+ T cells (Fig. 2D, 2E).

With regard to viral factors, we tested whether miR-146a was subject to regulation by the cell culture supernatant of HepG2.2.15 cells, which are HepG2 cells stably transfected with the HBV genome, constitutively express all viral proteins, and secrete virions. As shown in Fig. 2F, the miR-146a level in primary CD4+ T cells was significantly increased when treated with the supernatant of HepG2.2.15 cells compared with that of HepG2 cells, whereas no obvious change of miR-146a levels was observed in CD8+ T cells. These results are consistent with the observation in Fig. 1D that miR-146a levels in CD4+ but not CD8+ T cells are moderately correlated with the viral load in CHB. In addition, significantly higher serum TNF-α levels (∼2-fold) were found in IA than in IT patients (p < 0.01) (Fig. 2G), indicating that the upregulation of miR-146a in CD8+ T cells correlated with higher levels of TNF-α in IA patients. These data support our hypothesis that inflammatory cytokines and virus factors in CHB may both contribute to upregulation of miR-146a in T cells.

Upregulation of miR-146a leads to decreased antiviral cytokine production and cytotoxicity of T cells by targeting Stat1

To assess the potential role for miR-146a in CHB, we examined anti-HBV cytokine production and the cytotoxicity of CD4+ and CD8+ T cells.
CD8+ T cells. Transfection of T cells with a miR-146a mimic resulted in a dramatic decrease of IFN-γ, TNF-α, and IL-2 [the major cytokines that suppress HBV gene expression and replication (29)] at both the mRNA and protein levels, whereas a miR-146a inhibitor greatly promoted the expressions of these cytokines (Fig. 3A). The efficiency of miR-146a mimic and inhibitor was determined by real-time PCR analysis demonstrating that transfection of a miR-146a mimic or inhibitor resulted in ~3-fold increase or 70% reduction, respectively, in the miR-146a levels in T cells (data not shown). Only moderate effects were observed for intracellular levels of perforin (Fig. 3B), GrA (Fig. 3C), and GrB (Fig. 3D) by flow cytometric analysis of CD4+ and CD8+ T cells treated with the miR-146a mimic or inhibitor. We further examined the effect of miR-146a on the lytic function of CD8+ T cells using HepG2 cells as target cells. Both gain-of-function and loss-of-function analyses verified a moderate suppressive effect of miR-146a on the cytotoxicity of CD8+ T cells (Fig. 3E).

We then measured anti-HBV cytokine production and lytic protein levels of T cells in CHB patients. As shown in Fig. 4A and 4B, significantly lower expression of IFN-γ, TNF-α, and IL-2, as well as lower intracellular levels of perforin, GrA, and GrB, in CD4+ and CD8+ T cells were observed in CHB patients compared with healthy controls. Moreover, inhibition of miR-146a in CD4+ and CD8+ T cells from CHB patients greatly increased anti-HBV cytokine production and lytic protein levels (Fig. 4C, 4D), indicating that elevated miR-146a levels contribute to impaired T cell functions in CHB.

Next, we explored the miR-146a target mRNAs that might modulate antiviral cytokine production and the cytotoxicity of T cells. Because IFN-γ–Stat1 signaling is critical for T cell differentiation and function (30) and a recent study identified Stat1 as a miR-146a target in human PBMCs (31), it is possible that decreased Stat1 expression in miR-146a upregulated T cells in CHB may account for the impaired T cell function observed in HBV-infected patients. A putative miR-146a complementary region in

**FIGURE 3.** miR-146a decreases antiviral cytokine production and inhibits the cytotoxicity of T cells. (A) Primary T cells from healthy subjects were transfected with 5 nmol/ml miR-146a mimic (left), its randomized oligonucleotide as a mock, the miR-146a inhibitor (right), or its randomized oligonucleotide as a mock, and cultured in the presence of 1 μg/ml anti-CD3 for 48 h. The mRNA levels of IFN-γ, IL-2, and TNF-α were assessed by real-time PCR using β-actin as an endogenous control. The levels in mock-transfected cells were arbitrarily set as 1.0. The IFN-γ, IL-2, and TNF-α levels in the supernatants were analyzed by ELISA. (B–D) PBMCs from healthy subjects were transfected with 5 nmol/ml miR-146a mimic, its inhibitor, or a mock and cultured in the presence of 1 μg/ml anti-CD3 for 48 h. The intracellular expression levels of perforin (B), GrA (C), and GrB (D) in CD4+ and CD8+ T cells were analyzed by flow cytometric analysis. (E) Primary T cells from HLA-A2–positive healthy subjects were treated with 5 nmol/ml miR-146a mimic, its inhibitor, or a mock. Forty-eight hours after treatment, CFSE-labeled HepG2 cells (as the target cells) were added at an E:T ratio of 10:1, and the lytic function of the CD8+ T cells was determined by cytometric analysis. Error bars, means ± SD of five independent experiments. *p < 0.05, **p < 0.01 compared with the mock.
the Stat1 gene sequence (position 776–783 in the Stat1 3′-UTR) is shown in Fig. 5A. Furthermore, miR-146a reduced the activity of a firefly luciferase reporter using the wild-type (STAT1 wt) but not mutant (STAT1 mut) 3′-UTR of Stat1. As shown in Fig. 5B, miR-146a T cells displayed a decrease in Stat1 expression, whereas the depletion of endogenous miR-146a by its inhibitor led to an increase in Stat1 levels. We then determined the expression levels of Stat1 in T cells from CHB patients; a representative anti-Stat1 Western blot is shown in Fig. 5C. A dramatic decrease in Stat1 expression was observed in the T cells of CHB compared with healthy controls.

Next, we examined the effect of Stat1 knockdown on cytokine production and the cytotoxicity of T cells. T cells from healthy subjects were cotransfected with the Stat1-specific siRNA si-Stat1. Significant decreases in IFN-γ, TNF-α, and IL-2 production were detected in T cells after treatment with si-Stat1 (all \( p < 0.01 \)) (Fig. 5D), and a significant decrease in intracellular levels of perforin was also observed in both CD4+ and CD8+ T cells (Fig. 5E). Accordingly, flow cytometric analysis showed that, compared with the mock, Stat1 RNAi treatment reduced the lytic function of CD8+ T cells (Fig. 5F).

We further investigated whether overexpression of Stat1 could restore the impaired T cell function from CHB patients. Dramatic increases of IFN-γ, TNF-α, and IL-2 expression were detected in both CD4+ and CD8+ T cells from IA CHB patients after transfection of the Stat1 expression vector (Fig. 5G), and a significant increase in intracellular levels of perforin was also observed in T cells (Fig. 5H). These data indicate that downregulation of Stat1 by miR-146a dampens T cell function in CHB.

**Blockage of miR-146a improves the antiviral capacity of T cells from CHB patients**

Given that the HBV-specific T cell immunoresponse plays a key role in virus control and clearance, we next investigated whether...
inhibition of miR-146a could restore the impaired T cell function from CHB patients. As shown in Fig. 6A, transfection with the miR-146a inhibitor led to significantly increased T cell responses to two major Ags of HBV (HBsAg and HBcAg) as determined by IFN-γ ELISPOT assays, whereas only low virus-specific T cell immunity was detected in CHB patients, indicating the hyporesponsiveness of HBV-specific T cells in these patients. In addition, the capacity of PBMCs from CHB patients to express the antiviral cytokine IL-2 under viral Ag stimulation was greatly improved by transfection with the miR-146a inhibitor (Fig. 6B).

Discussion

In the current study, we investigated the functional relevance of miR-146a for impaired T cell function in chronic HBV infection. We found that miR-146a levels were significantly increased in both CD4+ and CD8+ T cells of patients infected with HBV, which may be due to chronic inflammation and/or viral replication. Moreover, our findings revealed that downregulation of Stat1 by miR-146a resulted in inhibition of T cell antiviral responses. Importantly, the blockage of miR-146a could reverse, at least in part, the functional impairment of HBV-specific T cells in CHB. Therefore, we
was not observed in CD8 + T cells between IA and IT CHB patients, where protein levels in T cells than healthy controls, but a similar trend played significantly lower anti-HBV cytokine production and lytic activity. It is expected, CHB patients with higher miR-146a expression displays a blockage of miR-146a reverses virus-specific T cell activity and enhances antiviral cytokine production in CHB (Figs. 4, 6). As a transcription factor, Stat1 plays an important role in antiviral responses by inducing expression of antiviral IFN-stimulated genes and epitope-specific CTLs, and Stat1-null mice exhibit enhanced susceptibility to bacterial and viral infections (32–34). Based on these studies, together with a recent study showing that miR-146a impairs both AP-1 activity and IL-2 expression induced by TCR engagement (13), our current work suggests that upregulation of miR-146a in CHB may lead to decreased expression of antiviral cytokines, antiviral IFN-stimulated genes, and the lytic function of T cells by targeting Stat1, thus promoting T cell functional deficiency and immune tolerance to HBV infection.

Responses to IFN-α therapy, the first drug licensed to treat HBV infection, vary greatly in CHB patients, but the mechanisms underlying this phenomenon are still unclear. Inhibition of the key IFN-α signal transducer Stat1 by HBV has been suggested to antagonize the IFN response (35, 36). In this study, we found that miR-146a, which targets Stat1, is significantly upregulated in the T cells of patients with CHB, which could shed light on the mechanism of the limited effectiveness of therapeutic IFN-α in CHB.

Several unanswered questions remain. For example, what are the mechanisms involved in miR-146a upregulation in T cells by HBV infection? We speculate that both chronic inflammation and HBV replication are involved in this process. As for inflammation, we found that IA patients had much higher miR-146a levels than IT patients, and there was a high correlation between miR-146a levels and ALT levels in CD8+ T cells. Moreover, additional experiments demonstrated that TNF-α, IFN-α, and IL-6 significantly induced miR-146a expression in CD4+ and CD8+ T cells. Furthermore, IL-1β, TGF-β1, and LPS also stimulate miR-146a expression through activation of the transcription factor NF-κB or PU.1 (37). With regard to viral factors, we found that both IA and IT patients had higher miR-146a levels than healthy controls in CD4+ T cells, and miR-146a levels in CD4+ but not in CD8+ T cells were correlated with HBV loads. Furthermore, HBcAg-positive patients had higher miR-146a levels than HBcAg-negative patients in both IT and IA subsets. This indicates that active viral replication and HBV load are correlated with miR-146a upregulation, as in general patients with positive HBcAg tend to have higher HBV DNA load than patients with negative HBcAg (but the degree of hepatic inflammation is not significantly different). Indeed, our next experiment revealed that viral factors can stimulate miR-146a expression in CD4+ T cells, but not in CD8+ T cells. Conceivably, the activation of NF-κB by HBV proteins (38) may be one of the contributing reasons for miR-146a upregulation in T cells. The intriguing observation that viral factors induce miR-146a expression in CD4+ but not CD8+ T cells may be explained by the different expression profile of TLRs and retinoic acid–inducible gene between CD4+ and CD8+ T cells, which are involved in the recognition of HBV-specific components and subsequent induction of NF-κB. These data also highlight that regulation of miR-146a expression can be exquisitely cell-type specific, which is consistent with a previous study (37). Another intriguing question arises from our observations: could systemic interference with miRNA-146a potentially be a therapeutic approach for HBV infection? Although blockage of miR-146a improved HBV-specific T cell activity (Fig. 6), it may also boost nonspecific or other Ag-specific T cell responses, which could lead to immune-mediated liver injury and pathogenesis. Indeed, a recent study demonstrates that miR-146a inhibits T cell hyperresponsiveness after TCR stimulation by targeting the NF-κB signaling transducers TNFR-associated factor 6 and IRAK1, acting as a critical constituent of

present a new model in which miR-146a acts as viral-specific T cell suppressor during CHB, and its upregulation in T cells by HBV infection may contribute to impaired T cell responses and hamper the development of the virus-specific immune response. To our knowledge, this is the first report of deregulation of miR-146a in any human viral infection.

In this study, we identified that Stat1 was a miR-146a target and involved in the regulation of T cell function in CHB. Two recent studies suggest that miR-146a regulates downstream IFN-γ receptor signaling in Treg- and IFN-γ-mediated Th1 pathobiology by targeting Stat1 in both mouse models and patients with autoimmune disease (14, 31). This intrigued us to further explore the negative regulation of miR-146a on HBV-specific T cell function. We found that, aside from acting as a negative regulator of the production of key anti-HBV cytokines, miR-146a inhibits the expression of granzymes and perforin and suppresses the cytotoxicity of T cells. Thus, miR-146a upregulation in CHB contributes to intrinsic defects in T cell hyperresponsiveness. Indeed, the blockage of miR-146a reverses virus-specific T cell activity and enhances antiviral cytokine production in CHB (Figs. 4, 6). As expected, CHB patients with higher miR-146a expression displayed significantly lower anti-HBV cytokine production and lytic protein levels in T cells than healthy controls, but a similar trend was not observed in CD8+ T cells between IA and IT CHB patients (Fig. 4A, 4B), although the miR-146a levels were higher in IA patients (Fig. 1A). We assume that factors other than miR-146a in IA and IT patients may also be involved in the regulation of IFN-γ, TNF-α, IL-2, perforin, GrA, and GrB expression, which could counteract the effect of miR-146a on these antiviral cytokines and cytolytic proteins.
the negative feedback network regulating TCR signaling to NF-κB (39). It is possible that miR-146a may exert beneficial effects by preventing overactivation of inflammation and T cell responses in CHB. More studies are needed to dissect the immunoregulatory mechanisms of miR-146a in various states of HBV infection and its use as a potential prognostic marker in disease progression.

In conclusion, this study indicates chronic inflammation and/or viral factors may induce miR-146a expression in T cells during chronic HBV infection, and miR-146a upregulation may subsequently lead to suppression of the antiviral function of T cells by targeting Stat1. Given the broad function of miR-146a in innate and adaptive immune responses, our work provides valuable insight for the functional implications of miR-146a in HBV infection, indicating that miR-146a upregulation by viral infection may contribute to the functional impairment of HBV-specific T cells and viral persistence. Understanding miR-146a function in the complex regulation networks that orchestrate immune defects and immunopathogenesis in chronic viral infections will allow us to identify novel therapeutic targets for treatment of CHB.

Disclosures
The authors have no financial conflicts of interest.

References