

Interleukin-33-induced immune tolerance is associated with the imbalance of memory and naïve T-lymphocyte subsets

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Abstract. The current study aimed to investigate the distribution of memory and naïve T cell (TN) subsets in hepatitis B virus (HBV)-infected patients at different immune stages and investigate the effect of interleukin 33 (IL-33) on the regulation of the T-cell subsets. The distributions of memory and naïve T cells were detected by flow cytometry. ELISA was conducted to assess the levels of IL-4, IL-5, IL-10, IL-12, interferon γ and tumor necrosis factor α . The expression levels of IL-33 and

HBV x protein (HBx) were measured by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. By detecting TNs, central memory T cells (TCM) and effector memory T cells (TEM), it was identified that the proportions of TCM and TEM in CD4+ T cells were increased in patients with HBV. The trend observed for levels of CD8+ TCM and TEM was similar to that of CD4+ T cells in the immune tolerance and immune activation groups, however CD8+ TCM and TEM were significantly reduced in patients who underwent treatment. The CD8+ TEM cells appeared to be more sensitive to HBV activation and drug therapy. In addition, IL-33 stimulation was observed to induce imbalances of CD8+ TN and CD8+ TEM, and while the imbalances were directly regulated by HBx, IL-33 was not a key factor for the expression of HBx. CD8+ TEM cells may be a sensitive marker to assess the immune state of patients with HBV and the effect of clinical therapy. Treatment targeting IL-33 may be a potential method to reverse HBV-induced immune tolerance.

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBx, HBV x protein; Th, T helper cell; Treg, regulatory T cells; ST2, suppression of tumorigenicity 2; IFN- γ , interferon γ ; TNF- α , tumor necrosis factor α ; IL-33, interleukin 33; HC, healthy controls; PBMCs, peripheral blood mononuclear cells; IT, immune tolerance; IA, immune activation; RP, responders with hepatitis B surface antigen seroconversion; AST, aspartate aminotransferase; ALT, alanine transaminase; AFP, α -fetoprotein; PBS, phosphate-buffered saline; ELISA, enzyme-linked immune-sorbent assay; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Abs, antibodies

Key words: HBV, IL-33, immune tolerance, T-lymphocyte subsets

Introduction

Hepatitis B virus (HBV) is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC), which is a severe threat to patient health. Approximately 400 million people have been diagnosed in worldwide, and in addition, the risk of HBV-associated mortality is greater than 15% (1-5). Although several therapies targeting virus invasion and replication have exhibited some curative effects, it remains difficult to thoroughly eradicate HBV. This is due to the fact that the cccDNA reservoir persists in infected cells, and in addition that the immunosuppressive environment induced by HBV infection counteracts the antiviral response of the local innate immune system and impairs the specific immune response, resulting in defective immune surveillance and a chronic viral infection (6-9). The HBV-induced systemic immune tolerance (IT) generally presents with the characteristics of a shift of T helper

cell (Th) 1/Th2 balance, a deficient cytotoxic T-lymphocyte response to hepatitis B surface antigen (HBsAg) or hepatitis B core antigen and an increased proportion of regulatory T cells (Tregs) (10-16). However, the alterations to T cell-mediated specific immunity in HBV infection, and how HBV induces the immunosuppression remain unclear.

Interleukin 33 (IL-33), a novel member of the IL-1 family, has been identified as the special ligand for the receptor suppression of tumorigenicity 2 (ST2), which is selectively expressed on Th2 cells however not on Th1 cells. IL-33 was initially recognized as a specific Th2 effector, inducing the production of IL-4, IL-5, IL-6, IL-10 and IL-13, and leading to the IT of Th2-associated diseases including asthma, atopic dermatitis and anaphylaxis (17-19). Further investigations indicated that the IL-33/ST2 axis was not regulating the Th2 response alone, however was additionally acting as an important component in Th1/Th17 and innate inflammation (20,21). In addition, IL-33 has been reported to exhibit various protective effects in cardiovascular conditions including atherosclerosis and cardiac remodeling, in addition to obesity and type 2 diabetes (22-25). In cardiomyocytes and hepatocytes, IL-33 has been reported to protect against apoptosis (26). The mechanism of IL-33 on regulating T-cell subsets is complex, and whether IL-33 participates in the regulation of T-cell mediated IT during HBV infection remains to be investigated.

In the current study, the distribution of different T-lymphocyte subsets in was investigated in patients with HBV at different immune phases, and in addition, the effect of IL-33 on the regulation of T subset distribution and the levels of relative inflammatory cytokines was assessed. Furthermore, the interaction between IL-33 and HBV x protein (HBx) was also explored. The results of the current study suggested that clinical therapy targeting IL-33 may be a potential method to reverse IT in patients with HBV.

Materials and methods

Patients. A total of 50 patients with chronic HBV and 20 vaccinated healthy controls (HCs) were recruited from the Department of Hepatology of the First Hospital of Jilin University (Changchun, China). Patients with chronic HBV were categorized according to the disease phase: IT phase (n=17) and immune activation (IA) phase (n=18). The remaining 15 patients were interferon (IFN)- α therapy-induced responders with HBsAg seroconversion (RP). None of the patients had received antiviral treatment within the previous 6 months. All individuals were negative for other infectious diseases, autoimmune disorders and malignancies. The clinical characteristics of these participants are listed in Table I. The levels of serum aspartate aminotransferase (AST), alanine transaminase (ALT) and α -fetoprotein (AFP) in participants were detected using the Biochemistry Automatic Analyzer (Roche Diagnostics, Lewes, UK). The Ethical Committee of the First Hospital of Jilin University had approved the experiment, and all participants had provided written informed consent.

Cell culture and transfection. HepG2.2.1.5 (derived from HepG2 cells transfected with a plasmid carrying two head-to-tail copies of the HBV genome DNA serotype ayw) cell line was originally obtained from the American Type Culture Collection

(Manassas, VA, USA) and were maintained at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). To silence the HBx gene, the specific short hairpin RNA (shRNA) was inserted into the shRNA pSIREN expression vector. The shRNA sequences targeting HBx were as follows: HBx-shRNA forward 5'-GGATCCAGGTCTTTGTAC TAGGAGGCTCCACCAGCCTCCTAGTACAAAGACCTT-3'; reverse 5'-GAATTCAAGGTCTTTGTACTAGGAGGCTGG TGGAGCCTCCTAGTACAAAGACCT-3'. Then the recombinant vector was transfected into HBV-persistent HepG2.2.1.5 cells. The day before plasmid transfection, HepG2.2.1.5 (2x10⁶) were seeded onto 6-well plates (Costar; Corning Incorporated, Corning, NY, USA) at 80% confluency. Following 24 h incubation at 37°C, cells were transfected with pSIREN-HBx-shRNA or empty pSIREN vectors separately according to the manufacturer's instructions of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were collected using centrifugation at 1,100 x g for 10 min at room temperature 48 h subsequent to transfection. A total of 50 ng/ml IL-33 (PeproTech, Inc., Rocky Hill, NJ, USA) was used to stimulate HepG2.2.1.5 and HBx-deficient HepG2.2.1.5 cells *in vitro*. In order to inhibit the IL-33 relative signaling, the ST2 blocking antibody (sc-18687P; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used to inhibit the normal binding of IL-33 to its receptor ST2L.

Peripheral blood mononuclear cell (PBMC) isolation and flow cytometry. PBMCs from venous blood samples were isolated by Ficoll-Paque (GE Healthcare Life Sciences, Chalfont, UK) density-gradient centrifugation at 1,100 x g for 30 min at room temperature. For analyzing the distribution of T-lymphocyte subsets, separated PBMCs were stained with different fluorescein-labeled antibodies (Abs). Surface staining was performed using the following monoclonal Abs: Anti-human CD4-phycoerythrin (PE; 12-0049-42), anti-human CD8-fluorescein isothiocyanate (9011-0087), anti-human CD45RA-allophycocyanin (17-0458), anti-human CCR7-PE-cyanine7 (25-1979) and the corresponding fluorescence-conjugated immunoglobulin G isotypes (all antibodies from eBioscience, Inc., San Diego, CA, USA). A minimum of 50,000 cells prepared for phenotypic analysis were collected using a FACSCalibur (BD Pharmingen, San Diego, CA, USA) analytical instrument and were analyzed by FlowJo software, version 7.6 (Tree Star, Inc., Ashland, OR, USA).

Enzyme-linked immunosorbent assay (ELISA). Venous blood (10 ml) from patients with HBV and HCs were collected and centrifuged at 1,100 x g for 10 min at room temperature. Subsequently, the blood plasma samples were used to detect inflammatory factors using ELISA. The ELISA kit (Roche Diagnostics) was used to detect the serum or supernatant levels of IL-4, IL-5, IL-10, IL-12, IFN- γ and tumor necrosis factor α (TNF- α) according to the manufacturer's instructions. The absorbance of the plates was read at 450 nm using an Automated Microplated Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA from HepG2.2.1.5 and HBx-deficient HepG2.2.1.5 cells were isolated with TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription

Table I. Clinical characteristics of participants in the study.

Group	HC n=20	IT n=17	IA n=18	RP n=15
Sex (male/female)	10/10	12/5	15/3	11/4
Age (years)	28 (25-35)	33 (18-49)	36 (24-52)	37 (20-49)
ALT (U/l)	24 (13-27)	26 (16-37)	292 (66-985) ^a	36 (16-43)
AST (U/l)	22 (15-32)	25 (19-32)	328 (47-875) ^a	19 (12-38)
AFP (ng/ml)	7 (2-11)	64 (42-87) ^a	194 (12-723) ^a	28 (9-47)
HBV DNA (100 IU/ml)	ND	8.2 (1.8-8.9)	6.8 (3.3-8.8)	LDL

Normal values: AFP, <25 ng/ml; ALT, <40 IU/l; AST, <40 IU/l. ^aP<0.05 vs. HC. Data presented as the median and the range. HC, healthy controls; IT, immune tolerance; IA, immune activation; RP, responders with hepatitis B surface antigen seroconversion; ALT, alanine transaminase; AST, aspartate aminotransferase; AFP, α -fetoprotein; HBV, hepatitis B virus; ND, not determined; LDL, lower detection limitation.

was conducted using 500 ng total RNA with the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.).

IL-33-specific RT-qPCR amplification was performed with Power SYBR Green Master Mix (containing SYBR[®] Green I Dye, AmpliTaq Gold[®] DNA Polymerase, dNTPs, passive reference and optimized buffer) using ABI 7300 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, and all experiments were repeated three times. Relative gene expression was calculated with the 2^{- $\Delta\Delta C_q$} method (27) following normalization to the expression of GAPDH. The primers used were as follows: IL-33, sense 5'-CACCCCTCAAATGAATCAGG-3' and antisense 5'-GGAGCTCCACAGAGTGTTC-3'; HBx, sense 5'-CGACCGACCTTGAGGCATAC-3' and antisense 5'-TTAGGCAGAGGTGAAAAAGTTG-3'.

Western blotting. HepG2.2.1.5 and HBx-deficient HepG2.2.1.5 cells were harvested and then lysed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China). Following centrifugation at 10,000 x g for 15 min at 4°C, whole cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (GE Healthcare Life Sciences). Sequentially, the membranes were incubated with the indicated antibodies. The primary antibodies for IL-33 (sc-130625), HBx (sc-57760) and GAPDH (sc-32233), in addition to the goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (sc-2302) were all purchased from Santa Cruz Biotechnology, Inc.

Statistical analysis. All data are representative of three independent experiments and are expressed as the median \pm range or mean \pm standard deviation as indicated. Statistical analysis was conducted using Student's t-test. P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using GraphPad software, version 5.0 (GraphPad, Inc., La Jolla, CA, USA).

Results

T-lymphocyte subset distribution in patients with HBV at different immune stages. To investigate the frequencies of

different T-cell subsets in patients with HBV at different immune stages; IT, immune clearance (IC), IA and RP (28); PBMCs were isolated. Subsequently, the proportional changes of naïve T cells (TN; CD45RA+CCR7+), central memory T cells (TCM; CD45RA-CCR7+) and effector memory T cells (TEM; CD45RA-CCR7-) were assessed (29,30). As presented in Fig. 1, there was no significant difference in the frequencies of CD4+ TN and CD8+ TN in patients and HC. The proportions of CD4+ TCM and CD4+ TEM were markedly increased in patients with HBV compared with HC, however appeared at similar levels within the three patient groups. Similar to the results of CD4+ TCM and CD4+ TEM, the frequencies of CD8+ TCM and CD8+ TEM were increased in IT and IA compared with HC. However, different to the results of CD4+, the CD8+ TCM and TEM subsets were significantly reduced in the RP group compared with that of IT and IA. In particular, the proportion of CD8+ TEM was lower in RP than in HC, and levels were statistically greater in IA compared with IT. This data indicated that levels of CD8+ TEM cells were associated with the immune state of patients with HBV. The results indicated that CD8+ was more sensitive to HBV activation and IFN- α based therapy. The frequency of CD8+ TEM cells may be an effective marker to assess the immune state of patients with HBV and the effect of clinical therapy.

Additionally, the serum levels of IL-4, IL-5, IL-10, IL-12, IFN- γ and TNF- α were measured in order to evaluate the immune status of the participants. As presented in Fig. 2, the levels of Th2-associated factors (IL-4, IL-5 and IL-10) were all increased in IT and IA, however a clear reduction was observed in the RP group, which may have been induced by an IFN- α based therapy. The concentrations of Th1-associated factors (IL-12, IFN- γ and TNF- α) appeared in general at similar low levels in IT and IA, while the patients who had been treated exhibited increased levels. The data indicated that there was a clear Th2-dominant immune response in patients with HBV, and the IFN- α based therapy predominantly improved the Th1 response while inhibiting the Th2 reaction.

The effect of IL-33 on the distribution of T-lymphocyte subsets. As demonstrated by a previous study, serum IL-33

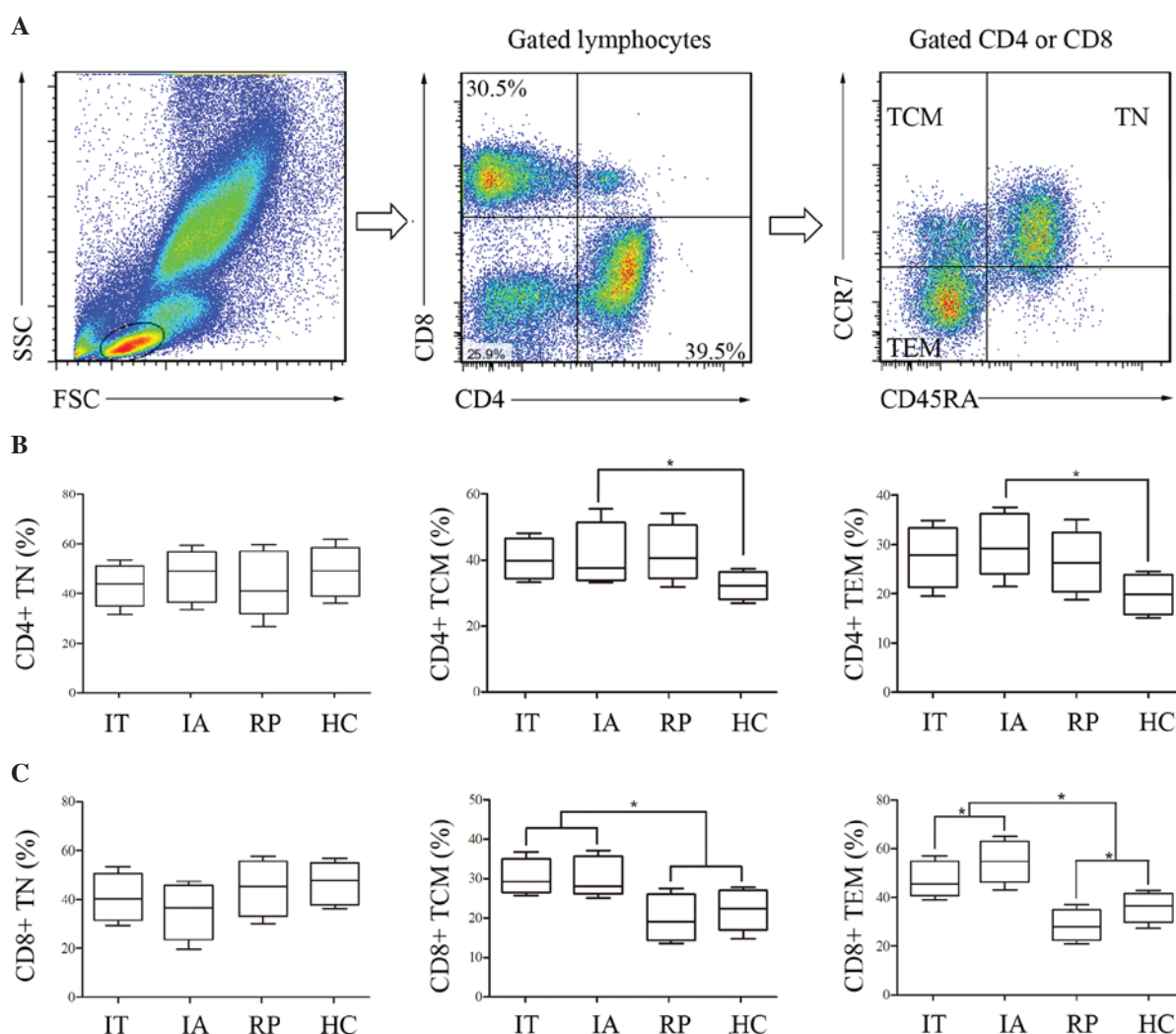


Figure 1. Differential distribution of circulating CD8+ and CD4+ T-lymphocyte subsets in HBV-infected patients at various immune phases. PBMCs from patients with HBV and HCs were isolated and categorized as TN (CD45RA+CCR7+), TCM (CD45RA-CCR7+) and TEM (CD45RA-CCR7-). (A) The flow cytometry chart represented the gating strategy. Detailed proportions of naïve and memory (B) CD4+ T cells, in addition to (C) CD8+ T subsets in the participants were presented in the form of a box plot. The boxes represented the 5th, 25th, 75th and 95th percentiles, and the solid line indicates the median value of each subset. * $P < 0.05$. HBV, hepatitis B virus; PBMCs, peripheral blood mononuclear cells; HC, healthy controls; TN, naïve T cells; TCM, central memory T cells; TEM, effector memory T cells; IT, immune tolerance; IA, immune activation; RP, responders with hepatitis B surface antigen seroconversion.

levels were closely associated with liver damage in patients with chronic hepatitis B (31); meanwhile, IL-33 could enhance humoral immunity against HBV infection through activating T follicular helper cells (32). In the current study, in order to investigate the effect of IL-33 on T-cell subsets, PBMCs were isolated from patients with HBV at the IT stage (since the frequency of CD8+ TEM was already at a very high level in the IA phase), and were stimulated with 50 ng/ml IL-33 for 0, 2, 6 and 12 h respectively. As presented in Fig. 3A, the percentages of CD4+ TN, TCM and TEM cells were not significantly altered obviously following stimulation with IL-33. The frequency of CD8+ TCM also seemed not to be influenced by IL-33, however the proportion of CD8+ TEM was reduced while CD8+ TN increased over time of IL-33 treatment. The concentrations of Th2-secreting cytokines were additionally upregulated along with the duration of IL-33 treatment, which indicated a trend towards Th2 response (Fig. 3B). These results demonstrated that IL-33-induced IT may be mediated by the modulation of CD8+ TN and CD8+ TEM cells.

The interaction between IL-33 and HBx. As a key risk factor involved in HBV chronic infection, the HBx protein is able to bind directly to DNA and perform transcriptional activation. HBx has been demonstrated to accelerate the progress of HCC in numerous aspects, including involvement in apoptosis, proliferation, inflammation, angiogenesis, immune responses and multi-drug resistance (33,34). In order to investigate whether IL-33 could be regulated by HBx, a vector containing HBx-gene-silencing shRNA was constructed, and it was then transferred into HepG2.2.1.5 cells. Subsequently, the expression of IL-33 was detected in HepG2.2.1.5 and HBx-deficient HepG2.2.1.5 cells, respectively. The expression levels of IL-33 were observed to be at a higher level in HepG2.2.1.5 than in HBx-deficient HepG2.2.1.5 cells at both transcriptional and translational levels (Fig. 4A). To clarify whether IL-33 could additionally influence HBx, the ST2 blocking antibody was used in order to antagonize IL-33-mediated signaling transduction. As presented in Fig. 4B, although the levels of HBx exhibited certain changes in the

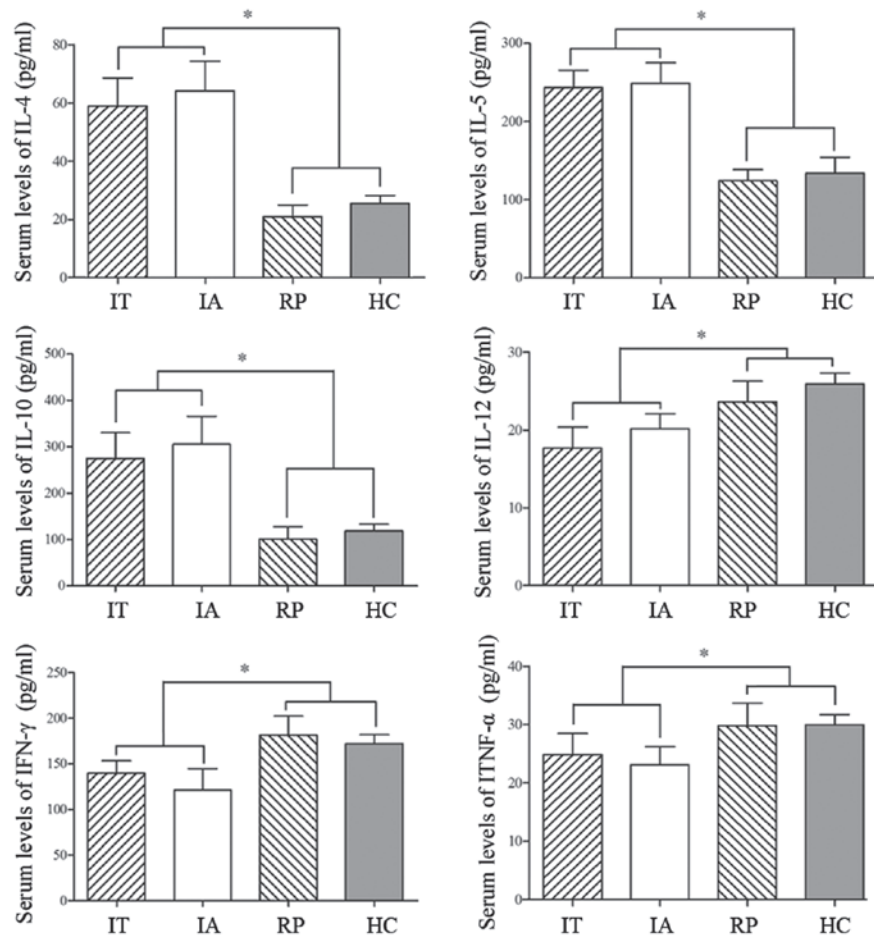


Figure 2. The immune state in HBV patients at different immune stages. Venous blood (10 ml) from patients with HBV and HCs was separated to detect inflammatory factors using ELISA. The levels of IL-12, IFN- γ and TNF- α indicated a Th1-mediated immune response, while IL-4, IL-5 and IL-10 are associated with the Th2-mediated immune response. The data are expressed as the mean \pm standard deviation. * $P < 0.05$. HBV, hepatitis B virus; IL-12, interleukin 12; IFN- γ , interferon γ ; TNF- α , tumor necrosis factor α ; Th, T helper; IT, immune tolerance; IA, immune activation; RP, responders with hepatitis B surface antigen seroconversion; HC, healthy controls.

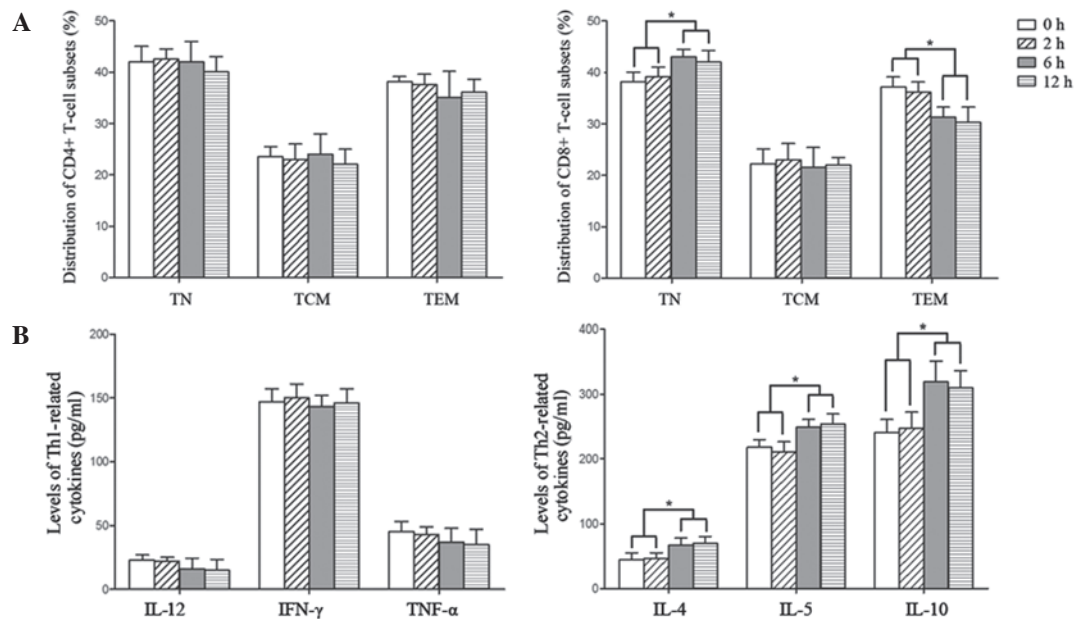


Figure 3. The frequency changes of T-cell subsets induced by IL-33. PBMCs from healthy controls were isolated and stimulated with 50 ng/ml IL-33 for 0, 2, 6 and 12 h. (A) Significant increases in CD8+ TN and reductions in CD8+ TEM were observed following IL-33 stimulation for 6 h. (B) Expression of Th2-associated cytokines were increased while Th1-associated factors were reduced as a result of IL-33 treatment. The data are presented as the mean \pm standard deviation. * $P < 0.05$. IL-33, interleukin 33; PBMC, peripheral blood mononuclear cells; TN, naïve T cells; TCM, central memory T cells; TEM, effector memory T cells; Th, T helper; IFN- γ , interferon γ ; TNF- α , tumor necrosis factor α .

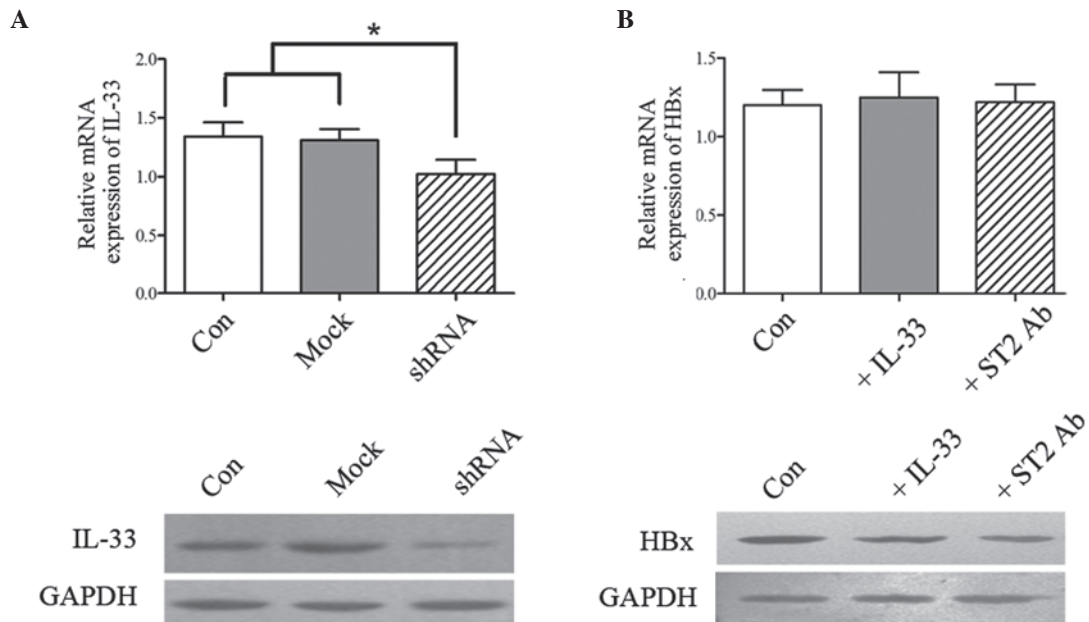


Figure 4. The interaction between IL-33 and HBx protein. (A) Total RNA from HepG2.2.1.5 and HBx-deficient HepG2.2.1.5 were isolated to detect the level of intracellular IL-33 at a transcriptional level by quantitative polymerase chain reaction, while these cells cultured *in vitro* were harvested to analyze expression of IL-33 at a translational level by western blot analysis. (B) To clarify whether HBx was influenced by IL-33, the expression of HBx was tested in IL-33-stimulated and ST2 antibody-blocked HepG2.2.1.5 cells at transcriptional and translational levels using the above-mentioned methods. Con, HepG2.2.1.5; Mock, HepG2.2.1.5 transfected with empty plasmid; shRNA, HepG2.2.1.5 transfected with shRNA targeted HBx. * $P < 0.05$. IL-33, interleukin 33; HBx, hepatitis B virus x protein; ST2, suppression of tumorigenicity 2; shRNA, short hairpin RNA; Con, control.

IL-33 stimulating and ST-2 blocking groups when compared with the untreated group, the differences had no statistical significance. These data demonstrated that HBx may directly regulate IL-33, however, IL-33 was not a key factor to affect the expression of HBx. HBx production is clearly modulated by more complex cell signaling networks, which remain to be fully elucidated.

Discussion

HBV-induced systemic IT generally inhibits the innate or adaptive immune response, resulting in a life-long chronic viral infection (6-9). In the current study, the distributions of TN, TCM and TEM cells were initially investigated in patients with HBV at the IA, IT and RP immune stages, respectively. Neither CD4⁺ TN nor CD8⁺ TN were identified to be associated with the immune phase in participants. Meanwhile, although the proportions of CD4⁺ TCM and CD4⁺ TEM were greater in IT, IA and RP than in HC, there were no significant alterations within the three patient groups. The phenomenon that there were no clear changes of CD4⁺TN/TCM/TEM cells may be associated with the various compositions of Th cells, including immune-promoted CD4⁺ T cells and immune-suppressive types such as Tregs. The integrated effects of these Th subsets may lead to the frequency of stable CD4⁺ T cells in patients with HBV at different immune stages. By contrast, although the frequencies of CD8⁺ TCM and CD8⁺ TEM cells were identified to be increased in the IA and IT groups, these high levels could be restored in patients at the RP stage who had received clinical treatment. The percentage of CD8⁺ TEM cells was greater in IA when compared with IT, and it was

reduced to a lower level in the RP group compared with the HC group. This indicated that CD8⁺ TEM, rather than other naïve and memory CD4⁺ or CD8⁺ T subsets, may be a more sensitive marker to evaluate the HBV activation and the effect of clinical therapy. Furthermore, the levels of IL-4, IL-5 and IL-10 were detected, which represented the Th2 immune response, in addition to IL-12, IFN- γ and TNF- α , which are highly expressed in the Th1 response. The data demonstrated that the IFN- α based therapy resulted in a marked reversal of the dominant Th2 response, and an increase in the Th1-associated factors, resulting in a Th2 to Th1 shift.

Although there previous studies have focused upon the role of IL-33 in mediating the Th2-associated immune response, its effect on different T-lymphocyte subsets remains unclear. Thus, in the current study, PBMCs were stimulated with IL-33. The main effect of IL-33 identified was the regulation of CD8⁺ TEM and CD8⁺ TN cells, with little influence on CD4⁺ T subsets and CD8⁺ TCM cells. Considering the Th1 to Th2 shift induced by IL-33, it was concluded that IL-33-mediated IT partially resulted from the imbalance of CD8⁺ TN and CD8⁺ TEM, particularly the inhibition of the main cytotoxic lymphocytes, the CD8⁺ TEM cells.

Considering HBx was a key transcriptional activator in HBV, the association between IL-33 and HBx was further investigated. The level of IL-33 was demonstrated to be directly diminished subsequent to silencing of the HBx gene. However, the expression of HBx was not significantly altered following IL-33 treatment or the blocking of IL-33/ST2 signaling. Thus the IT induced by HBV may be associated with the effect of HBx promoting IL-33, which has been recognized as an inducer of Th2. Furthermore, the expression of HBx appears

to act independently of IL-33, however requires additional cytokines.

In conclusion, the current study identified that HBV-induced IT may be mediated via the regulation of IL-33 through HBx, leading to the imbalance of CD8⁺ TEM and CD8⁺ TCM cells, in addition to a Th2-dominant response. Thus, it is suggested that clinical therapy targeting IL-33 may be a potential method to enhance the immune response of patients with HBV. Further longitudinal studies focussing upon the immunosuppression caused by HBV and the associated inflammatory cytokines should be conducted in order to further understand these processes.

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