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TIPE2 protein negatively regulates HBV-specific CD8$^+$ T lymphocyte functions in humans

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ABSTRACT

Cytotoxic T cell-mediated killing of virus-infected hepatocytes is an important pathogenic process of hepatitis B. However, its underlying molecular mechanisms are not fully understood. TNFAIP8L2 (TIPE2) is a newly described anti-inflammatory protein that is essential for maintaining immune homeostasis. In this study, we found that the protein levels of TIPE2 in PBMCs of hepatitis B patients were significantly reduced and negatively correlated with the sera values of aminotransferases. Importantly, TIPE2 protein was downregulated preferentially in cytotoxic CD8$^+$ T cells, not CD4$^+$ helper T cells. The CD8$^+$ T cells with low TIPE2 expression were more activated and produced higher levels of perforin, granzyme B, and IFN-γ. As a result, their cytolytic activity was markedly enhanced. Interestingly, HBc_{18-27} peptide stimulation could reduce TIPE2 expression in PBMCs. These results indicate that TIPE2 plays an important role in regulating HBV-specific CD8$^+$ T cell functions in patients with hepatitis B.

1. Introduction

TIPE2, the tumor necrosis factor (TNF)-α-induced protein 8-like 2 (also known as TNFAIP8L2), is a newly identified protein of the TNFAIP8 (a.k.a TIPE) family. It plays an essential role in maintaining immune homeostasis by negatively regulating T cell receptor and Toll-like receptor (TLR) signaling (Sun et al., 2008, 2012). TIPE2 was originally identified as an inflammation-related gene which is expressed primarily by lymphoid and myeloid cells (Sun et al., 2008; Zhang et al., 2009, 2010a,b). Recent studies revealed that TIPE2 is a negative regulator of not only inflammation but also carcinogenesis (Sun et al., 2008; Gus-Brautbar et al., 2012). TIPE2 deficiency in mice causes fetal inflammatory diseases (Sun et al., 2008) and its abnormal expression in humans is associated with hepatocellular carcinoma (Gus-Brautbar et al., 2012; Cao et al., 2013), diabetic nephropathy (Zhang et al., 2010a,b), stroke (Zhang et al., 2012), and atherosclerosis (Zhang et al., 2013; Lou et al., 2013).

Hepatitis B virus (HBV) infection is a worldwide problem that can cause hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The molecular mechanisms underlying the HBV-induced pathogenesis are not fully understood. Recent studies suggest that HBV is not directly cytopathic for the infected hepatocytes, but the host immune response plays a critical role in the outcome of HBV infection (Ferrari et al., 1990; Penna et al., 1997; Rehermann et al., 1995). It is now established that HBV-specific CD8$^+$ T cell response plays a fundamental role in viral clearance and the pathogenesis of liver disease, either by direct killing of infected hepatocytes or cytokine-mediated noncytolytic mechanisms (Nayersina et al., 1993; Missale et al., 1993; Phillips et al., 2010). Our previous studies showed that the levels of TIPE2 mRNA in peripheral blood mononuclear cells (PBMC) of chronic hepatitis B patients were downregulated significantly compared to normal controls and negatively correlated to sera levels of ALT, AST, and HBV DNA (Xi et al., 2011), suggesting that TIPE2 plays an important role during HBV infection by regulating cellular immunity. However, it is unclear which PBMC cell type downregulates TIPE2 in chronically infected patients. We report here that downregulation of TIPE2 in CD8$^+$ cytotoxic T cells but not CD4$^+$ helper T cells are associated with HBV-induced inflammation.

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2. Materials and methods

2.1. Mice

All experiments with animals were performed according to the guidelines of the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001) and were approved by the Animal Ethical Committee of Shandong University. C57BL/6 mice were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Science. HBV transgenic C57BL/6 mice were purchased from Air Force General Hospital of Guangzhou (China). TIPE2 deficient C57BL/6 mice (*TIPE2*$^{-/-}$) were generated as previously described (Sun et al., 2008). All the animals were kept in Shandong University Laboratory Animal Center under pathogen-free conditions and were used for experiments at about 8–10 weeks of age.

2.2. Human specimens

A total of 55 hepatitis B patients with a mean age of 37 years who were admitted to the Shandong Provincial Hospital between February 2012 and July 2013 were studied. Twenty two healthy individuals were used as controls. The diagnosis of hepatitis was made in accordance with the criteria approved by the Third National Conference of Infectious and Parasitic diseases held in Beijing in 1995. Routine laboratory methods were used to determine serum alanine aminotransferase (ALT) and aspartate aminotranseferase (AST) levels. The HBV DNA level was measured by real-time PCR. Clinical characteristics of hepatitis B patients were summarized in Table 1. All procedures were preapproved by the Institutional Review Board (IRB) of the Shandong University.

2.3. PBMCs and HBV-specific CD8$^+$ T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from CHB (chronic hepatitis B) patients or healthy controls by density gradient centrifugation using ficoll–hypaque solution (Sigma, Saint Louis, USA). HBV core antigen 18–27 epitope (HBc_{18-27}, FLPSDFFPSV) was used as the immunodominant epitope (Webster et al., 2004). PBMCs cultured at 3×10^5 well^{-1} in supplemented RPMI 1640 (Gibco, Invitrogen Coperation, USA) with 10% human AB serum (Sigma), were stimulated with 10 μg/ml synthetic HBc_{18-27} (Shanghai Qiangyao Bio-technology Company, Shanghai, China). IL-2 (PeproTech, Rocky Hill, USA) was added to a final concentration of 30 U/ml on days 3 and 6. After 10 days, cells were harvested and tested.

2.4. Human hepatoma cell line 2.2.15 culture

The human hepatoma cell line 2.2.15, derived from HepG2 cells, is stably transfected with HBV DNA genome and can secret viral Ags and infectious virions (Sells et al., 1988). For cell culture, 2.2.15 cells were seeded in 24-well plates (Corning Incorporated, USA) under positive selection with 0.4 mg/ml G418 (Invitrogen, California USA) in DMEM (Gibco, Invitrogen Coperation, USA) containing 10% FCS (Gibco), 100 μg/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma).

Table 1
Clinical characteristics of hepatitis B patients.

Hepatitis B patients		
Total number	55	
Age in years (range)	37 ± 14.87 (12–61)	
Male/female ratio	45/10	
ALT levels	<47 (n = 12)	35.14 ± 7.946 (23–45)
	>47 (n = 43)	298.3 ± 360.8 (60–1563)
AST levels	<47 (n = 12)	36.29 ± 8.939 (24–47)
	>47 (n = 43)	182.9 ± 178.5 (66–880)

2.5. Cytotoxicity of human HBV-specific CD8$^+$ T cells

PBMCs were adjusted to 6×10^5 ml^{-1} in RPMI1640 medium contained 10% human AB serum and stimulated with 10 μg/ml synthetic HBc_{18-27} peptide. 30 U/ml hrIL-2 was added every three days. Cells were cultured at 37 °C, 5% CO_2 for 10 days, and were used as effector cells.

HBc_{18-27} peptide stimulated CD8$^+$ T cells (effectors cells) were added to the confluent 2.2.15 cells (target cells). They were then cocultured for 24 h using an E:T ratio of 50:1. As the controls, 2.2.15 cells were cultured alone, and CD8$^+$ T cells were cultured without HBc_{18-27} peptide. Cytotoxicity assay was performed using 96-well plates (Corning). The results were expressed as OD450 values which were measured using CCK-8 kit (Dojindo, Japan) according to the manufacturer's instructions. The percentage of cytotoxicity was calculated using the following equation: percentage of cytotoxicity = [1 − (OD450 value of cocultured cells − OD450 value of effector cells)/OD450 value of target cells] × 100%.

2.6. Hepa 1–6 cell culture and transfection

Hepa 1–6 cells (purchased from the Type Culture Collection of the Chinese Academy of Science, Shanghai, China) were cultured in 24-well plates (Corning) in RPMI-1640 (Gibco) medium with 10% fetal bovine serum (Gibco), 100 μg/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) in an incubator at 37 °C containing 5% CO_2 for 24 h. Cells were transfected with recombinant plasmid pcDNA3-HBV1.1 (Xi et al., 2011) and used as the target cells for cytolytic killing experiments. cDNA of HBcAg was detected by RT-PCR to determine transfection efficiency. Qiagen Plasmid Maxi Kit (Qiagen, Hamburg, Germany) was used to extract and purify pcDNA3-HBV1.1.

2.7. Cytolytic activity of HBV-immunized splenic lymphocytes

TIPE2-deficient mice and WT controls were immunized twice with pcDNA3-HBV1.1 by injecting into tibialis anterior muscle. Mice were sacrificed two weeks post-injection. The spleens were isolated to harvest HBV-sensitized lymphocytes. The lymphocytes (effectors cells) were added to Hepa 1–6 cells transfected with pcDNA3-HBV1.1 (target cells) using an E:T ratio of 50:1 and cocultured for 24 h. Cytotoxicity assay was performed using CCK-8 kit using method the same as described above.

Furthermore, HBV-immunized splenic lymphocytes from *TIPE2*$^{-/-}$ mice or WT controls were transferred into HBV transgenic mice to induce liver injury (Gao et al., 2004). Liver specimens were fixed in 10% formalin solution and embedded sections were stained by hematoxylin–eosin (HE) to determine tissue inflammation (Gao et al., 2004; Xi et al., 2011).

2.8. Flow cytometry

For TIPE2 expression, peripheral blood from hepatitis B patients and healthy controls were stained firstly with FITC-conjugated anti-human-CD4 Ab (eBioscience) and PE-conjugated anti-human-CD8a Ab (eBioscience) for 20 min at 4 °C. Subsequently, the cells were permeabilized and fixed with cytopern (eBioscience) for 20 min at 4 °C, stained with mouse anti-human-TIPE2 antibodies (Sigma Aldrich, primary antibody). After washing, the cells were stained with Alexa Fluor 647-conjugated donkey anti-mouse-IgG(H + L) (Invitrogen, secondary antibody), and then analyzed by flow cytometry. The frequency or MFI of CD8$^+$ T or CD4$^+$ T cells which express TIPE2 were determined. The correlations between the levels of