Inhibitory Function of Tregs via Soluble FGL2 in Chronic Hepatitis B

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Summary: CD4⁺CD25⁺CD127dim⁻ regulatory T cells (Tregs) have been implicated in suppressing T cell immune responses to hepatitis B virus (HBV), but the inhibition mechanism has not being clear yet. This study investigated the effects of soluble FGL2 (sFGL2) secreted by Tregs on immune suppression in chronic HBV-infected patients. We verified that sFGL2 protein and mRNA were highly expressed in Tregs. The separated Tregs by using magnetic beads from peripheral blood mononuclear cells (PBMCs) in 20 patients with chronic hepatitis B were co-cultured with PBMCs at a ratio of 1:3 with anti-CD3 stimulating antibody or FGL2 blocking antibody. The proliferation index of CD8⁺T cells after blocking FGL2 was higher than that in blank group (3.58±0.18 vs. 3.28±0.17, P=0.034) in 18 of 20 samples, and lower than that in CD3 stimulation group (3.82±0.19, P=0.026) in 16 of 20 samples. The IFN-γ secreted in the mixed culture in the absence of Tregs was higher than that in the culture in the presence of Tregs, but it could be abolished by FGL2 blocking antibody. These results suggest that sFGL2 protein secreted by Tregs suppresses the proliferation and function of CD8⁺T cells in chronic hepatitis B.

Key words: soluble FGL2 protein; regulatory T cells; CD8⁺ T cells; chronic hepatitis B

Chronic hepatitis B (CHB) is a common and serious infectious disease of the liver caused by hepatitis B virus (HBV). About 350 million patients worldwide are chronically infected and become HBV carriers. 10% of adults and 90% of children become persistent HBV carriers after the infection and 1-2 million people died annually as the consequence of infection with the virus, such as liver cirrhosis and hepatocellular carcinoma[1, 2]. The frequency of both circulating and liver CD4⁺CD25⁺ regulatory T cells (Tregs) is increased in CHB patients, indicating that they play an important role in viral persistence by modulating virus-specific immune responses. Circulating CD4⁺CD25⁺ Tregs have been implicated in suppressing CD8⁺ T cell immune responses to viral infections including HBV[3-8].

FGL2/fibroleukin is a member of the fibrinogen-related protein superfamily[7, 8]. The protein exists as both a type II transmembrane protein on the surface of macrophages and endothelial cells and a soluble protein constitutively secreted by both CD4⁺ and CD8⁺ T cells[9]. Membrane bound FGL2 (mFGL2) has been shown to play an pivotal role in innate immunity as an immune coagulant expressed by activated reticuloendothelial cells (macrophages and endothelial cells)[10-12] and has been implicated in the pathogenesis of several inflammatory disorders, including viral hepatitis[13-16], allo- and xenograft rejection[17-19], and cytokine-induced fetal loss[20, 21]. In the mouse model, soluble FGL2 (sFGL2) can be secreted by Foxp3⁺ Tregs and contributes to Foxp3⁺ Treg cells immunoregulatory function of suppressing T cells proliferation and bone marrow-derived dendritic cells maturation through FGL2-FcgammaRIIB pathway[9]. In the murine autoimmune disease model sFGL2 contributes to Tregs activity and inhibits the development of disease. In human, previous study also demonstrated that patients with chronic HCV infection had significantly higher plasma levels of sFGL2 than healthy controls and patients with inactive end-stage alcoholic cirrhosis[20]. In addition, previous studies have confirmed that Tregs can express FoxP3⁺ molecules and have suppressive function of human CD4⁺ Tregs[27].

Based on previous studies, we propose the hypothesis that sFGL2 protein is one of the key factors secreted by circulating Tregs and suppressing proliferation and activity of CD8⁺ T cells in CHB, and Tregs or sFGL2 may be one of the potential predictive molecules to evaluate the effect of antiviral therapy and phase of immune status in CHB patients in the immediate future.

1 MATERIALS AND METHODS

1.1 Patients and Peripheral Blood Mononuclear Cells

Peripheral blood from 20 patients with CHB was collected with informed consent. The study was approved by the Ethics Committee of Tongji Medical Col-
1.4 Proliferation of CD8+ T Cells

In order to analyze proliferation index (PI) of CD8+ T cells, total PBMCs and PBMCs depleted of Tregs were labeled with CFSE (Invitogen, USA) according to the manufacturer’s instructions. The cells were washed with RPMI-1640 supplemented with 10% FBS and parts of them were stained with PE-anti human CD8a (Biologend, USA) and analyzed by flow cytometry to set the primary fluorescence intensity of parent generation, while the rest were seeded in U-bottomed 96-well plates and allowed to grow for 5 days in RPMI-1640-10% FBS media (methods as follows). The cells were stained by PE-anti CD8 and then collected for flow cytometry and the PI was determined using the Becton-Dickinson ModFit software.

Total PBMCs, PBMCs depleted of Tregs and Tregs+PBMCs (at a ration of 1:3) were separately cultured with 1 μg/mL anti-CD3 antibody (CD3 stimulation group), 5 μg/mL FGL2 antibody (FGL2 blocking group) and blank (blank group). The cells in a density of 5×10^5 to 1×10^6 cell/well were cultured with RPMI-1640-10% FBS including recombinant IL-2 (R&D Systems, USA) (2 U/well) in the U-bottomed 96-well plate. Each group had 2 duplicates. After culture for 2 days, the culture supernatants were collected for detecting interferon (IFN)-γ level by using ELISA, then fresh RPMI-1640-10% FBS medium was added for further culture, and the stimulants were added as before. After culture for 5 days, we collected the cells for CD8+ T cell proliferation analysis by using flow cytometry (BD FACS Canto II).

1.5 Real-Time Quantitative RT-PCR and Western Blotting for FGL2 Expression

Total RNA was isolated using TRIZOL reagent (Invitrogen, USA) according to the manufacturer’s instructions. cDNA was synthesized from total RNA using First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, USA). Real-time PCR was performed with a SYBR green quantitative RT-PCR kit (Sigma, USA). The PCR primers were as follows: FGL2 (169 bp), forward (F), 5′ACTGTGACATGGACGACATG3′, reverse (R), 5′CTTACTTTGCTGAGAAG3′; β-actin (180 bp), F, 5′CTTACGAAACTGGAAAACG3′, R, 5′AAGCCACCTGACTAAGATG3′. All PCRs were run in triplicate. Specificity of PCR was verified by melting curve analysis and agarose gel electrophoresis. The mRNA levels were detected by using the comparative threshold cycle (Ct) method and normalized to β-actin[28].

1.6 Detection of IFN-γ Level via ELISA

The IFN-γ levels in the culture supernatants in CD3 stimulation group, FGL2 blocking group and blank group were detected by using ELISA kit (Dakewe, Beijing)[29]. Standard cure was drawn according to the standard concentration dilution and corresponding absorbance (A) value.

1.7 Statistical Analysis

Data of CD8+ T cell proliferation and FGL2 expression were expressed as $\bar{x} \pm s$, and analyzed by unpaired Student’s t-test. The SPSS statistical software program was used to test correlations between quantitative variables by the establishment of non-parametric linear regression. The level of significance was set at $P<0.05$. 