

Original Article

Anti-CD200 attenuates concanavalin A induced hepatitis via modulating the imbalance of CD4⁺ T lymphocyte differentiation in mice

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Abstract: Hepatitis occurs in critical ill patients with bad morbidity and mortality. It is known that imbalance of Th1 and Th2 lymphocytes differentiations plays a key role in its mechanisms. Recent studies indicated that type 1 membrane glycoprotein CD200 serves as co-inhibitory molecule, negatively regulating the immune response. In regard of this, we used Concanavalin A (Con A) induced liver injury model to research the effect of CD200 on the differentiation of CD4⁺ T lymphocyte and found that the expression of CD200 on CD4⁺ T was significantly higher in hepatitis mouse. The apoptosis of CD4⁺ T cell in Con A induced liver injury was significantly attenuated by anti-CD200. The concentration of soluble IL-2 and IFN- γ was reduced by anti-CD200, in addition, the expression of T-bet, GATA3 and FoxP3 mRNA were all attenuated by anti-CD200. The phosphorylation of SH-2 containing inositol 5' polyphosphatase 1 (SHIP1) was significantly increased in Con A induced liver injury and reduced by anti-CD200. We hypothesized that, anti-CD200 inhibited the phosphorylation of SHIP1, the expression of T-bet, GATA3 and FoxP3 mRNA and CD4⁺ T differentiation to protect the liver from autoimmune hepatitis.

Keywords: CD200, Hepatitis, CD4⁺ T lymphocyte differentiation, mice

Introduction

Hepatitis is a chronic disease that impacts on morbidity and mortality of patients. Autoimmune hepatitis (AIH) is a rare liver disease caused by an auto-reactive immune response against the patient's own liver [1]. AIH may start with an episode of acute hepatitis but usually runs a chronic course. A proportion of patients are kept at an individualized low dose maintenance therapy or even with liver transplanted. In studying AIH, Con A induced hepatitis is a well-established mouse model of immune-mediated liver injury [2], in addition, the model is well-established for investigating T cell dependent liver injury in mice, which closely mimics the pathogenic mechanisms and pathological changes of patients with AIH [3, 4]. The Con A administration could provoke T cells activated and result in production and secretion of series of inflammatory cytokines which will exacerbate the recruitment and activation of immune

cells infiltrating, leading to severe hepatitis [5]. CD200 is a type-1 transmembrane with potent immunosuppressive function through interaction with its receptor, which had been found in several autoimmune or inflammatory associated disorders and diseases [6]. It had been reported that CD200 played an important role in regulating immune tolerance and inflammatory responses [7, 8]. Therefore, the aim of the present study was to investigate the immunomodulatory effect of CD200 and explore its potential mechanisms in the murine model of Con A induced hepatitis.

Material and methods

Chemicals and reagents

CD200 and anti-CD200 were purchased from Shanghai Wanyi Medicine Science and Technology Development Co., Ltd. (Shanghai, China). Con A was purchased from Solarbio Corporation (Beijing, China). All of the other chemicals

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and reagents were standard commercially available biochemical quality. Deionized water was purified with a Milli-Q purification system and was used to prepare all solutions.

Animals

C57BL/6 male mice (aged 6-8 weeks; 20-25 g) were obtained from the Animal Experimentation Center of Fudan University (Shanghai, China). Mice were housed under specific pathogen-free condition and provided a standard laboratory chow and water freely one week before experiment. All experiments were performed in accordance with the guidelines of Institutional Animal Ethics Committee of Fudan University (Shanghai, China).

Experimental design

Mice were administrated with normal saline (100 μ L) or anti-CD200 (5 μ g/kg, 100 μ L) through the tail vein according to the groups, following a dose of Con A (20 mg/kg, 100 μ L) intravenously in Con A group and anti-CD200 group 1 h later, respectively. The protocol methods and the antibody of anti-CD200 were carried out following previous studies [9]. All dosages were determined by preliminary experiments. Blood and liver tissue were harvested 12 h after Con A administration.

Liver function and cytokines assay

Twelve hours after Con A administration, mice were anesthetized by sevoflurane and blood was collected via cardiac puncture into heparinized syringes and centrifuged, and plasma samples were separated after centrifugation at 300 g for 5 min. Levels of ALT and AST were measured by automatic dry biochemical analyzer (Hitachi Auto Analyzer 7170, Japan). The concentrations of IL-2, and IFN- γ , were detected by using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D system, USA).

Histopathology assay

Liver tissues were harvested 12 h after Con A administration intravenously. Liver samples were fixed in 4% buffered paraformaldehyde for at least 24 h. Sections (4-5 μ m) on slides were deparafnized in xylene, rehydrated in decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E). All sections were graded blindly by three pathologists under light

microscopy according to the following criteria: 0, none; 1, individual cell necrosis; 2, \leq 30% lobular necrosis; 3, \leq 60% lobular necrosis; 4, >60% lobular necrosis [5].

RNA isolation and real-time PCR analysis

Total RNA was isolated from the homogenate of the liver with Trizol reagent (Invitrogen) at 12 h after Con A stimulated. Cellular RNA was treated with DNase I and then primed with a dT oligonucleotide and reverse transcribed with Superscript II. For real-time assays, PCR reactions were prepared in SYBR Green PCR Master Mix. DNA targets were amplified and analyzed with a Chromo Real-Time PCR Detection System (Bio-Rad Life Sciences). The murine primer sequences are shown as follows.

Mouse Foxp3 (Forward, F): 5-CCAGCTCTACTCTGCACCTT-3 and (Reverse, R): 5-GCCTTGCCCTTCTCATCCAG-3, Tbet-F: 5-TCCTTGGATCCTTCGCCTAC-3, R: 5-ACTCTCAGCTTCCCAGACAC-3, GATA3, F: 5-ACATCGATGGTCAAGGCAAC-3, R: 5-GTGGATGGACGTCTTGGAGA-3, IL-2, F: 5-AGCAGCTGTTGATGGACCTA-3, R: 5-AAATCCAGAACATGCGCAG-3, IFN- γ , F: 5-TTCTTCAGCAACAGCAAGGC-3, R: 5-ACTCCTTTTCCGCTTCTCTGA-3. Total RNA was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first-strand using reverse transcription system. Reverse transcription was carried out as follows: 42°C for 60 min, 70°C for 10 min, and first-strand cDNA was stored at -20°C. Real-time PCR was performed in a 20 μ L of reaction solution containing SYBR Premix Ex Taq, primers, and cDNAs. The cycles for PCR were as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 20 s. Melting curves were determined by heat-denaturing PCR products over a 35°C temperature gradient at 0.5°C/s from 65 to 99.5°C. GAPDH was used as an internal control [10]. The relative amount of mRNA was determined using the $\Delta\Delta$ CT technique as described previously [11]. The levels of mRNA were expressed as fold-changes after normalization to GAPDH.

Western-blotting analysis of SHIP

Livers were carefully excised and homogenized into lysis buffer (Termo, USA) to yield a homogenate. After centrifugation (12000 g for 10 min) at 4°C, protein concentration was detected by Bradford protein assay kit (Termo, USA) with bovine serum albumin as standard. Equal

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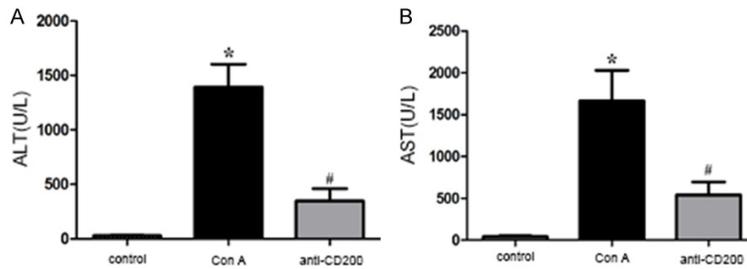


Figure 1. Anti-CD200 ameliorates liver damage following Con A administration. Plasma was separated after centrifugation at 300 g for 5 min. Levels of ALT and AST were measured by automatic dry biochemical analyzer. Both ALT (A) and AST (B) were increased significantly in Con A group and reduced by anti-CD200 (n=12 mice per group); *P<0.01 compared to control group, #P<0.01 compared to Con A group.

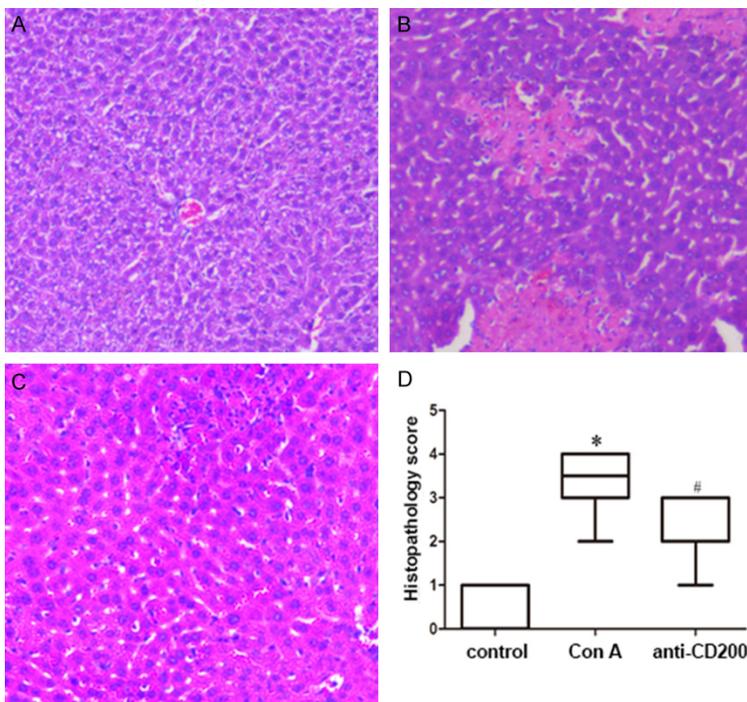


Figure 2. Histopathological changes of livers in mice. Liver tissues were harvested 12 h after Con A administration intravenously. Liver samples were fixed in 4% buffered paraformaldehyde for at least 24 h. Sections (4-5 μ m) on slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E). All sections were graded blindly by three pathologists under light microscopy according to the following criteria: 0, none; 1, individual cell necrosis; 2, \leq 30% lobular necrosis; 3, \leq 60% lobular necrosis; 4, >60% lobular necrosis. A-C represented the microscopic appearance of liver tissue in control, Con A and anti-CD200 group. D represented the severity of liver injury scored by three blinded Pathologists. *P<0.01 compared to control group, #P<0.01 compared to Con A group.

amounts of protein extracts separated discontinuously onto 10% polyacrylamide gels (Life Technologies, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (Life Technologies). After blockade of nonspecific binding

sites, membranes were incubated with various antibodies against SHIP1 (Cell Signaling Technology, Danvers, MA, USA) for 2 h at room temperature. Membranes were developed by chemiluminescence using an Amersham prime ECL Plus detection system (ChemiDoc-I_t 600 Imager, Ultra-Violet Product Ltd, UK). Signals were densitometrically assessed and normalized to the GAPDH signals (Image J, NIH) [5].

Flow cytometry analysis

Single-cell suspensions were obtained 12 h after Con A administration. Cells were then stained with fluorescencelabeled antibody (anti-CD4 APC, code: 17-0041-82; clone: GK-1.5, eBioscience USA); anti-CD200 PE, code: 11-0081-85; clone: 53-6.7, eBioscience USA). The counts of CD4⁺ T lymphocytes infiltrating in the liver were analyzed by flow cytometry (Miltenyi, Germany).

Statistical analysis

All results were expressed as mean \pm SD. All statistical analyses were performed by Prism 6.0 (GraphPad Software, USA). All comparisons (such as cell counting, cytokines) among groups were performed by one-way analysis of variance (ANOVA). P<0.05 was regarded as statistically significant.

Results

Anti-CD200 ameliorates the injuries of Con A-induced hepatitis

The experimental hepatitis was established by Con A on C57BL/6 male mice. Liver function was measured by the plasma levels of ALT and AST in the present study. Compared with control group, ALT and AST levels were extremely

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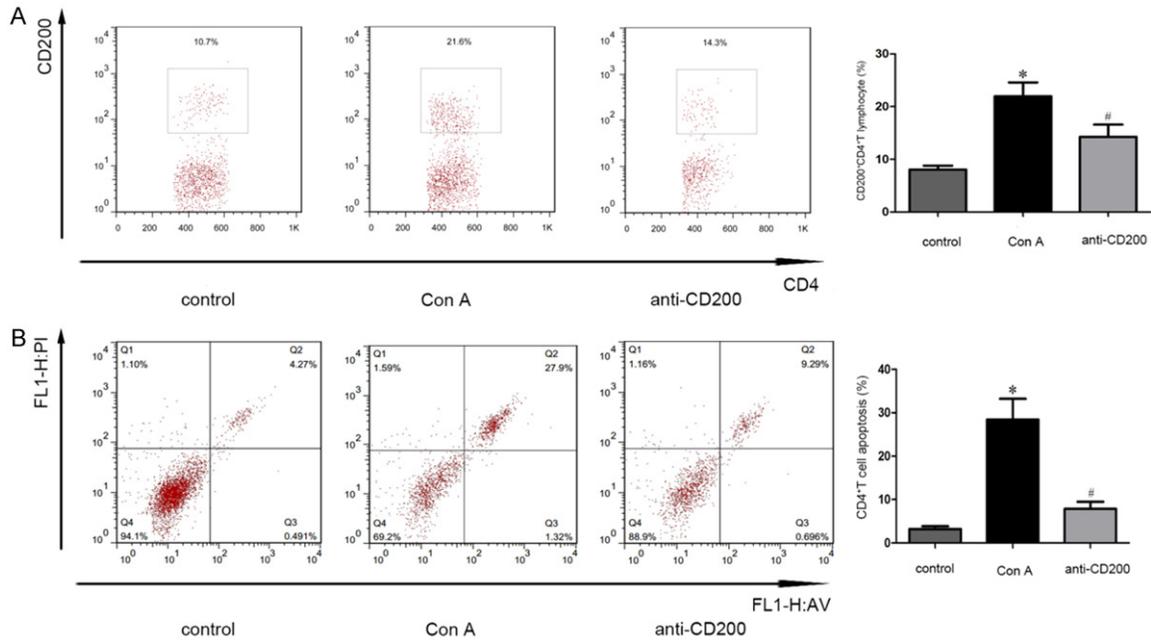


Figure 3. The expression of CD200 on CD4⁺ T lymphocyte and the apoptosis of CD4⁺ T lymphocyte in hepatitis. Single-cell suspensions were obtained 12 h after Con A administration. Cells were then stained with fluorescencelabeled antibody. The counts of CD4⁺ T lymphocytes infiltrating in the liver were analyzed by flow cytometry. The expression of CD200 on CD4⁺ T lymphocyte was extremely aggrandized in Con A induced hepatitis and alleviated by anti-CD200 ($P<0.01$), the apoptosis of CD4⁺ T lymphocyte was also attenuated by anti-CD200 in Con A induced hepatitis (n=12 mice per group); * $P<0.01$ compared to control group, # $P<0.01$ compared to Con A group).

elevated response to Con A and decreased by anti-CD200 (Figure 1A and 1B).

Morphological analysis of livers was also applied to further confirm the protective effect of anti-CD200 on Con A induced hepatitis. The liver response to Con A was characterized by massive hepatocyte necrosis and disorder of hepatic sinusoids structure (Figure 2B). However, the hepatocyte necrosis and T lymphocyte infiltration were extremely alleviated by anti-CD200 (Figure 2C). The pathological scores calculated by three blinded Pathologists were excessively higher induced by Con A but attenuated by anti-CD200 (Figure 2D).

The relationship between CD200 and CD4⁺ T lymphocyte in hepatitis

Con A induced hepatitis was associated with various CD4⁺ T lymphocyte infiltration in the liver [5]. The expression of CD200 on CD4⁺ T lymphocyte was extremely aggrandized in hepatitis and alleviated by anti-CD200 ($P<0.01$) (Figure 3A). The activation of CD4⁺ T lymphocyte was the crux of the liver function, however, the apoptosis of CD4⁺ T lymphocyte was elevat-

ed excessively. We subsequently found in the present study, the apoptosis of CD4⁺ T lymphocyte was attenuated extremely by anti-CD200 ($P<0.01$) (Figure 3B).

The anti-inflammatory effect of anti-CD200 on Con A induced hepatitis

Various production of proinflammatory cytokines including IL-2 and IFN- γ was the symbol of hepatitis. We also found the elevated levels of IL-2 and IFN- γ induced by Con A and reduced by anti-CD200 in the present study ($P<0.01$) (Figure 4A and 4B). We subsequently investigated the effect of anti-CD200 on the expression of IL-2 and IFN- γ mRNA in the liver. The results demonstrated mRNA levels of IL-2 and IFN- γ were induced significantly higher in Con A group and attenuated by anti-CD200 ($P<0.01$) (Figure 4C and 4D).

Anti-CD200 suppressed phosphorylation of SHIP1

The production of proinflammatory cytokines including IL-2 and IFN- γ was regulated by the differentiation CD4⁺ T lymphocyte depended on

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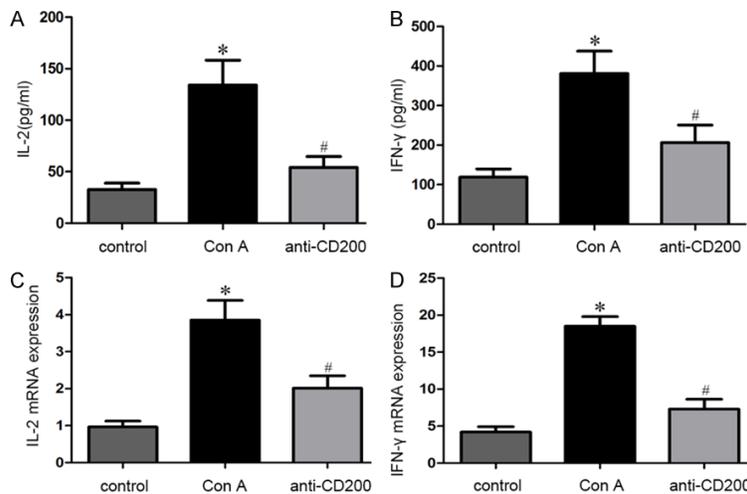


Figure 4. The anti-inflammatory effect of anti-CD200 in Con A induced hepatitis. Single-cell suspensions were obtained 12 h after Con A administration. Cells were then stained with fluorescencelabeled antibody. Total RNA was isolated from the homogenate of the liver with Trizol reagent at 12 h after Con A stimulated. Cellular RNA was treated with DNase I and then primed with a dT oligonucleotide and reverse transcribed with Superscript II. For real-time assays, PCR reactions were prepared in SYBR Green PCR Master Mix. DNA targets were amplified and analyzed with a Chromo Real-Time PCR Detection System. The levels of mRNA were expressed as fold-changes after normalization to GAPDH. The soluble expression and mRNA of IL-2 and IFN- γ were extremely elevated and lowered by anti-CD200 (n=12 mice per group); *P<0.01 versus control group, #P<0.01 versus Con A group.

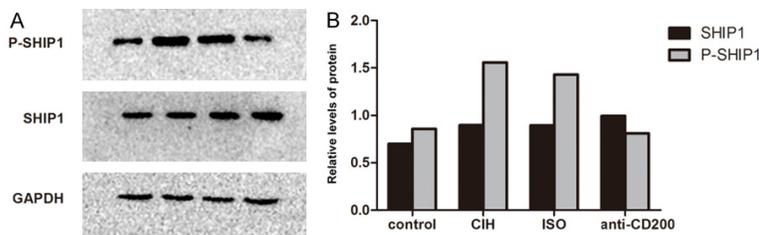


Figure 5. The potential mechanism of CD200 in Con A induced hepatitis. Livers were carefully excised and homogenized into lysis buffer to yield a homogenate. After centrifugation at 4°C, protein concentration was detected by Bradford protein assay kit with bovine serum albumin as standard. Equal amounts of protein extracts separated discontinuously onto 10% polyacrylamide gels and transferred to nitrocellulose membranes. After blockade of nonspecific binding sites, membranes were incubated with various antibodies against SHIP1 for 2 h at room temperature. Membranes were developed by chemiluminescence using an Amersham prime ECL Plus detection system. Signals were densitometrically assessed and normalized to the GAPDH signals. The phosphorylation of SHIP1 was elevated in Con A induced hepatitis and depressed in anti-CD200 group. CIH, Con A induced hepatitis.

the expression of T-bet, GATA3 and FoxP3. Furthermore, the expression of T-bet, GATA3 and FoxP3 was regulated by SHIP1 partly. We then found anti-CD200 depressed the phosphorylation of SHIP1 compared with Con A (Figure 5A). The relative levels of proteins also demonstrated the analogical tendency (Figure 5B).

Anti-CD200 inhibited T cells differentiation via regulating the expression of T-bet, GATA3, and FoxP3

It is well known that the activation of CD4⁺ T lymphocyte was expressed on the differentiation, that were TH1, TH2 and Treg. The differentiation was depended on the expression of T-bet, GATA3 and FoxP3. We found the mRNA expression of T-bet, GATA3 and FoxP3 were suppressed by anti-CD200 in the present study (P<0.01) (Figure 6A-C). Therefore, we speculated the activation of CD4⁺ T lymphocyte was inhibited by anti-CD200.

Discussion

Autoimmune hepatitis is a rare chronic inflammatory disorder [12], little methods could be applied to therapy autoimmune hepatitis and the optimal salvage strategy remains unclear. Alternative immunosuppressive with high-dose of prednisolone are recommended as the front-line salvage therapy [13], however, disease activity in 10-20% of patients were poorly tolerated or controlled [14], and immunosuppressive agents were considered as the second-line therapies for these patients [15, 16]. Anti-CD200 was used to prolong the survival of allografts, which indicated its immunosuppressive activity [17].

Con A induced hepatitis was the best model to simulate autoimmune hepatitis. The severity and pathological changes of liver in Con A group in the present study were extremely altered, which further illuminate the Con A model could mirror the hepatitis. While the immunopathogenesis of AIH is incompletely understood, current opinion is that AIH could develop from an interaction between an envi-

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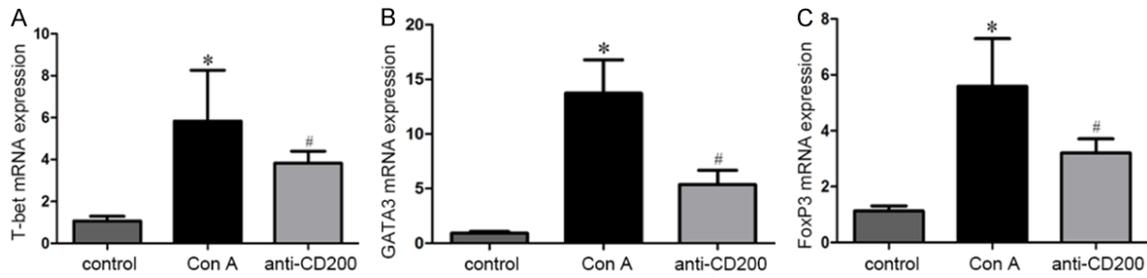


Figure 6. The presentation of CD4⁺ T cells differentiation. Total RNA was isolated from the homogenate of the liver with Trizol reagent at 12 h after Con A stimulated. Cellular RNA was treated with DNase I and then primed with a dT oligonucleotide and reverse transcribed with Superscript II. For real-time assays, PCR reactions were prepared in SYBR Green PCR Master Mix. DNA targets were amplified and analyzed with a Chromo Real-Time PCR Detection System. The levels of mRNA were expressed as fold-changes after normalization to GAPDH. The mRNA expression of T-bet, GATA3 and FoxP3 were inhibited by anti-CD200 in Con A induced hepatitis (n=12); *P<0.01 versus control group, #P<0.01 versus Con A group.

ronment trigger(s) and genetic factors in a generically susceptible host(s) [18, 19]. Regardless of the triggers, initiation of the autoimmune attack is mediated via the presentation of an autoantigenic peptide within a major histocompatibility complex (MHC) molecule by antigenpresenting cells (APCs) to undifferentiated CD4 effector cells [20]. Naïve CD4⁺ T cell differentiation was encouraged with the presence of IFN- γ , while in turn resulted in IFN- γ secretion. In addition, IFN- γ resulted in up regulation of MHC class I and induction of aberrant class II expression by hepatocytes that in turn further exacerbated liver injury via further activation of CD4⁺ T cells [18]. Current evidence indicates that auto-antibody production may contribute to hepatocyte injury via antibody-mediated cellular cytotoxicity and complement activation [21]. CD200 was considered “tolerance signaling molecule” [22], and it was strongly expressed on the surface of lymphocytes [23]. It had been reported that CD200 suppressed secretion of the pro-inflammatory cytokine and enhanced the expression of anti-inflammatory cytokine [24]. This may suggest that modification with CD200 leads to a response that simultaneously prevented inflammation and enhanced phagocytosis. In the present study, the concentration of dissociate IL-2 and IFN- γ were induced extremely higher by Con A, but decreased by anti-CD200. In addition, the expression of T-bet, GATA3 and FoxP3 mRNA were also attenuated by anti-CD200, which indicated that the differentiations of CD4⁺ T lymphocyte were suppressed by anti-CD200 and protected the liver from damaged by autoimmune hepatitis.

It had been well described that Th1 immune response can be correlated with the induction of cellular immunity by secreting cytokines and Th2 immune response controlling humoral immune response through mediating B-cell proliferation, differentiation and production of specific antibodies. Cytokines produced by mature Th cells not only promote their own differentiation, but also inhibit the proliferation of each other. Individuals chronically with AIH were associated with the host immune responses, particularly the cellular immune response [25]. The hepatitis could trigger secretion of Th1 cytokines, which had been considered as a possible approach to blocking or terminating persistent of AIH [26]. Unfortunately, a number of studies have demonstrated that hepatitis patients often had an imbalance of Th1/Th2 function, in which Th1 cells were defective [27-29]. In general, the immunopathogenesis of hepatitis persistence was associated with host immune reactions biased towards Th2 response. However, the mechanism involved in this process was still not clear. SHIP1 signal way played an important role in negatively regulating the activation of immune cells [30], here we demonstrated that SHIP1 was regulated by CD200. In conclusion, hepatitis was induced by the differentiations of CD4⁺ T lymphocyte via the expression of T-bet, GATA3 and FoxP3, which was regulated by the phosphorylation of SHIP1. Hence, we speculated that the mechanism of hepatitis was related by SHIP1 via the expression of CD200 partly.

Conclusion

Anti-CD200 ameliorated Con A induced hepatitis and suggested that the differentiation of

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CD4⁺ T lymphocytes was modulated via SHIP1 signaling pathway served to attenuate the inflammatory response. These findings raised the potential effect of anti-CD200 as the second-line therapies for hepatitis.

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Disclosure of conflict of interest

None.

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