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Original Paper

Comparison of Concanavalin a-Induced Murine Autoimmune Hepatitis Models

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Key Words
Autoimmune hepatitis (AIH) • Concanavalin A (ConA) • Mouse models • Immune cells • Cytokines

Abstract
Background/Aims: Autoimmune hepatitis (AIH) is a chronic necroinflammatory disease of the liver whose pathogenic mechanisms have not yet been elucidated. Moreover, the current treatment used for the vast majority of AIH patients is largely dependent on immunosuppressant administration and liver transplantation. However, research on the pathogenesis of AIH and effective new treatments for AIH have been hampered by a lack of animal models that accurately reproduce the human condition. ***Methods:*** AIH models created by concanavalin A (ConA) injections at different times and doses. The levels of ALT, AST, LDH and inflammatory cytokines were examined at various times after 20 mg/kg ConA was administered by ELISA using commercially available kits. Moreover, liver pathological changes were observed by flow cytometry (FCM) and H&E staining. ***Results:*** Our experiments demonstrated that the levels of ALT, AST, LDH and several inflammatory cytokines, including TNF-α, IFN-γ, and IL-6, were higher in the 20 mg/kg 12 h ConA group than in the other groups. Importantly, the numbers of activated $CD4^+$ and $CD8^+$ T lymphocytes in the blood, spleen and liver were calculated. These results showed that ConA (20 mg/kg for 12 h)-induced hepatitis was similar to that in clinical AIH patients. Furthermore, we found that the number of MDSCs in the blood was significantly increased in the ConA (20 mg/kg for 12 h) group compared with controls. Our findings indicated that ConA (20 mg/kg for 12 h)-induced hepatitis could be used as an experimental murine model that mirrors most of the pathogenic properties of human type I AIH. ***Conclusion:*** This model [ConA (20 mg/kg for 12 h)] provides a valuable tool for studying AIH immunopathogenesis and rapidly assessing novel therapeutic approaches.

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Introduction

Autoimmune hepatitis (AIH) is a chronic necroinflammatory disease of the liver with unclear etiology. AIH mainly affects patients and rapidly leads to cirrhosis and end-stage-liver disease if left untreated [1]. It is characterized serologically by increased aminotransferase levels, interface hepatitis, hypergammaglobulinemia with high IgG levels and the presence of characteristic autoantibodies [2, 3]. AIH is historically classified as type 1 or type 2 on the basis of the autoantibody profile and according to seropositivity: AIH-1 is characterized by the presence of anti-smooth muscle and/or anti-nuclear (anti-SMA/ANA) autoantibodies, and AIH-2 is characterized by the presence of liver kidney microsomal antibody type 1 (anti-LKM-1) and/or anti-liver cytosol type 1 (anti-LC1) autoantibodies [4-6]. The latter is mainly a pediatric condition and has a higher risk of acute liver failure [7]. AIH is a worldwide health problem and a significant cause of mortality; it has been estimated that AIH has an annual incidence of approximately 2 in 100, 000 individuals and a prevalence of 15 cases per 100, 000 persons worldwide [8]. Without treatment, nearly 50% of patients with severe AIH die in approximately 5 years [9, 10]. The incidence rate is also different between men and women. It has been reported that women are more vulnerable to AIH [11]. Standard clinical therapy for AIH consists of a combination of corticosteroids and azathioprine and is the mainstay therapy for AIH [2]. Despite the availability of this effective treatment, AIH also poses therapeutic problems. Patients are commonly treated with steroids, but this therapy is not effective in all cases; more than 10-20% of AIH patients are refractory [2, 12]. Furthermore, the prolonged use of steroids may cause significant side effects, such as immunosuppression, osteoporosis, and sodium retention, and the discontinuation of steroid treatment is followed by disease relapse in most patients [13-15]. Considering these factors, it is significantly important to develop new, safe and effective drugs. However, the development of novel therapeutic strategies has long been hampered by a lack of valid animal models. Animal models are the basis for drug discovery and development. Therefore, a good preclinical animal model that can replicate the main features of AIH is urgently needed.

AIH is triggered by autoreactive T cells. Animal models are needed to elucidate the early pathogenic events in this process, namely, the priming of autoreactive T cells [16]. Many animal models for AIH have been developed since the early 1970s [8]. The early models of AIH were based on the administration of crude liver homogenates of heterologous origin and complete Freund's adjuvant, with no knowledge of the target antigens responsible for the disease [8, 17, 18]. The second phase began in 1983 when Kuriki et al. established transient hepatitis in mice by immunizing them with syngeneic liver homogenates or liver-specific lipoproteins with polysaccharides from *Klebsiella pneumonia* [19]. Then, many follow-up studies used inbred or neonatal thymectomy mice to establish the T cell-responsive AIH model [20, 21]. During the last 20 years, several animal models of AIH have been developed [22-25]. For example, an alternative model of AIH was created by vaccinating mice with dendritic cells (DCs) loaded with well-differentiated murine hepatocellular carcinoma cells (Hepa1-6) and administering interleukin-12 [26]. Moreover, a recent study has reported that low and transient expression of transgenic IL-12 in hepatocytes could cause a loss of tolerance to hepatocellular antigens that leads to chronic hepatitis resembling human type 1 AIH [12].In addition, murine ConA-induced AIH may be prevented by the IL-12 antibody and exacerbated by exogenous IL-12 [27].

Although several murine models of AIH have been described, none of them are completely satisfactory [28]. As the most commonly used AIH research model, ConA-induced hepatitis is a well-established T cell-mediated murine model that mimics human AIH. ConA is a type of lectin that is purified from the crude extract of *Canavalia ensiformis* seeds. It can agglutinate blood erythrocytes and predominantly stimulates T cells. In addition, immune cells are activated after a ConA injection; many cytokines are released and cause activated lymphocyte infiltration to aggravate liver injury [29]. Moreover, the ConA AIH model is easy to generate, inexpensive, convenient and replicable [30]. Therefore, this model is well-

established in mice for investigating T cell- and macrophage- dependent liver injuries, which closely mirror most of the pathogenic properties of AIH patients [31].

The absence of valid animal AIH models is considered to be the main reason for the lack of a simple and efficient cure. Drug candidate activity could be evaluated rapidly by using an optimal ConA AIH model. The aim of this study was to establish a unified standard model of ConA AIH by comparing ConA injections at different times and doses.

Materials and Methods

AIH patients and healthy donors

A total of 12 peripheral blood samples were obtained from patients with AIH between May 2016 and September 2016 at the Division of Gastroenterology & Hepatology, West China Hospital, Sichuan University (Sichuan, China). Ten peripheral blood samples were obtained from healthy volunteers for use as normal controls. The present study was approved by the Ethics Committee of West China Hospital, Sichuan University, and written informed consent was obtained from each subject. Table 1 shows the clinical characteristics of the AIH patients who were included in the study.

Animals

C57BL/6 female mice (8-12 weeks; 20-25 g) were obtained from Beijing HFK Bioscience Co., Ltd., Beijing, China. The mice were housed in a specific-pathogen-free (SPF) facility with a consistent room temperature and humidity. They were provided with free access to standard laboratory chow and water for one week before the experiments. All animal experiments were approved by the Institutional Animal Care and Treatment Committee of Sichuan University in China (Permit Number: 20161208).

Experimental design

First, the mice were intravenously injected with ConA at different concentrations ranging from 2.5 to 25 mg/kg for 18 h to test their survival rates. To evaluate ConA-induced AIH models, the mice were divided into three groups 1) Mice were given a single intravenous injection of normal saline as a vehicle control. 2) Mice were given a single intravenous injection of ConA (Sigma-Aldrich, St. Louis, MO) at a dose of 20 mg/kg body weight and were sacrificed at various times (2 h, 4 h, 6 h, 8 h and 12 h) after ConA administration. 3) Mice were given a single intravenous injection of ConA at a dose of 15 mg/kg body weight and were sacrificed 12 h after ConA administration.

Liver function and cytokine assay

Retro-orbital blood samples were collected from the mice. The plasma was separated by centrifugation at 300 g for 10 min. ALT and AST levels were measured by automatic dry biochemical analyzer (Hitachi Auto Analyzer 7170, Japan). The levels of TNF-α, IFN-γ and IL-6 in the murine plasma were analyzed by ELISA using commercially available kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Histopathology assay

Liver tissues were harvested after intravenous ConA administration. Liver samples were fixed in 4% buffered paraformaldehyde for 48 h. Sections (4-5 μm) on slides were deparaffinized with xylene, rehydrated with decreasing concentrations of ethanol, and stained with hematoxylin and eosin (H&E). Then, all sections were graded blindly under a light microscope according to the following criteria: 0, none; 1, individual cell necrosis; 2, ≤30% lobular necrosis; 3, ≤60% lobular necrosis; and 4, >60% lobular necrosis [9].

Flow cytometry (FCM) analysis

Venous blood was collected aseptically from patients and healthy volunteers. Red blood cells in the peripheral blood and spleens were lysed and washed twice with phosphate-buffered saline (PBS). In addition, single-cell suspensions of blood, liver, and spleen were obtained 12 h after ConA administration by using mechanical and enzymatic dispersion as described previously [32]. In general, the peripheral blood, spleen and liver were harvested. Red blood cells in the peripheral blood and spleen were lysed. A single-cell suspension of the liver was mechanically disrupted and then enzymatically digested with 1 mg/mL

collagenase I. Then, 1×10^6 freshly prepared cells were suspended in 100 µl of PBS and stained with different combinations of fluorochrome-coupled antibodies for CD11b, CD8, CD4, CD69 and Gr-1. The cells were collected by FCM, and the data were analyzed using FlowJo software.

Statistical analysis

All experiments were performed at least three times. The results are expressed as the mean ± SD, and *P* values for comparisons were determined by 2-tailed Student's t tests. Statistically significant *p* values are labeled as follows: **p*<0.05; ***p*<0.01; ****p*<0.001. $^{\#}p$<0.05; $^{\#\#}p$<0.01; and $^{\#\#\#}p$<0.001.

Results

Clinical and laboratory features of AIH patients at the time of the study

Similar to many other autoimmune diseases, AIH is prevalent in women. Twelve consecutive patients with AIH-1 [median age=50.5 years (range=41-53.5 years), 91.7% female] were enrolled between May 2016 and September 2016; ten healthy volunteers served as healthy controls [HCs; median age=42 years (range=27-54 years), 80% female]. Patient clinical and laboratory features are summarized in Table 1. All patients had high aminotransferase gamma-glutamyl transpeptidase (GGT), bilirubin, gamma globulin and immunoglobulin G (IgG) levels. As shown in Table 1, ANAs were present in all 12 patients, and AMAs were present in 5 patients (41.75). At diagnosis, all patients met the diagnostic criteria of the International Autoimmune Hepatitis Group [33].

Table 1. Clinical characteristics of the patients and healthy subjects who were recruited

Characteristics	Amount
Sex (Female, N%)	12 (91.7%)
Age (years)	50.5 (41.0, 53.5)
Liver function indexes	
TB (µmol/L)	37.6 (9.6, 137.6)
DB (µmol/L)	27.0 (6.4, 112.2)
ALB (g/L)	37.0 (34.4, 42.9)
GLB (g/L)	42.6 (37.1, 47.8)
ALT (IU/L)	255.5 (144.8, 438.8)
AST (IU/L)	375.0 (180.0, 568.2)
ALP (IU/L)	144.0 (123.5, 155.0)
GGT (IU/L)	135.0 (61.3, 260.3)
Immunoglobulin	
IgG (g/L)	26.3 (22.8, 32.8)
IgM (mg/L)	2660 (1887.5, 3975.0)
Autoantibody	
ANA (+, N%)	12 (100.0%)
AMA (+, N%)	5 (41.7%)

Percentages of T cell populations

T cell-mediated immune responses are thought to play a key role in causing of autoimmune liver damage. Therefore, we assessed the T cells in the peripheral blood from AIH patients and HCs by FCM using CD3, CD4 and CD8 antibodies. As shown in Fig. 1a, the results showed that the percentage of $CD4^+$ $CD3^+$ T cells was higher in AIH patients than in HCs. The levels of activated $CD4^+$ T cells in the HC group were reduced by ~1.6-fold compared with those in the AIH group. Furthermore, using the FCM data, we found an ~1.4-fold reduction in $CD8^+CD3^+$ T cell infiltration in the AIH group compared with that in the control group (Fig. 1b).

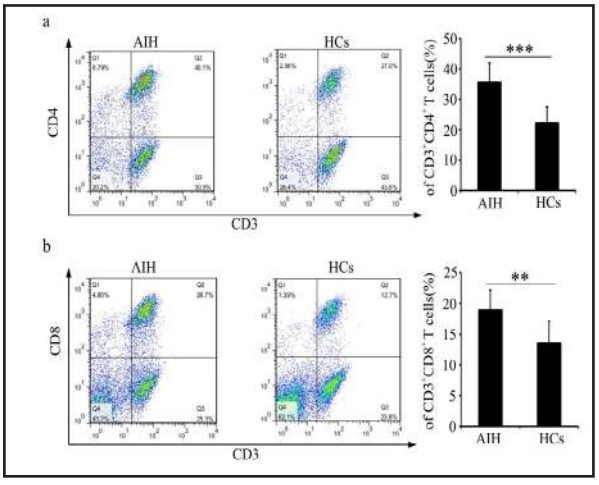

Fig. 1. Analysis of T cells from AIH patients and HCs. (a) Single-cell suspensions prepared from the peripheral blood of AIH patients and HCs (HCs represent healthy human controls) were analyzed for the presence of $CD4^+CD3^+$ cells by FCM. Bars show the mean±SD; ***p<0.001. (b) Single-cell suspensions prepared from the peripheral blood of AIH patients and HCs were analyzed for the presence of $CD8^+CD3^+$ cells by FCM. Bars show the mean±SD; **p<0.01.

Dose dependence of ConA-induced liver injury

AIH is chronic and more prevalent in females and genetically predisposed individuals. In fact, the gender ratio is 3.6:1, with women being more

affected by AIH than men [34]. In addition, mice with a Th2-biased immune response, such as BALB/c inbred mice, require higher ConA doses of up to >30 mg/kg body weight to induce AIH. In contrast, animals with an immune response of more Th1-like T helper cells, such as C57BL/6 mice, are more susceptible to ConA and typically require only approximately 15-20 mg/kg body weight [35]. Therefore, we chose female C57/6 mice to our study. First, we examined mice that were i.v. injected with ConA at different concentrations of 5-25 mg/kg for 18 h. As shown in Table 2, the 5 to 10 mg/kg doses of ConA at 18 h were safe for the mice, whereas the 15 mg/kg 18 h dose induced death 20% of the mice. Thus, we used 12 h as the longest time for our study.

Table 2. Various concentrations of ConA ranging from 5 to 25 mg/kg for different times, The subscript represents the number of deaths in mice

Doses\Time	8h	12h	18h
5mg/kg	10_0	10_0	10_0
10mg/kg	10_0	10_0	10_0
15mg/kg	10_0	10_0	10_2
20mg/kg	10_0	10_1	10_4
25mg/kg	10_1	10_3	10_6

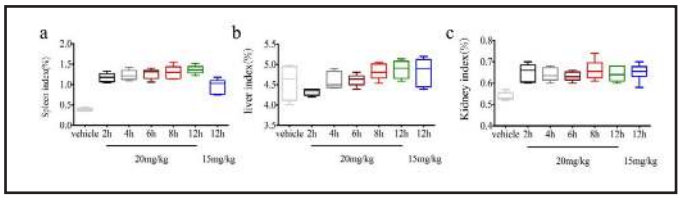

Fig. 2. Organ indexes in ConA-induced hepatitis. Mice (n=5 for each group) were injected with ConA (20 mg/kg) at different times. Each group was sacrificed at the following time points: 2 h, 4 h, 6 h, 8 h, 10 h and 12 h after ConA injection, and the other group was injected with ConA for 12 h. (a) Spleen index of the different groups. (b) Liver index of the different groups. (c) Kidney index of the different groups.

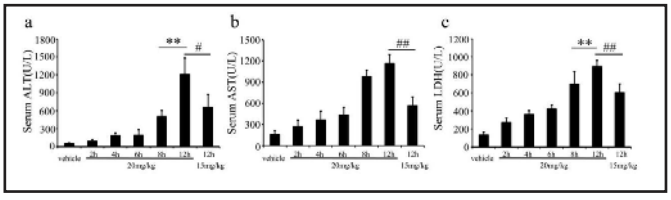

Fig. 3. ALT、AST and LDH levels were determined in ConA-induced hepatitis. (a) Serum transaminase activities (ALT) were determined at different time after intravenous injection of 20 mg/kg or 15 mg/kg ConA into C57BL/6 mice. (b) AST levels were determined at different times after intravenous injection of 20 mg/kg or 15 mg/kg ConA into C57BL/6 mice. (c) Serum LDH levels were determined at different times after intravenous injection of 20 mg/kg or 15 mg/kg ConA into C57BL/6 mice. *p<0.05; **p<0.01; #p<0.05; ##p<0.01.

Hepatic injury after ConA administration

We first successfully developed mouse models of AIH by using ConA treatment for different times. As illustrated in Fig. 2a, the ConA-treated groups had significantly higher spleen indexes than the control group. (The spleen index was calculated as the relative spleen weight ratio (spleen weight/body weight) for the experimental animals and controls). However, there were no significant differences in the liver indexes between the ConA-treated group and the control group (Fig. 2b). Interestingly, as shown in Fig. 2c, the ConA-treated groups had significantly higher kidney indexes than the control group. Moreover, compared with the control-treated mice, the mice injected with ConA developed acute hepatitis as indicated by their elevated serum ALT and AST levels. As shown in Fig. 3a-c, the serum levels of ALT, AST and LDH in mice were elevated significantly after ConA injection at 2 h, and reached their peak levels at 12 h. Moreover, the levels of ALT, AST and LDH in the group treated with ConA 20 mg/kg for 12 h were higher than those in other groups. Furthermore, light microscopy showed dramatic inflammatory cell infiltration, massive hepatocyte necrosis, blood vessel congestion and dilatation and disordered hepatic sinusoid structures in ConA-treated mice. The pathological scores showed that liver injury in the ConA (20 mg/kg for 12 h) group was significantly more severe than that in the other groups (Fig. 4h). Therefore, the pathological evidence demonstrated that this model had the characteristics of AIH.

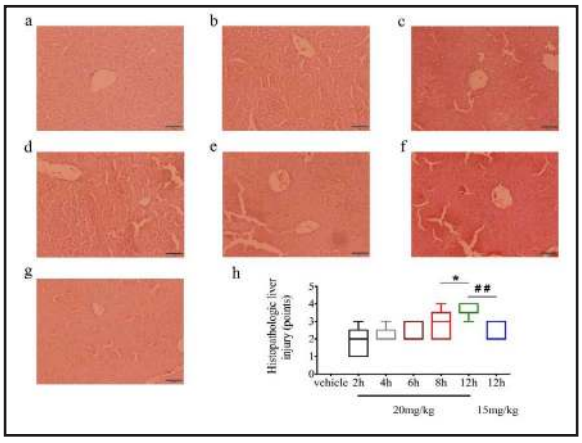

Fig. 4. Liver damage after ConA treatment. Representative H&E sections of the liver. (a) Vehicle control group. (b) 20 mg/kg 2 h group. (c) 20 mg/kg 4 h group. (d) 20 mg/kg 6 h group. (e) 20 mg/kg 8 h group. (f) 20 mg/kg 12 h group. (g) 15 mg/kg 12 h group. (h)Pathological scores of the different groups. *p<0.05; **p<0.01; #p<0.05; ##p<0.01.

Changes in the expression levels of relevant cytokines

ConA-induced hepatitis is associated with the production of various pro-inflammatory cytokines. Therefore, we next measured a series of cytokines, including TNF-α, IFN-γ, and IL-6, by ELISA. As shown in Fig. 5a-c, after ConA administration, the levels of the pro-inflammatory cytokines TNF-α, IFN-γ, and IL-6 were elevated in the plasma compared with those in the control groups. Moreover, TNF-α and IL-6 levels robustly increased after ConA (20 mg/kg) administration. Moreover, previous data revealed that the ConA (20 mg/kg for 12 h) group was significantly more severe than that in the other groups. Therefore, we use the ConA (20 mg/kg for 12 h) group for the next experiments.

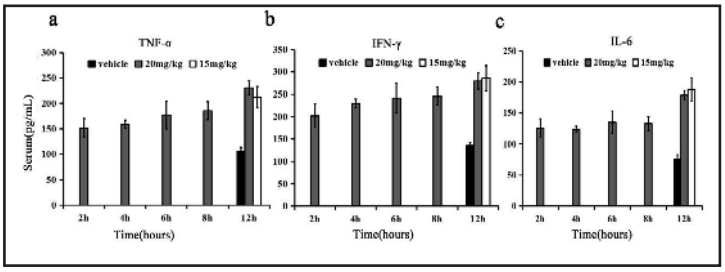

Fig. 5. ELISA analysis of cytokines in ConA-induced hepatitis. C57BL/6 mice (n=5) were injected with PBS or ConA at different times and doses. Blood was collected after the indicated times, and serum cytokine levels were determined by ELISA.

ConA-induced inflammatory cell recruitment and activation

AIH is caused by activated T lymphocytes that infiltrate and destroy the liver parenchyma, thus leading to liver injury. To further confirm the role of T cells in the development of AIH in our model, lymphocytes were isolated from the blood after ConA treatment (20 mg/kg for 12 h). We used the CD4 and CD69 antibodies to identify active $CD4^+$ T cells. Fig. 6 shows that after treatment with ConA, the number of active $CD4^+$ T cells was increased in the blood. Notably,

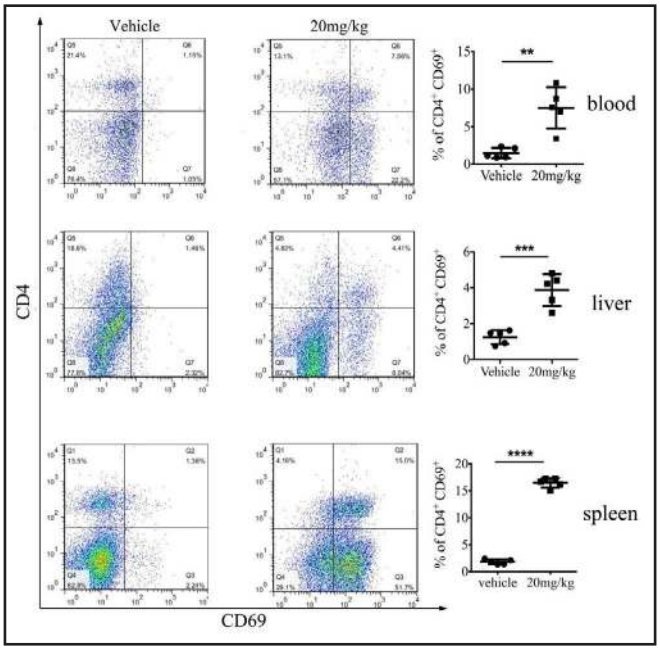

Fig. 6. Effects of ConA on host immunity. Single-cell suspensions prepared from the peripheral blood, livers and spleens of the ConA 20 mg/kg 12 h group and the vehicle group were analyzed by FCM for the presence of $CD4^+CD69^+$ cells. Bars show the mean±SD; **p<0.01,***p<0.001.

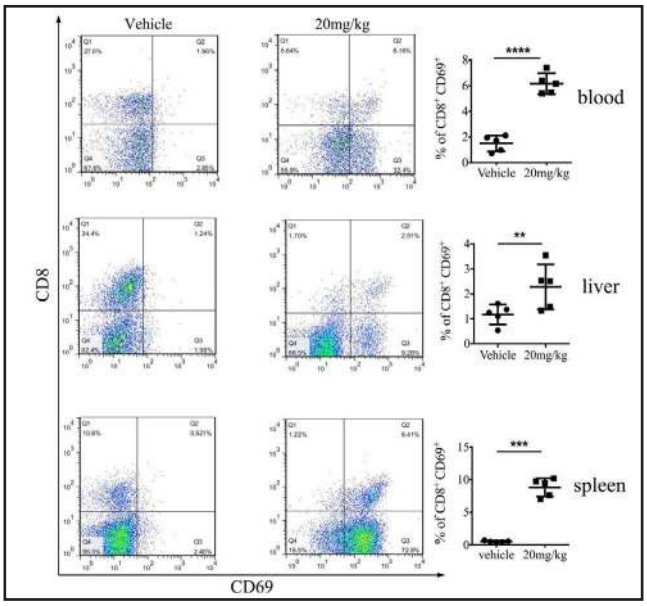

Fig. 7. Active CD8⁺T cells were determined after ConA treatment. Single-cell suspensions prepared from the peripheral blood, livers and spleens of the ConA 20 mg/kg 12 h group and the vehicle group were analyzed by FCM for the presence of CD8⁺CD69⁺cells. Bars show the mean±SD; **p<0.01,***p<0.001.

the liver infiltration of active CD4⁺ T lymphocytes was increased in the ConA-treated group compared with that in the control groups. Similarly, the active CD4⁺ T lymphocytes in the spleen were significantly increased in the ConA group. Moreover, we investigated the levels of active CD8⁺ T lymphocytes in organs. As shown in Fig. 7, active CD8⁺ T lymphocyte infiltration was higher in the ConA-treated group than in the control groups. Taken together, these results implied that intravenous injection of ConA into mice could increase the number of activated T cells. These results were consistent with those from the clinical AIH patients.

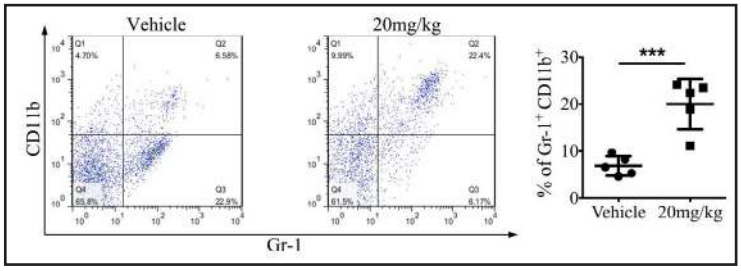

Fig. 8. Gr-1⁺CD11b⁺ myeloid cells were increased after ConA treatment. Gr1⁺CD11b⁺ cells were gated and analyzed by FCM for the presence of MDSCs. Statistical results from each group. Compared with vehicle treatment, treatment with ConA 20 mg/kg 12 h significantly increased the number of MDSCs. Values represent the mean±SD (n=5); ***p<0.001.

ConA increased the number of myeloid-derived suppressor cells (MDSCs)

MDSCs are a heterogeneous cell population consisting of immature myeloid cells and myeloid progenitor cells that can suppress T cell responses by a variety of mechanisms [32]. Therefore, we measured the number of MDSCs, which were identified as CD11b⁺ and Gr-1⁺ double-positive myeloid cells by FCM, to investigate the blood myeloid cell infiltration. As shown in Fig. 8, the percentage of MDSCs was significantly higher in the ConA-treated group than in the control group.

Discussion

AIH is a chronic inflammatory liver disease of unknown etiology and is associated with interface hepatitis, the presence of autoantibodies and regulatory T cell (Tregs) dysfunction [36]. Moreover, AIH severity varies widely among patients; some cases will develop confluent hepatocellular necrosis that leads to acute liver failure or advanced liver cirrhosis that requires liver transplantation [12]. Therefore, AIH is a complex polygenic disease that remains a major clinical challenge, and novel effective therapies need to be explored for clinical use [37]. Furthermore, proper animal models that replicate human AIH are required to achieve this goal.

ConA can activate T cells to secrete cytokines that cause liver injury. Therefore, ConA-induced hepatitis is used as a mouse model of immune-mediated liver injury and resembles

AIH in humans [38].In this study, we used ConA to induce AIH in mice and established a unified standard model of the ConA AIH model. The levels of ALT, AST and LDH increased from 2 h and reached their peaks at 12 h. Moreover, the pathological scores were significantly higher in the ConA 20 mg/kg 12 h group than in the other groups. It is a commonly thought that hepatitis progression is associated with pro-inflammatory cytokines, such as TNF-α, IL-6 and IFN-γ [37, 39]. Therefore, we measured the serum levels of TNF-α, IL-6 and IFN-γ. The levels of these cytokines increased after ConA treatment and were highest at 12 h.

TNF-α plays a major role in the pathogenesis of ConA-induced AIH. In the plasma, TNF-α first peaked after 1 h in the ConA model of BALB/c mice [30]. Moreover, in the Male Naval Medical Research Institute (NMRI) ConA model in albino mice [40], the levels of TNF-α peaked 2 h after ConA injection. Furthermore, an earlier report showed that the plasma levels of TNF-α were highest at 2 h after an LPS challenge in neonatal mice challenged with 25 mg/kg S. enteriditis [41]. In addition, in a type 1 diabetes model in NOD mice, *in vivo* injection with anti-CD3 mAb induced T cells to release several cytokines, such as TNF-α and IFN-γ, which appear in the circulation with different kinetics. Because the release of TNF-α from mononuclear cells is faster than that of IFN-γ, TNF-α was found in the blood at 2 h but not at 6 h after administration [42]. However, in our study, we did not examine the levels of TNF-α 1 h after ConA injection. TNF-α levels should peak 1 h after ConA injection in female C57/6 mice. The reason for this discrepancy is not known, although different strains of mice and environmental factors may be involved.

During AIH, self-tolerance is defective and results in T lymphocytes dysfunction, including highly activated CD4 and CD8 T cells, which mediate autoimmune liver injury [38, 43]. These findings suggest that $CD4^+$ T helper (Th) cells were involved in liver injury. In our research, T cell-mediated liver injury was detected in the group receiving intravenously injected ConA (20 mg/kg 12 h). Previous studies have indicated that immune cells are activated after ConA injection and that, as a result, a number of cytokines that aggravate liver injury are released [44].

The $CD11b^+Gr\text{-}1^+$ MDSCs are receiving increasing attention as one of the main regulatory cells of the immune system [45]. The frequency of MDSCs has been reported to be involved in the immune response, not only in inflammation associated with cancer but also in AIH [43]. Moreover, the frequency of MDSCs in AIH patients has been reported to be significantly higher than that in HCs. Moreover, the frequency of MDSCs in the peripheral blood has been positively correlated with ALT and AST levels in AIH patients [43]. Furthermore, administration of ConA resulted in an increase in both the percentage and absolute number of hepatic MDSCs. ConA+ cannabidiol treatment resulted in further robust induction of MDSCs when compared to the ConA+vehicle treatment group [43]. In this study, we found that the number of MDSCs in the peripheral blood of ConA-treated mice was higher than that in the vehicle-treated mice. These results were consistent with the AIH clinical data. Therefore, MDSCs are important for ConA-induced AIH and may provide a possible method for immune intervention.

Conclusion

In summary, this study provides a reliable mouse model of ConA-induced AIH. The ConA (20 mg/kg 12 h) animal model, which is a typical T cell and marcrophage-dependent model, mimics the mechanisms and characteristics of clinical AIH. Therefore, we believe this is a good mouse model for studying the mechanisms of AIH and developing new therapeutic drugs.

Abbreviations

AIH (Autoimmune hepatitis); ConA (Concanavalin A); FCM (Flow cytometry); AST (Aspartate aminotransferase); ALT (Alanine aminotransferase); LDH (Lactate dehydrogenase);

MDSCs (myeloid-derived suppressor cell); HE (hematoxylin-eosin); PBS (Phosphate-buffered saline).

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Disclosure Statement

The authors declare no conflict of interest.

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