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Original Article

Hepatoprotective effect of capsaicin against concanavalin A-induced hepatic injury via inhibiting oxidative stress and inflammation

Hui Zhang[1*], Yang Bai[2*], Min Gao[3], Junfeng Zhang[1], Guanjun Dong[1], Fenglian Yan[1], Qun Ma[1], Xingqin Fu[1], Qingqing Zhang[1], Chunxia Li[1], Hui Shi[1], Zhaochen Ning[1], Jun Dai[1], Zhihua Li[1], Jiankuo Ming[1], Qingjie Xue[1], Chuanping Si[1], Huabao Xiong[4]

[1]Institute of Immunology and Molecular Medicine, Jining Medical University, Jining 272067, Shandong Province, China; [2]Department of Urology, [3]Clinical Laboratory, Jining First People's Hospital, Jining 272011, Shandong Province, China; [4]Department of Medicine, Precision Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA. [*]Equal contributors.

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Abstract: Immune-mediated liver injury plays a crucial role in the pathogenesis of liver diseases, which can result from viral infections, autoimmunity, alcohol intake, and drug use. Concanavalin A (Con A)-induced hepatitis is a well-characterized murine model with similar pathophysiology to that of human viral and autoimmune hepatitis. Capsaicin, a selective agonist of the transient potential vanilloid subfamily member 1 (TRPV1) receptor, exhibits anti-inflammatory effects on various causes of inflammation. In the present study, we investigated the effect of capsaicin on Con A-induced hepatitis. Capsaicin (1 mg/kg body weight) was administered by intraperitoneal injection, after which (30 minutes), the mice were challenged intravenously with Con A (20 μg/g body weight). We collected serum for plasma transaminase analysis. Pro-inflammatory cytokine levels and hepatocyte apoptosis were assayed by ELISA and TUNEL, respectively. Liver samples were collected for real-time PCR, hematoxylin and eosin staining, and measuring oxidative stress and myeloperoxidase levels. Activation of splenocytes and hepatic mononuclear cells was analyzed by flow cytometry. Compared with control, the capsaicin-treated group showed significantly decreased aminotransferase levels and markedly prolonged mouse survival. Capsaicin pretreatment also attenuated hepatocyte apoptosis and oxidative stress. Furthermore, tumor necrosis factor-α and interferon-γ levels in serum and liver were significantly suppressed, while the percentage of myeloid-derived suppressor cells increased after capsaicin pretreatment. Our findings indicate that capsaicin pretreatment protects mice from Con A-induced hepatic damage and is partially involved in inhibiting hepatocyte apoptosis, oxidative stress, and inflammatory mediators as well as regulating activation and recruitment of intrahepatic leukocytes.

Keywords: Concanavalin A, hepatitis, capsaicin, inflammation, oxidative stress

Introduction

Liver diseases, including viral and autoimmune hepatitis as well as drug-induced liver damage, are a major threat to human health worldwide. Although there are heightened concerns regarding acute and chronic liver diseases, the immunological pathogenesis is not yet well understood. Concanavalin A (Con A)-induced hepatitis, established by Tiegs and colleagues in 1992, has been widely used as a model for acute immune-mediated hepatitis in mice [1]. In contrast to other models for acute hepatitis, Con A-induced hepatic injury is primarily T cell-mediated hepatic damage [1, 2]. After intravenous injection of Con A, T cells, natural killer (NK) cells, natural killer T (NKT) cells, and other inflammatory cells are activated and recruited to the liver where they secrete various hepatotoxic cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), which subsequently induce severe liver inflammation and massive hepatocyte apoptosis/necrosis accompanied by highly elevated levels of serum transaminases [1, 3-8].

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), an active compound responsible for the spicy

flavor of chili peppers, is a selective agonist of the transient potential vanilloid subfamily member 1 (TRPV1) receptor [9]. Previous studies have addressed a variety of physiological activities of capsaicin in the treatment of cardiovascular disease, arthritis, weight loss, and cancer as well as an analgesic and antipruritic [10-12]. TRPV1 expression has been demonstrated in practically all types of mammalian immune cells, including lymphocytes, dendritic cells (DCs), macrophages, NK cells, and neutrophils [13, 14]. Subsequently, capsaicin was shown to play a pivotal role in inflammation and immunity by downregulating the expression of proinflammatory cytokines and chemokines and inhibiting immune cell function [15]. On the other hand, some studies have found that the inhibitory function of capsaicin on proinflammatory molecules is independent of TRPV1, indicating the involvement of an alternative mechanism [16].

Several studies have reported that treatment with capsaicin can ameliorate liver redox status and mitochondrial bioenergetic functions of mice fed a high fat diet [17, 18]. Furthermore, a recent study found that capsaicin protected mice from alcohol-induced acute liver injury via modulation of matrix metalloproteinases [19]. However, the effects of capsaicin on Con A-induced hepatitis, which closely resembles the pathogenic process of human autoimmune hepatitis, remain poorly defined. Based on the anti-inflammatory activity of capsaicin, our study focused on its protective effects and probable mechanisms on Con A-induced hepatitis.

Materials and methods

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Jining Medical University, Jining, China. Adult male C57BL/6 mice weighing 20-25 g were used throughout the experiments and were purchased from Pengyue Experimental Animal Breeding Co. Ltd. (Jinan, China). The mice were housed in a specific pathogen-free facility at Jining Medical University.

Reagent

Con A (Type IV; C-2010) and capsaicin were purchased from Sigma-Aldrich (St. Louis, MO). Murine FITC-conjugated anti-CD3, PE-conjugated anti-NK1.1, APC-conjugated anti-CD69, FITC-conjugated anti-CD11b, APC-conjugated anti-Gr-1, PE-conjugated anti-Ly6G, APC-conjugated anti-Ly6C, and isotype control antibodies were purchased from BioLegend (San Diego, CA). Primers for real-time PCR were synthesized by Thermo Fisher Scientific (Waltham, MA).

Mouse model

Capsaicin dissolved in a solution of 10% ethanol/10% Tween 80/80% pyrogen-free phosphate-buffered saline (PBS) was administered by intraperitoneal injection at 1 mg/kg of body weight. Thirty minutes later, the mice were challenged intravenously with Con A (20 μg/g body weight). At the indicated time points after Con A injection, serum was collected for plasma cytokine and transaminase analysis. Liver samples were collected for RNA isolation, reverse transcription (RT)-PCR, hematoxylin and eosin (H&E) staining, and immunofluorescence. Lethal doses of Con A (25 μg/g body weight) were administered intravenously for survival experiments.

Analysis of plasma aminotransferases

Alanine aminotransaminase (ALT) activity was measured using a multiple biochemical analyzer (Cobas 8000; Roche, Basel, Switzerland), 12 h after Con A administration.

Histopathology

Liver tissues were fixed in 4% formalin, embedded in paraffin, and then cut into 5-μm-thick sections. The sections were then stained with H&E following a standard procedure [20]. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assays were performed according to the manufacturer's instructions (Sigma-Aldrich) [21].

Isolation of splenocytes and hepatic mononuclear cells (MNCs)

Spleens and livers were harvested and pressed through a 200-gauge stainless steel mesh. To obtain hepatic MNCs, cells from liver tissues were first resuspended in 40% Percoll (Sigma-Aldrich). Then, single-cell suspensions were gently overlaid onto 70% Percoll and centrifuged at 1,260 × *g* for 30 min at room temperature. Erythrocytes were removed using a lysis solution. After washing twice with PBS, the cells were resuspended in RPMI 1640 (Gibco, Waltham, MA).

Flow cytometric analysis

Splenocytes and liver MNCs (approximately 1 × 10^6 cells) were preincubated with an Fc receptor blocker (BioLegend) at 4°C for 10 min according to standard protocols [22]. Then, the cells were incubated with fluorescence-labeled antibodies (murine FITC-conjugated anti-CD3, PE-conjugated anti-NK1.1, APC-conjugated anti-CD69, FITC-conjugated anti-CD11b, APC-conjugated anti-Gr-1, PE-conjugated anti-Ly6G, APC-conjugated anti-Ly6C, or isotype control antibodies) at 4°C for 30 min. After washing twice with PBS, the cells were analyzed with a FACSCalibur flow cytometer (BD Bioscience, Franklin Lakes, NJ).

RNA isolation and real-time PCR

Total RNA was isolated from liver using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan). Real-time PCR was performed using a LightCycler® 480 System (Roche) with AceQ Universal SYBR qPCR Master Mix (Vazyme Biotechnology, Nanjing, China). Primers used for real-time PCR were as follows, TNFα forward, 5'-TTGGCTCCAGCATGTACCCT-3' and reverse, 5'-TCCTGCCCACTGAGTTCGTC-3'; IFN-γ forward, 5'-ACAGCAAGGCGAAAAAGGATG-3' and reverse, 5'-TGGTGGACCACTCGGATGA-3'; β-actin forward, 5'-ACTGCTGGGACTCTG-3' and reverse, 5'-TGATGGCGTAGAACAG-3'.

ELISA

Serum protein levels of IFN-γ and TNF-α were assayed with cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits (BioLegend) according to manufacturer's instructions.

Analysis of superoxide dismutase (SOD), myeloperoxidase (MPO), and malondialdehyde (MDA) levels in liver tissue

Liver tissues were homogenized to obtain 10% homogenate with normal saline and then SOD and MPO activity, as well as MDA content, were measured with kits according to manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

All data are expressed as the means ± standard deviation (SD) of three independent experiments. Student's *t*-test (two groups) or one-way analysis of variance (ANOVA; multiple groups) were used. All analyses were performed with GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when *P* values were less than 0.05.

Results

Capsaicin pretreatment alleviates Con A-induced hepatic injury

To examine the effect of capsaicin on Con A-induced hepatitis, capsaicin (1 mg/kg body weight) was administered to mice by intraperitoneal injection 30 min prior to Con A (20 μg/g body weight) injection. Serum ALT levels were determined 12 h after Con A injection. Consistent with previous reports, capsaicin alone had almost no effect on serum ALT levels, whereas ALT levels were significantly increased in Con A-treated mice. Surprisingly however, the Con A-induced elevation in ALT levels was markedly decreased in mice pretreated with capsaicin (**Figure 1A**). Furthermore, histological analysis revealed that massive necrosis was presented in the liver of Con A-treated mice, which was nearly abolished in animals pretreated with capsaicin (**Figure 1C**). Notably, capsaicin pretreatment also protected mice from a lethal dose of Con A (25 μg/g body weight)-induced death (**Figure 1B**). Based on the above observations, it can be concluded that capsaicin exerts protective effects on Con A-induced hepatic injury.

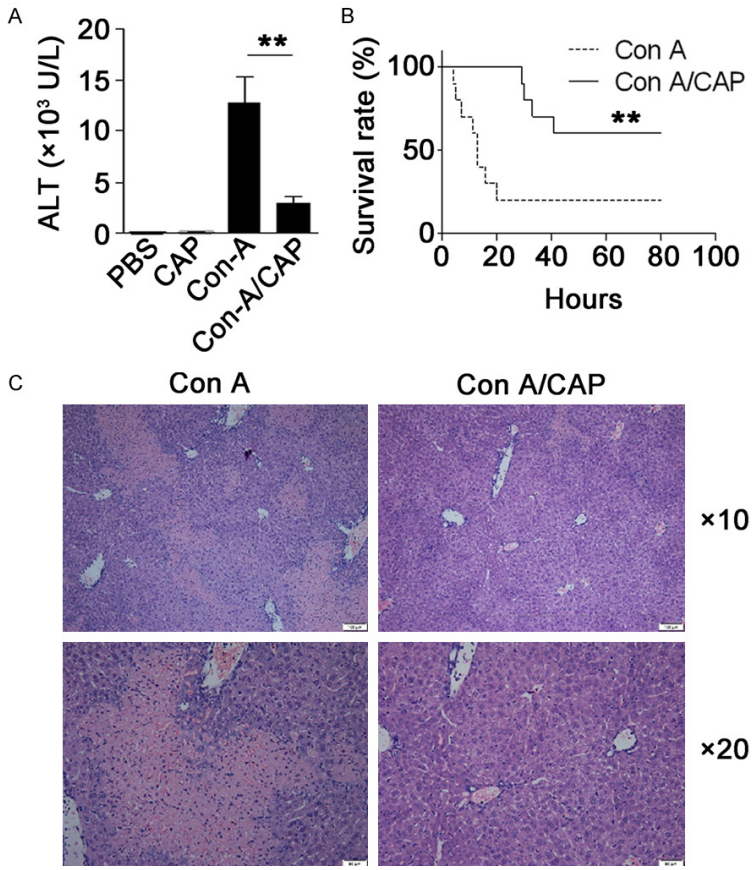

Figure 1. Capsaicin (CAP) pretreatment alleviated concanavalin A (Con A)-induced hepatic injury. Mice were treated with PBS or capsaicin 30 min before being challenged with Con A (20 μg/g body weight). Twelve hours after Con A injection, serum and livers were collected. A. Alanine aminotransaminase (ALT) levels in serum. The data represent the means ± standard deviation (SD; n = 6-8). **P < 0.01. B. Survival experiments were performed with mice treated with a lethal dose of Con A (25 μg/g body weight; n = 8-10). C. Liver sections (Con A or CAP/Con A group) were stained with hematoxylin and eosin (H&E). Original magnification, 100 × and 200 ×.

Capsaicin attenuates hepatocyte apoptosis in Con A-induced hepatitis

As previously reported, massive hepatocyte apoptosis was detected in the livers of mice treated with Con A, as shown in **Figure 2A** [23, 24]. However, capsaicin pretreatment significantly prevented the apoptosis induced by Con A. Meanwhile, the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax in the liver was examined by real-time PCR (**Figure 2B**) and the results indicated that Con A upregulated Bax expression [25]. In contrast, Bax mRNA levels were markedly downregulated while Bcl-2 mRNA levels were upregulated in the capsaicin pretreatment group. These findings suggest that capsaicin pretreatment alleviates Con A-induced hepatic injury and that capsaicin is partly involved in inhibiting the apoptosis of hepatocytes.

Capsaicin pretreatment suppresses oxidative stress and MPO levels in Con A-induced hepatic injury

Increasing evidence indicates that antioxidant enzymes including SOD play important roles in Con A-induced liver injury and human hepatitis [26]. Effects of capsaicin on SOD and MDA levels are shown in **Figure 3A** and **3B**. As expected, capsaicin remarkably improved SOD activity, especially 12 h after Con A injection. Moreover, compared with the Con A-only group, capsaicin pretreatment of Con A-challenged mice resulted in a significant decrease in MDA levels. MPO, which mainly exists in neutrophils, has been utilized as an indirect indicator for the recruitment of neutrophils to infected organs [27]. As shown in **Figure 3C**, capsaicin pretreatment significantly alleviated the high MPO levels observed in the Con A group.

Capsaicin administration inhibits cytokine release in Con A-induced hepatic injury

It has been reported that Con A-induced hepatic injury is accompanied by the production of various proinflammatory cytokines, including IFN-γ [7, 8] and TNF-α [6]. The effects of capsaicin on IFN-γ and TNF-α levels, at different time points (2, 6, 12, and 24 h) after Con A administration, were measured with ELISA. As shown in **Figure 4A**, Con A dramatically induced serum TNF-α and IFN-γ production, while capsaicin pretreatment significantly decreased serum IFN-γ levels at 12 h after Con A administration. Moreover, capsaicin also suppressed TNF-α secretion, especially at 2 h after Con A injection. We also analyzed liver mRNA levels of these cytokines by real-time PCR and found

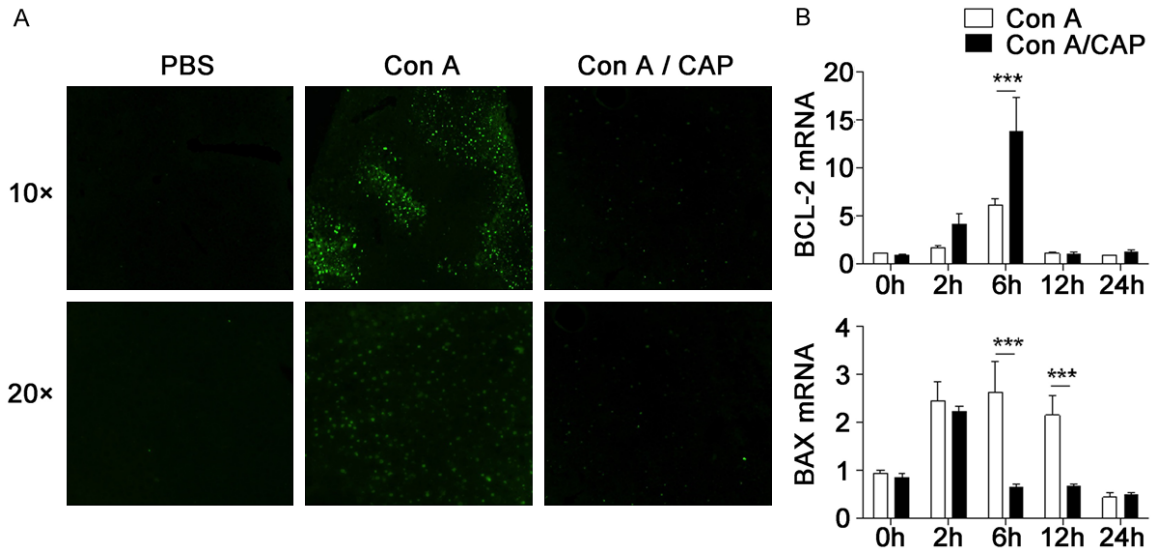

Figure 2. Capsaicin prevents hepatocyte apoptosis in Con A-induced hepatitis. Mice were treated with PBS or capsaicin, after which Con A was injected (20 μg/g body weight) 30 min later. A. Liver tissues were collected 12 h after Con A administration for TUNEL staining (original magnification, 100 × and 200 ×). B. Bcl-2 and Bax mRNA levels in the liver were assessed by real-time PCR. The data represent the means ± SD (n = 6-8). *P < 0.05; **P < 0.01.

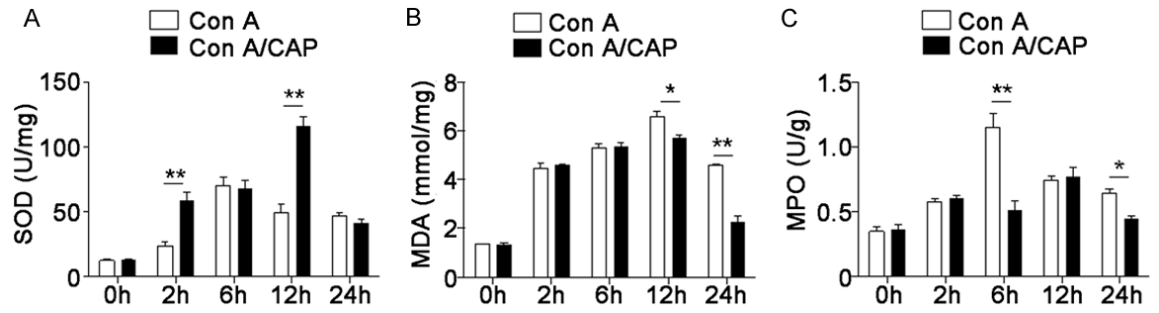

Figure 3. Effects of capsaicin on oxidative stress and myeloperoxidase (MPO) levels in liver tissue. Mice were treated with PBS or capsaicin 30 min before being challenged with Con A. Liver tissues were collected at the indicated time points after Con A injection. (A) Superoxide dismutase (SOD), (B) malondialdehyde (MDA), and (C) MPO levels in the liver were tested. The data represent the mean ± SD (n = 6-8). *P < 0.05; **P < 0.01).

that capsaicin pretreatment downregulated mRNA levels of both IFN-γ and TNF-α (**Figure 4B**). Therefore, capsaicin can effectively suppress the production of IFN-γ and TNF-α to ameliorate liver damage caused by Con A.

Capsaicin pretreatment inhibits lymphocyte activation and promotes myeloid-derived suppressor cell (MDSC) accumulation

Following Con A administration, Con A primarily leads to recruitment and activation of various lymphocytes, including T, NK, and NKT cells. These activated cells then secrete proinflammatory cytokines that subsequently induced hepatocyte cell death [1, 3, 4]. To further investigate the protective mechanisms of capsaicin against liver damage, we analyzed the effect of capsaicin pretreatment on the activation of inflammatory cells in the liver. As shown in **Figure 5A** and **5B**, capsaicin suppressed the activation of T cells ($CD3^+$), however, capsaicin pretreatment had no significant effects on the Con A-induced recruitment of these cells to the liver (**Figure 5C**, **5D**).

MDSCs represent a heterogeneous population of immature myeloid cells and are negative regulators in the maintenance of liver immune homeostasis [28]. Thus, we analyzed whether capsaicin affects the recruitment and infiltration of MDSCs under inflammatory conditions. The results showed that the percentage of $CD11b^+Gr\text{-}1^+$ MDSCs in the liver and spleen

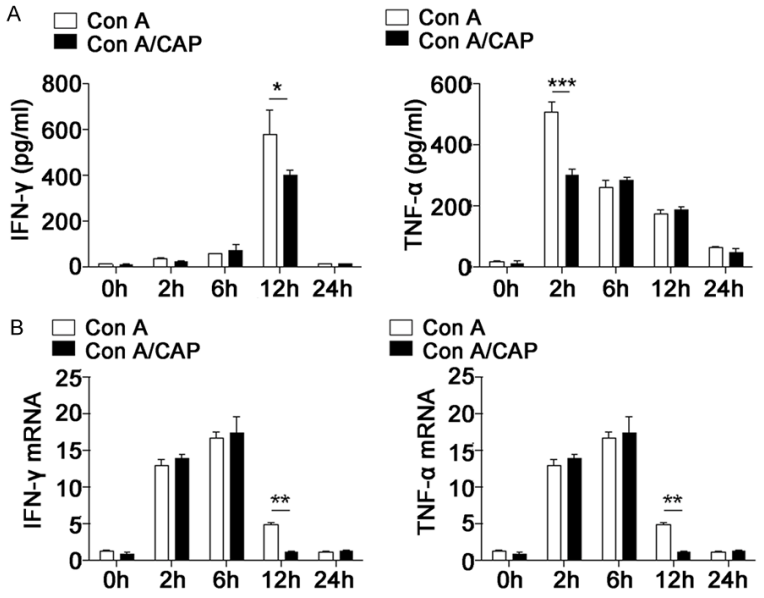

Figure 4. Capsaicin pretreatment inhibited cytokine release in Con A-treated mice. Mice were treated as described in **Figure 3**. Serum and liver tissue were collected at the indicated time points after Con A injection. A. TNF-α and IFN-γ levels in serum were measured by ELISA. The data represent the means ± SD (n = 6-8). *P < 0.05; **P < 0.01. B. mRNA levels of IFN-γ and TNF-α in liver tissues were determined by real-time PCR. The data represent the means ± SD (n = 6-8). *P < 0.05; **P < 0.01.

of capsaicin-pretreated mice was significantly higher than in the control group (**Figure 6A**, **6C**). Consistent with previous studies, these infiltrated cells were further subdivided into $Ly6C^{int}Ly6G^{high}$ and $Ly6C^{high}Ly6G^{low}$ subsets (**Figure 6B**, **6D**). These results suggest that capsaicin may recruit more MDSCs to reduce liver injury.

Discussion

In the present study, we investigated the effect of capsaicin, an active component accounting for the pungency of chili peppers, on Con A-induced hepatitis. The results showed that capsaicin pretreatment 30 min prior to Con A injection markedly downregulated serum ALT levels and inhibited hepatocyte apoptosis/necrosis induced by Con A administration. Moreover, capsaicin pretreatment significantly reduced the release of proinflammatory cytokines and our results also indicated that capsaicin inhibited lymphocyte activation. Therefore, our findings suggest that capsaicin has a protective effect on Con A-induced hepatitis.

Con A-induced hepatitis is a well-established mouse model with unique features with respect to its pathogenesis and important similarities to human autoimmune hepatitis and acute viral hepatitis [1, 2]. An increasing number of studies have shown that Con A-induced hepatitis is associated with the release of large amounts of proinflammatory cytokines, including IFN-γ, TNF-α, interleukin (IL)-6, and IL-1, leading to hepatocyte apoptosis/necrosis [5-8, 29]. Among these cytokines, IFN-γ and TNF-α have been reported as critical mediators for the pathogenesis of Con A-induced hepatitis; this was confirmed by the finding that that Con A-mediated liver damage may be prevented in mice with IFN-γ [7] or TNF-α deficiencies [6]. Meanwhile, several studies have indicated that capsaicin may act as an anti-inflammatory agent *in vivo* by attenuating cytokine levels, such as TNF-α, IFN-γ, IL-1β, and IL-12p40 [30, 31]. Consistent with previous experimental results, our study showed that capsaicin pretreatment significantly reduced TNF-α and IFN-γ levels in both serum and liver of Con A-challenged mice, which may contribute to the protective effects of capsaicin on Con A-induced hepatitis.

Superoxide, an essential effector in Con A-induced hepatitis, is produced by Kupffer cells and hepatocytes and promotes apoptosis and proinflammatory cytokine production [32]. SOD is an important factor in maintaining balance between oxidation and antioxidation by scavenging superoxide anion free radicals to protect cells from injury [33]. We found that capsaicin pretreatment significantly increased SOD activity in Con A-induced hepatic injury. We also tested the production of MDA, which is the most important end-product of lipid peroxidation and has been frequently regarded as a marker of cellular oxidation status [26]. As expected, capsaicin pretreatment significantly inhibited MDA production. Moreover, capsaicin markedly reduced MPO levels *in vivo* after Con A injection, indicating that capsaicin may suppress neutrophil liver infiltration during inflam-

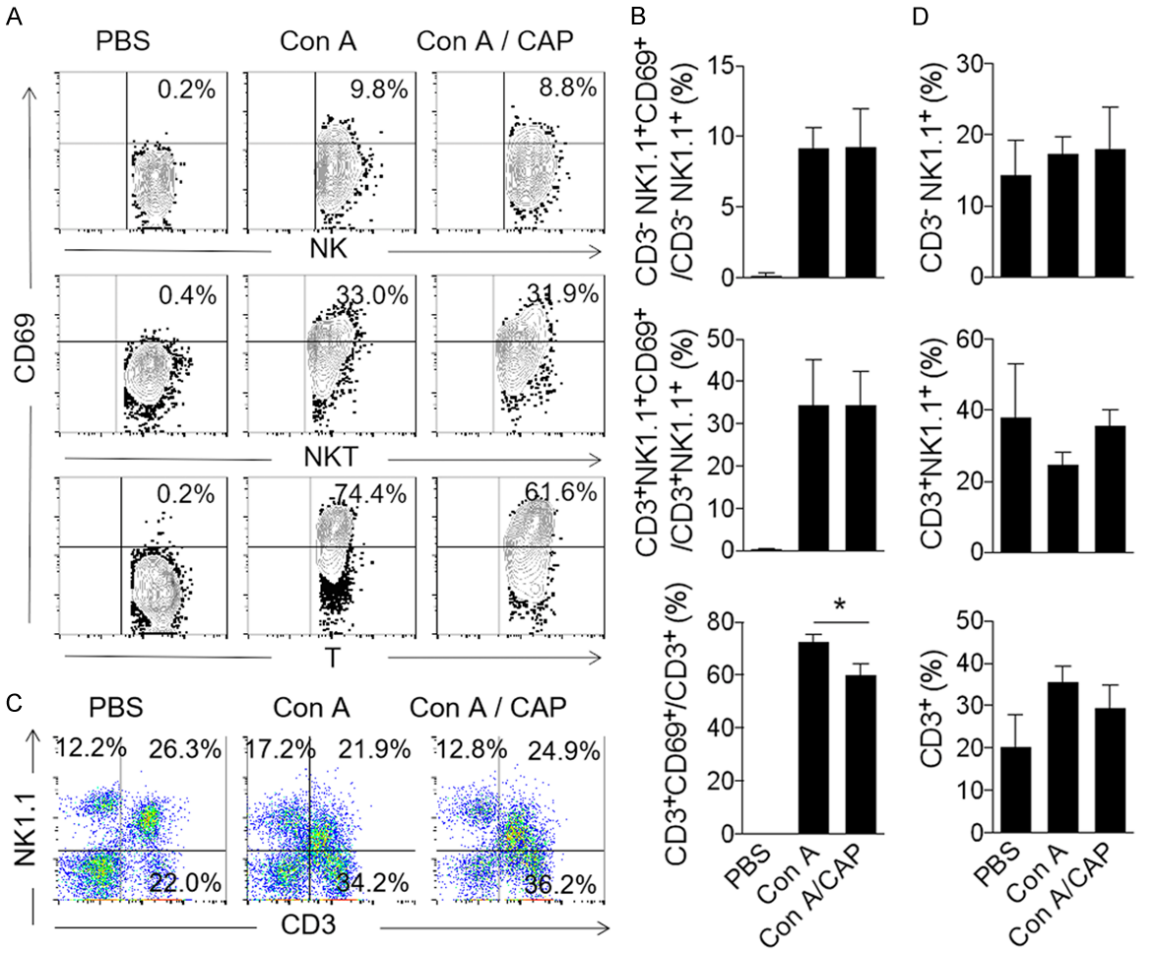

Figure 5. Capsaicin pretreatment inhibited T lymphocyte activation in the liver. Mice were treated as described in **Figure 3**. Hepatic mononuclear cells (MNCs) were prepared 12 h after Con A injection and were analyzed by FACS using PE-conjugated anti-CD3, FITC-conjugated anti-CD69, and APC-conjugated anti-NK1.1 antibodies. A, B. The effects of capsaicin on the percentage of $CD69^+$ T cells ($CD3^+$), NK cells ($CD3^-$ $NK1.1^+$), and NKT cells ($CD3^+$ $NK1.1^+$). C, D. Percentages of T cells ($CD3^+$), NK cells ($CD3^-$ $NK1.1^+$), and NKT cells ($CD3^+$ $NK1.1^+$). Data represent the means ± SD (n = 5-7). *P < 0.05; **P < 0.01.

mation. These findings, together with the fact that capsaicin downregulated proinflammatory cytokines, support the inhibition of apoptosis by capsaicin *in vivo*. Our results are consistent with previous reports that showed capsaicin conferring hepatoprotective effects to carbon tetrachloride-induced hepatic injury via antioxidant system induction, reducing MDA generation, and active caspase-3 inhibition.

It is well known that Con A-induced injury is primarily driven by the activation and recruitment of T cells. Besides T cells, other leukocytes such as NKT and NK cells are also involved in acute liver disease [1, 3, 4, 23]. After Con A exposure, these leukocytes are rapidly activated and recruited to the liver where numerous proinflammatory cytokines are secreted. Studies have revealed that depletion of NKT and NK cells by anti-NK1.1 antibody protected mice against Con A-induced hepatitis [34]. In the present study, pretreatment of mice with capsaicin partly suppressed the activation of T cells but did not show a discernable effect on the activation of NKT and NK cells. Furthermore, capsaicin also failed to decrease the accumulation of T and NKT cells in the liver.

We also examined the effect of capsaicin on MDSCs, which are a heterogeneous population of immature myeloid cells characterized by the co-expression of Gr-1 and CD11b. $Gr\text{-}1^+$ and $CD11b^+$ MDSCs are further subdivided into two major groups, $CD11b^+Ly6G^+Ly6C^{low}$ granulocyt-

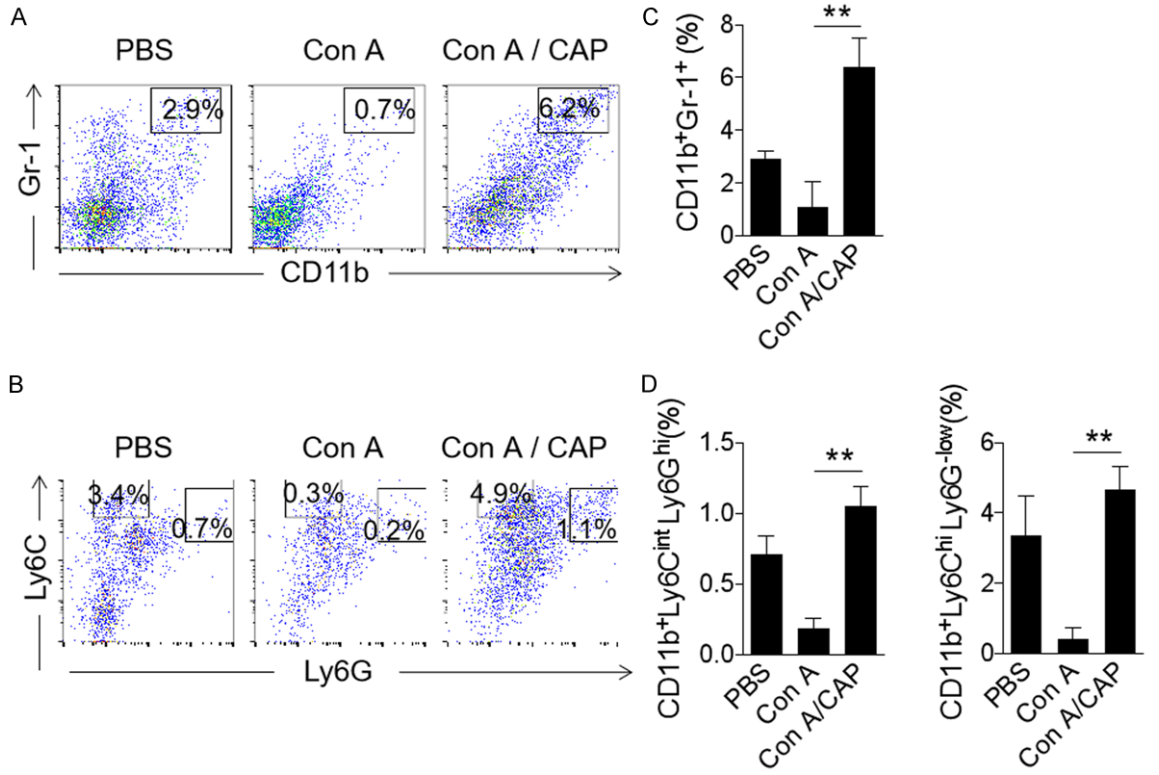

Figure 6. Capsaicin pretreatment increased the percentage of myeloid-derived suppressor cells (MDSCs) in the liver. Hepatic MNCs were isolated as described in **Figure 5** and were analyzed by FACS with FITC-conjugated anti-CD11b Ab, APC-conjugated anti-Gr-1 Ab, PE-conjugated anti-Ly6G Ab, and APC-conjugated anti-Ly6C antibodies. The percentages of (A, C) $CD11b^+Gr\text{-}1^+$ MDSCs as well as (B, D) $CD11b^+Ly6C^{int}Ly6G^{high}$ granulocytic and $CD11b^+Ly6C^{high}Ly6G^{low}$ monocytic MDSCs. Data represent the means ± SD (n = 5-7). *P < 0.05; **P < 0.01.

ic MDSCs and $CD11b^+Ly6G^-Ly6C^{high}$ monocytic MDSCs, both of which exhibit immunosuppressive activity [35]. Previous studies demonstrated that increasing the frequency of MDSCs by either adoptive transfer or glucocorticoid treatment alleviated Con A-induced liver injury, suggesting that MDSCs exert a direct protective role in T cell-mediated hepatitis [36]. Consistent with the results mentioned above, we found that capsaicin significantly increased the frequency of MDSCs in the liver. Similar results were reported by other researchers where cannabidiol, another activator of the vanilloid receptor/TRPV1, suppressed Con A-induced hepatitis, which involved the induction of MDSCs in the liver [37].

In summary, our study demonstrated that capsaicin can protect mice against acute liver injury induced by Con A. The protective effect of capsaicin was partially associated with its inhibition of hepatocyte apoptosis, oxidative stress, and inflammatory mediators as well as regulation of intrahepatic leukocyte activation and recruitment. Consequently, our findings highlight capsaicin as a potential therapeutic agent that can protect the liver from autoimmune hepatitis.

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Disclosure of conflict of interest

None.

Address correspondence to: Huabao Xiong, Department of Medicine, Precision Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA. Tel: +86 (212) 6599413; Fax: +86 (212) 8492525; E-mail: huabao.xiong@mssm.edu; Chuanping Si, Institute of Immunology and Molecular Medicine, Jining Medical University, Jining 27-2067, Shandong Province, China. Tel: +86 (537) 3616283; E-mail: chpsi@163.com

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