Resolvin D1 and E1 alleviate the progress of hepatitis toward liver cancer in long-term concanavalin A-induced mice through inhibition of NF-κB activity

HAOYU KUANG[1], XIAOLI HUA[2], JIANHUI ZHOU[1] and RUI YANG[3]

[1]Department of Laboratory, The Fifth Affiliated Hospital, Sun Yat-sen University, Zhuhai, Guangdong 519000; [2]Department of Pharmacy, Union Hospital of Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei 430022; [3]Department of General Surgery, Fifth Affiliated Hospital of Jianghan University, Wuhan, Hubei 430050, P.R. China

Abstract. Resolvins, an endogenous lipid mediator derived from eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) of fish oil, has been reported to have anti-inflammatory and antitumor effect in various pathogenic processes. However, there are no studies about the effects of resolvin D1 and E1 on concanavalin A-induced hepatitis. Hence, the present study is to illustrate whether resolvin D1 and E1 inhibit concanavalin A-induced liver injury, liver cancer and underlying mechanisms by which they may recover. C57BL/6 mice were pretreated with resolvin D1 and E1 for 4 h, and then injected with concanavalin A for 12 h. Subsequently, blood and liver tissue were collected at 0, 2, 4, 8 and 12 h for different analysis. Analysis of inflammatory cytokines indicated that the inhibition of necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2, IL-1β and IL-6 could be performed by resolvin D1 and E1. Moreover, Toll-like receptor (TLR) 4 expression, NF-κB and AP-1 activity also have been confirmed to have key roles in the development of liver injury. They were significantly suppressed in the treatment group, compared to model. In addition, resolvin D1 and E1 markedly downregulated CD4^{+} and CD8^{+} cell infiltration in the liver. A long-term concanavalin A treatment for 32 weeks was performed to analyze the changes of hepatitis to liver cancer and the antitumor effect of resolving D1 and E1. These results indicated that resolvin D1 and E1 prevent concanavalin A-induced liver injury and the changes of hepatitis to liver cancer in mice through inhibition of inflammatory cytokine secretion and NF-κB/AP-1 activity. Thus, they could be novel target to be considered in the process of finding sufficient drug to protect against various forms of hepatitis and liver cancer.

Introduction

Our liver, the largest internal organ and largest gland in the human body, plays an important role in many bodily functions from protein production and blood clotting to cholesterol, glucose and iron metabolism (1). The liver supports almost all organs in the body and is vital for survival. Due to its strategic location and multidimensional functions, the liver is also bringing about many diseases (2,3). Liver disease is also referred to as hepatic disease. Liver disease is regarded as a major public health problem worldwide due to serious morbidity and mortality (2,4). Liver disease can be caused by inherited disorders such as cystic fibrosis or hemochromatosis, a condition that involves having too much iron in your body or Wilson's disease, a disorder in which the body to retains too much cupric ion (5). Concanavalin A (Con A)-induced hepatitis in mice has been accepted as mature model that is very similar to the autoimmune hepatitis and virus caused pathogenic mechanisms and pathological changes in human body (6). Fulminant, T cell-dependent hepatitis and main inflammatory factors, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-6 cause liver damage, can be induced by a single intravenous (i.v.) injection of Con A (6,7).

A variety of studies demonstrated that Toll-like receptor (TLR) 4 signaling pathway was involved in the development and process of liver injury (7,8). Besides, Tu *et al* (8) found that TLR4 is the key between Con A-induced liver damage and inflammatory actions, and increasing evidence shows that TLR4/NF-κB pathway could be activated in Con A-induced liver injury (8). Hence, how to inhibit the activation of NF-κB pathway and prevent inflammatory cells or factors from mediating expression of TLR4 may be a target associated with autoimmune hepatitis.

Resolvins, an anti-inflammatory and endogenous lipid mediator family, have been reported to play an important role in the treatment of many diseases. Resolvin D1 and resolvin E1 (RvD1/RvE1) are compounds that are made by the human body

Correspondence to: Professor Xiaoli Hua, Department of Pharmacy, Union Hospital of Tongji Medical College of Huazhong University of Science and Technology, Wuhan, Hubei 430022, P.R. China
E-mail: huaxiaoli430022@126.com

from the ω-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (9-11). They are increasingly and continuously generated by COX-2 pathway especially in the presence of aspirin. Previous investigations of RvD1 or RvE1 exhibited many pharmacological effects such as anti-inflammation, neuroprotective actions and suppression of insulin resistance (12). However, to our knowledge, there are no related studies on whether RvD1 and RvE1 also provide a positive treatment to Con A-induced hepatitis in mice. In view of such a situation, in the present study we used Con A as inducer to construct autoimmune hepatitis model in order to investigate and evaluate the treatment actions of RvD1 and RvE1.

Materials and methods

Animals and drug administration. The 6-8 weeks old male C57BL/6 mice weighing 20-25 g were purchased from the Vital River Laboratory Animals Co., Ltd. (Beijing, China) and housed in a temperature and humidity-controlled environment (25±2°C, 50±5% humidity) with a standard 12 h on/off light cycle with food and water in cages. The Institutional Animal Care and Use Committee of the Sun Yat-sen University approved all the animal study protocols. RvD1 and RvE1 were purchased from Cayman Chemical Cooperation (Ann Arbor, MI, USA) and prepared in phosphate-buffered saline (PBS) before it was used. Con A (CAS 11028-71-0, purity ≥98%) was obtained from Santa Cruz Biotechnology Co., Ltd. (Shanghai, China). The mice were divided into 4 groups: i) control; ii) Con A group as model; iii) RvD1+Con A; and iv) RvE1+Con A. Mice were injected intravenously with PBS as a control. RvD1+Con A or RvE1+Con A group mice were pretreated with 10 µg/kg RvD1 or RvE1, without Con A treatment for 4 h. Then model and treatment group mice were injected intravenously with Con A (15 mg/kg) for 12 h. After Con A injection, blood and liver tissue were collected at 0, 2, 4, 8 and 12 h during the Con A administration.

ELISA measurement and biochemical analysis. After 12 h Con A administration and extracting the eyeball blood, the different period serum concentration of the inflammatory cytokines such as TNF-α, IFN-γ, IL-2, IL-1β and IL-6 were measured using ELISA kits according to the manufacturer's instructions (R&D Systems, USA). In addition, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also analyzed using a Diagnostics ALT and AST test kit from Sigma-Aldrich (St. Louis, MI, USA) (cat. nos. MAK052 and MAK055) and automatic biochemical analyzer (Hitachi Auto Analyzer 7170; Japan). Also, serum levels of RvD1, RvE1 and high mobility group protein B1 (HMGB1) were quantified using Cayman/MyBioSource resolvin ELISA kits and sandwich immunoassays from Biohelper Immune Test Corporation (Shanghai, China).

H&E staining and flow cytometric analysis. The collection of liver samples was subjected to hematoxylin and eosin (H&E) staining as previously described (8) and was examined for liver injury by light microscopy. For flow cytometric analysis, the lymphocytes were obtained through shearing liver tissue, separated by collagenase type II (Invitrogen, USA) digestion

and suspended in RPMI-1640 medium (Gibco-BRL). Cell suspensions were centrifuged at 1,000 rpm for 5 min to remove cellular debris and impurities. Then, the hepatic mononuclear cells (MNCs) were harvested and re-suspended in 70% Percoll (Sigma). MNCs were collected from the interphase, and washed twice in Hank's buffer (Gibco-BRL). According to the protocol of R&D Kit Systems for flow cytometry, anti-CD4 FITC and anti-CD8 FITC antibodies were added to the flow cytometry tube containing single-cell suspension, and these tubes were analyzed by Cytomics™ FC500 MCL of Beckman Coulter (USA).

Quantitative real-time PCR (qPCR). Total RNA was isolated from lymphocytes using the total RNA isolation system (Roboklon, Berlin, Germany). In brief, 2 µg of total RNA was reverse transcribed using the M-MLV-RT system (Promega). The system was performed at 42°C for 1 h and terminated by deactivation of the enzyme at 70°C for 10 min. PCR was performed using SYBR-Green kits (Bio-Rad) in ABI PRISM 7900HT detection systems (Applied Biosystems). Invitrogen Corporation produced all sequences of primers (TNF-α, IFN-γ, IL-6, TLR4, IκBα, IKKβ and NF-κB) for qPCR. The thermal cycling conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 sec, annealing at 58°C for 15 sec and extension at 72°C for 20 sec. Each gene expression level was standardized to the housekeeping gene (GAPDH) and expressed as a fold of control.

Western blot analysis. Equal amounts of total protein were subjected to 10 or 12% SDS-PAGE followed by immunoblotting using the following antibodies (1:1,000): rabbit anti-NF-κB, TLR4, IκBα, IKKβ, MyD88, JNK, ERK and p38 (Cell Signaling Technology, Inc., USA). Western blotting bands were visualized using GE Healthcare ECL Western Blotting Analysis system and exposed to Kodak X-ray film.

Statistical analysis. Data are expressed as means ± SD. Treated tissue and the corresponding controls were compared using GraphPad Prism (version 6.0; GraphPad Software, USA) by a one-way ANOVA with Dunn's least significant difference tests or Student's t-test. Differences between groups were considered significant at $p < 0.05$.

Results

RvD1 and RvE1 inhibit Con A-induced liver injury in mice. In order to investigate the role of RvD1/RvE1 in the Con A-induced liver injury, C57BL/6 mice were treated with 15 mg/kg Con A by intraperitoneal injection, followed by biochemical and histological evaluation of the liver injury. As anticipated, serum levels of ALT and AST, both of which are well-established markers of liver damage, were markedly increased in Con A group mice as early as 4 h after Con A injection (Fig. 1A-D). In addition, histological analysis also demonstrated representative and massive hepatocytes in Con A group. However, in RvD1 or RvE1 pretreated group, the extent of necrosis was diminished (Fig. 2A and B). Notably, the elevation of serum ALT and AST as well as the hepatic necrosis area in the model group were significantly higher than those in the treatment group. Whereas, the scale
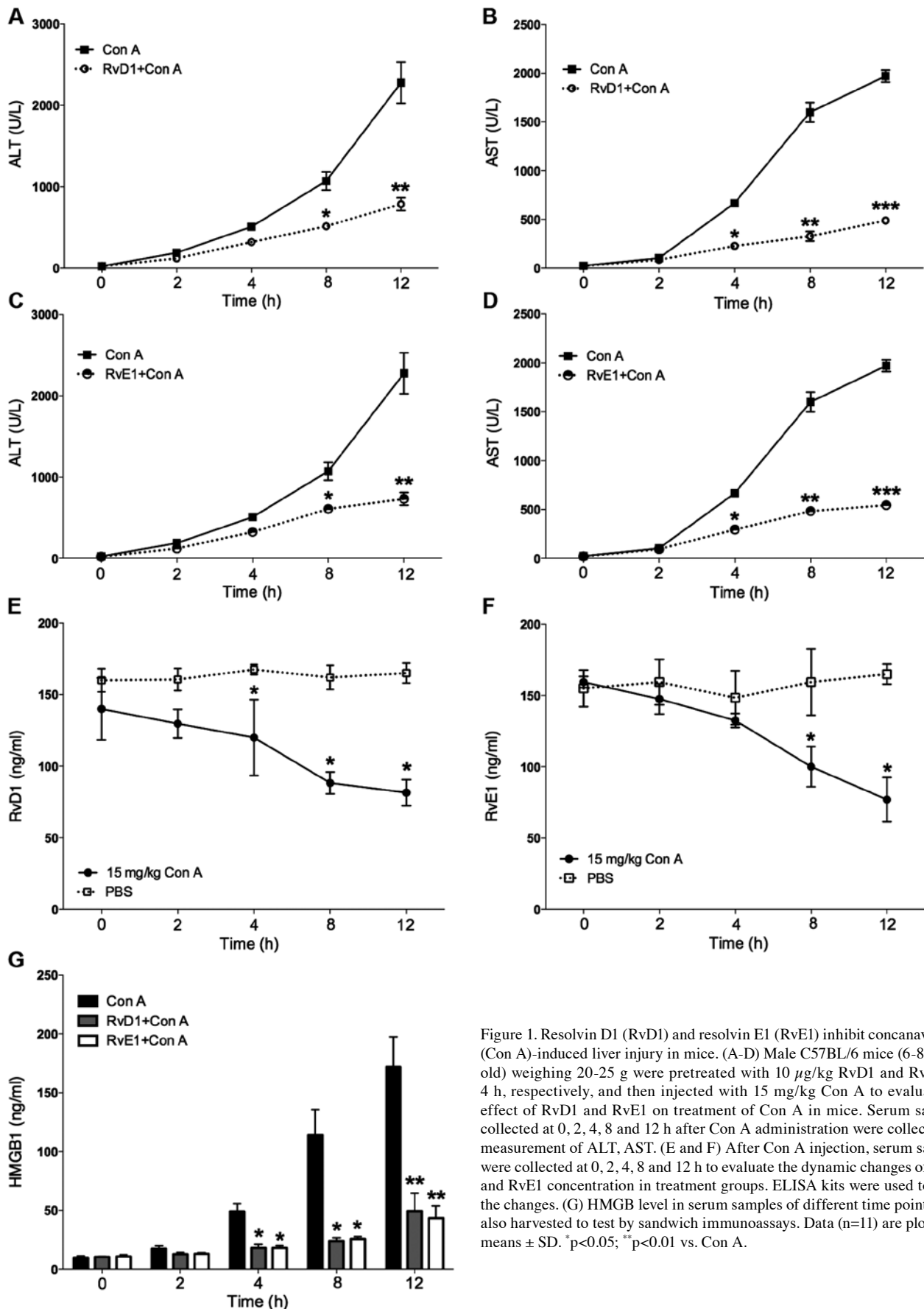
Figure 1. Resolvin D1 (RvD1) and resolvin E1 (RvE1) inhibit concanavalin A (Con A)-induced liver injury in mice. (A-D) Male C57BL/6 mice (6-8 weeks old) weighing 20-25 g were pretreated with 10 μg/kg RvD1 and RvE1 for 4 h, respectively, and then injected with 15 mg/kg Con A to evaluate the effect of RvD1 and RvE1 on treatment of Con A in mice. Serum samples collected at 0, 2, 4, 8 and 12 h after Con A administration were collected for measurement of ALT, AST. (E and F) After Con A injection, serum samples were collected at 0, 2, 4, 8 and 12 h to evaluate the dynamic changes of RvD1 and RvE1 concentration in treatment groups. ELISA kits were used to show the changes. (G) HMGB level in serum samples of different time points were also harvested to test by sandwich immunoassays. Data (n=11) are plotted as means ± SD. *p<0.05; **p<0.01 vs. Con A.

of increase in serum levels of the high mobility group protein B1 (HMGB1) in Con A injection group was higher than in the

mice in treatment group (Fig. 1G). We also investigated the dynamic changes of serum concentration of RvD1 and RvE1
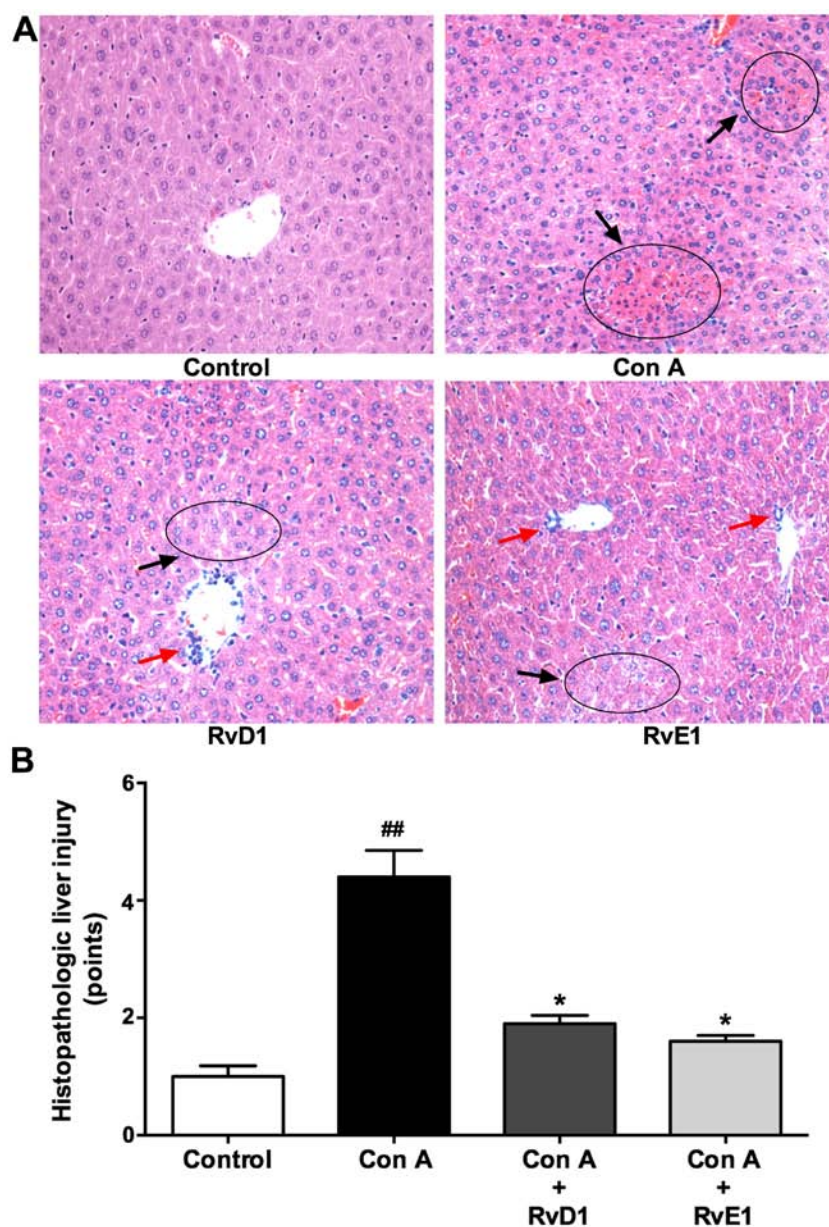
Figure 2. H&E staining analysis of liver sections at 12 h after concanavalin A (Con A) injection. (A) Liver samples was subjected to hematoxylin-eosin (H&E) staining and tested for liver injury by light microscopy. Histological sections of livers from Con A+RvD1 or Con A+RvE1 group mice indicated significantly suppressed injury compared with Con A group livers. (B) All the sections were classified by three pathologists according to the following criteria: 0, none; 1, individual cell necrosis; 2, ≤30% lobular necrosis; 3, ≤60% lobular necrosis; 4, >60% lobular necrosis. The black arrow indicates necrosis, and the red inflammatory cell infiltration. Data (n=9) are plotted as means ± SD. $^{\#\#}p<0.01$ vs. control; $^{*}p<0.05$ vs. Con A. RvD1, resolvin D1; RvE1, resolvin E1.

after Con A administration. RvD1 and RvE1 levels progressively decreased in response to Con A overdose (Fig. 1E and F).

Effect of RvD1 and RvE1 on the mRNA and production of pro-inflammatory cytokines in Con A-induced hepatitis. Pro-inflammatory cytokines play significant role in Con A-induced liver injury. As expected, our results in Fig. 3 show the dynamic changes of major pro-inflammatory cytokines in mRNA level. The increased intrahepatic expression levels of TNF-α, IFN-γ and IL-6 mRNA in response to Con A were suppressed by pre-treatment with the RvD1 and RvE1. Additionally, it is a common perspective that the progress of hepatitis is also associated with a series of pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-2, IL-1β and IL-6. Hence,

after Con A injection, ELISA was used to investigate the levels of the time points of TNF-α, IFN-γ, IL-2, IL-1β and IL-6 in serum production (Fig. 4). The inflammatory cytokines increased with Con A administration and the different factors were expressed at different time points, the production of TNF-α, IFN-γ, IL-2, IL-1β and IL-6 were inhibited with RvD1 and RvE1 pretreatment.

RvD1 and RvE1 prevent the NF-κB and MAPK signaling pathway in Con A-induced hepatitis. NF-κB or AP-1 signaling pathway mainly performs the production of pro-inflammatory factor release. In this regard, to determine whether RvD1 and RvE1 significantly suppressed NF-κB signal pathway activation in Con A-induced hepatitis, we observed the
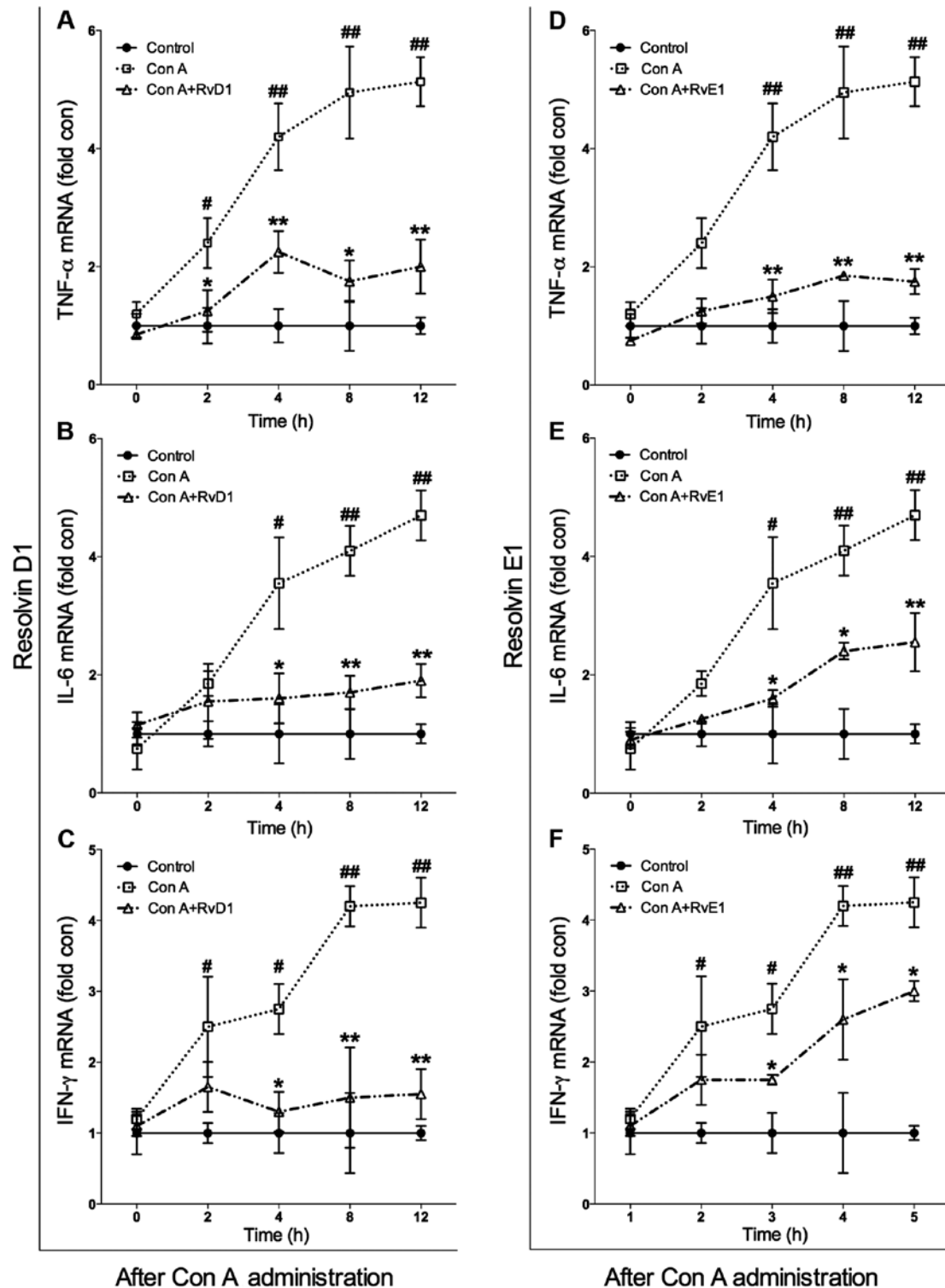
Figure 3. Effect of resolvin D1 (RvD1) and resolvin E1 (RvE1) on the mRNA level of pro-inflammatory cytokines in concanavalin A (Con A)-induced hepatitis. (A-F) Expression levels of TNF-α, IFN-γ and IL-6 mRNA in Con A-induced liver damage were measured by quantitative real-time PCR. Compared with control group, Con A+RvD1 or Con A+RvE1 markedly downregulated major inflammatory cytokines that represent factors within this model in Con A-induced liver damage. Data (n=9-11) are plotted as means ± SD. #p<0.05; ##p<0.01 vs. control; *p<0.05; **p<0.01 vs. Con A.

TLR4, NF-κB, IKKβ and IκBα expression in mRNA and protein level (Fig. 5). Both RvD1 and RvE1 pretreatment groups showed that the degradation of IKKβ and IκBα was obviously blocked by them at the mRNA level. Moreover, TLR4 has the key role in response to NF-κB pathway confirming that RvD1 and RvE1 contributed to the inhibition

of transcription. Furthermore, we investigated whether RvD1 and RvE1 could prevent JNK, ERK and p38 expression. As shown in Fig. 6, western blot analysis of NF-κB, TLR4, IκBα, IKKβ, MyD88, JNK, ERK and p38 found that these factors were downregulated in the presence of RvD1 and RvE1. Notably, JNK, ERK and p38, the major mediators involved
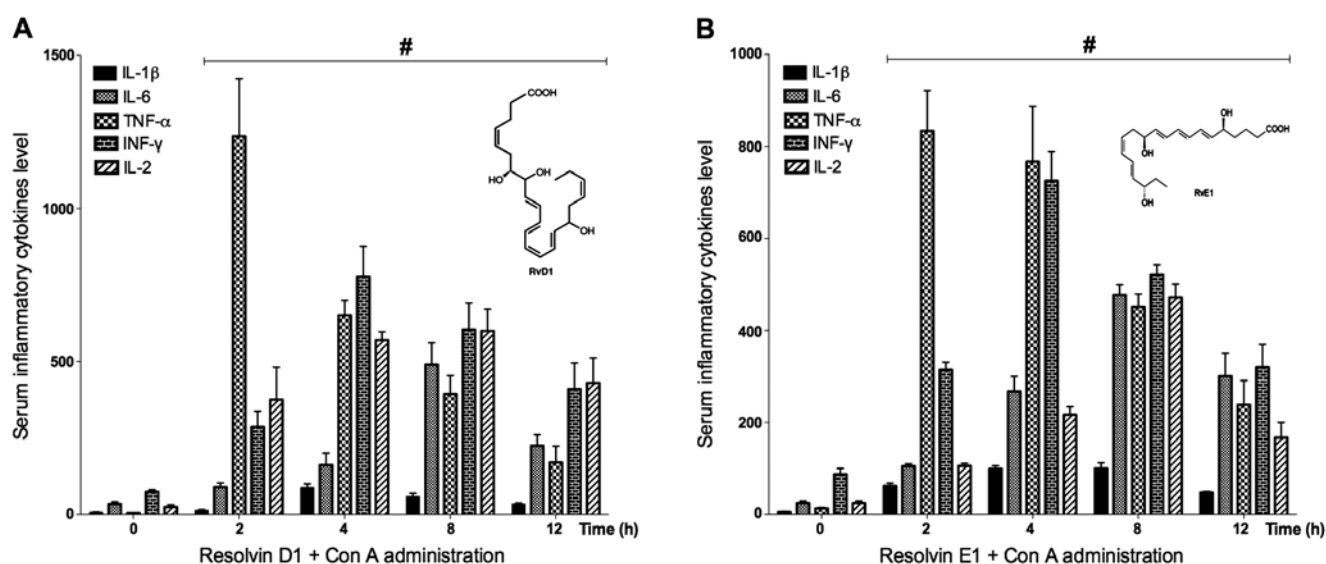
Figure 4. Effect of resolvin D1 (RvD1) and resolvin E1 (RvE1) on the production of pro-inflammatory cytokines in concanavalin A (Con A)-induced hepatitis. (A and B) ELISA kits were used to evaluate the expression of TNF-α, IFN-γ, IL-2, IL-1β and IL-6 in serum. Serum was collected at 0, 2, 4, 8, and 12 h to test the changes of RvD1 and RvE1 concentration in treatment groups. Importantly, apart from 0 h, in different time points or cytokines between 2-12 h, almost all showed upgrading first then descending tendency. Data (n=9) are plotted as means ± SD. #p<0.05 vs. control.
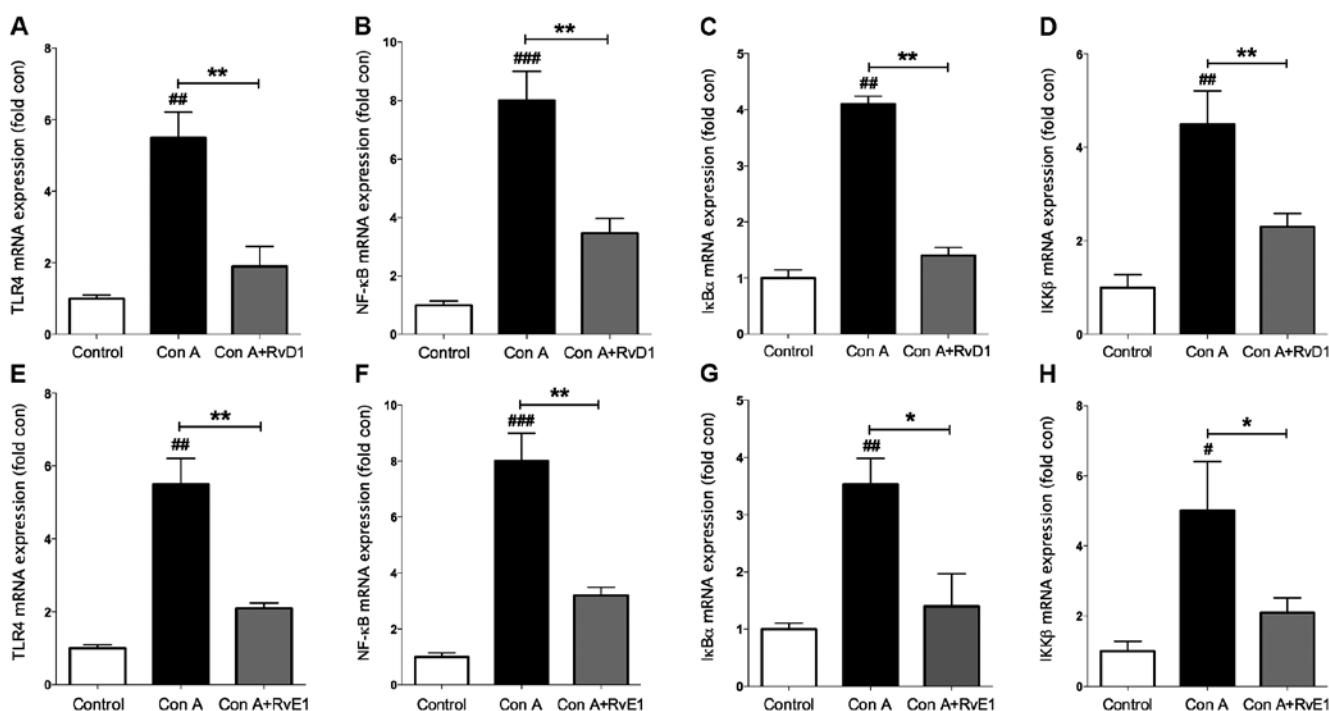

Figure 5. Effect of resolvin D1 (RvD1) and resolvin E1 (RvE1) on NF-κB and MAPK signaling pathway. (A-H) Quantitative real-time PCR measured the TLR4, NF-κB, IKKβ and IκBα mRNA level expression in concanavalin A (Con A)-induced liver injury. At 12 h point after Con A injection, tissue was collected to evaluate the possible signaling pathway involved in hepatitis. TLR4, NF-κB, IKKβ and IκBα as key links for NF-κB pathway were determined to take part in the development of inflammation. Data (n=9) are plotted as means ± SD. #p<0.05; ##p<0.01 vs. control; *p<0.05; **p<0.01 vs. Control.

in the AP-1 pathway, indicated that RvD1 and RvE1 may act directly or indirectly on cell signaling via inhibition of AP-1 transcriptional activity.

Effect of RvD1 and RvE1 on liver infiltrating CD4+ and CD8+ cells. We used flow cytometry to measure the CD4- and CD8-positive cells and the effect of pretreatment of RvD1 and RvE1 on the liver. The results in Fig. 7 show that

Con A significantly increased CD4+ and CD8+ markers. The percentage of positive cells in Con A group is ~18.96% (CD4+) and 14.61% (CD8+). Compared with model mice, RvD1 and RvE1 pretreatment significantly reduced CD4+ and CD8+ recruited into the liver. In addition, we further investigated the time-dependent changes of CD4+ and CD8+ T cells in Con A-induced hepatitis, and the CD4+/CD8+ ratio of T cells between control and RvD1, RvE1 treatment groups. As
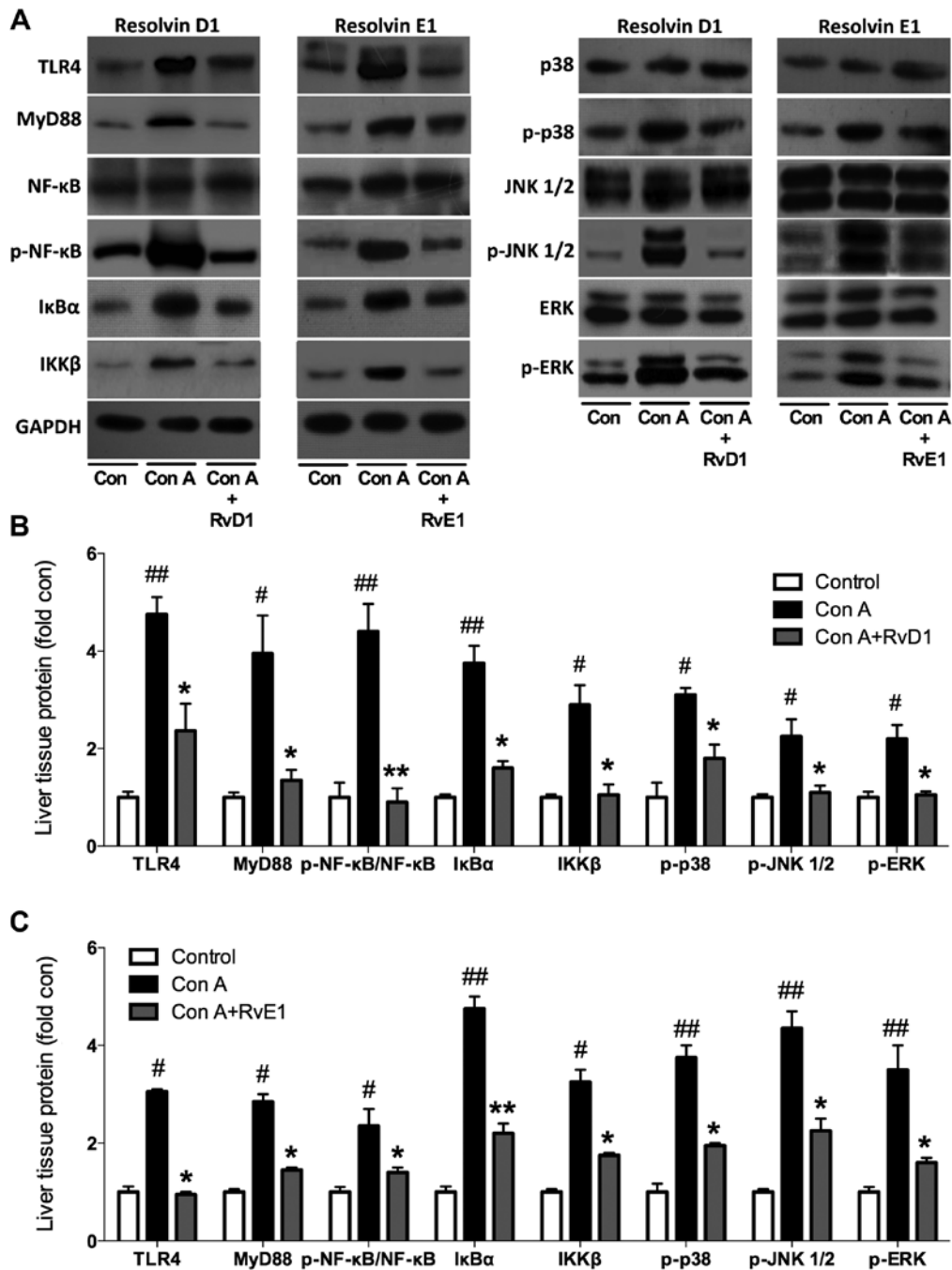
Figure 6. Resolvin D1 (RvD1) and resolvin E1 (RvE1) inhibit NF-κB and AP-1 activity. (A-C) Western blot analysis for NF-κB and AP-1 activity was used to evaluate the effect of RvD1 and RvE1 on concanavalin A (Con A)-induced liver injury and underlying mechanism. Liver tissues were harvested 12 h after Con A administration, NF-κB, TLR4, IκBα, IKKβ, MyD88, JNK, ERK and p38 protein levels were analyzed by western blotting with antibodies against TLR4, IκBα, IKKβ, MyD88, phospho-NF-κB p65, phospho-JNK1/2, phospho-ERK, phospho-p38 and JNK, ERK and p38. RvD1 and RvE1 pretreatment followed by Con A injection prevented IκBα, p65 and MAPK activation. Data (n=12) are plotted as means ± SD. $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs. control; $^{*}p<0.05$; $^{**}p<0.01$ vs. Con A.

expected, the data in Fig. 8 indicate that Con A administration has the ability to reduce CD4^{+} but partly increase CD8^{+} T cells with the extended treatment time in the blood. Also, the CD4^{+}/CD8^{+} ratio of T cells was enhanced with the RvD1 and RvE1, compared with 0 h.

RvD1 and RvE1 alleviate the progress of hepatitis toward liver cancer in long-term Con A-induced mice. As the results show in Fig. 9A, we tested the effect of a long-term Con A administration between 8 and 32 weeks on the changes of

hepatitis to liver cancer. The results showed that at 8 weeks, collagen deposition could be observed in the liver tissues; at 16 weeks, liver tissue hemorrhagic necrosis could be examined with H&E staining. In addition, at 32 weeks, the cancer cells were clearly observed. In contrast, the RvD1 and RvE1 were able to reverse these responses. As indicated in Figs. 9B and 10, we investigated the effect of RvD1 and RvE1 on IκBα expression and apoptosis in HepG2 cells. The results showed that RvD1 and RvE1 could inhibit IκBα degradation and enhance apoptosis of HepG2 in 48 h.
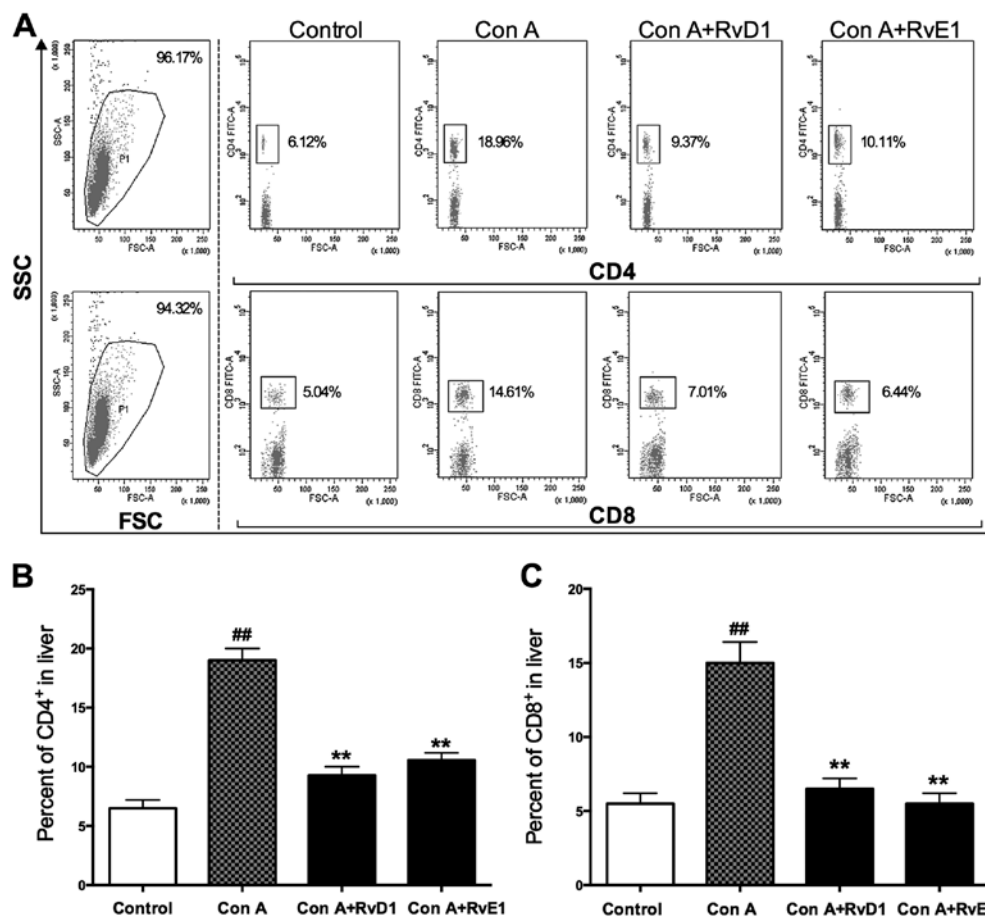
Figure 7. Effect of resolvin D1 (RvD1) and resolvin E1 (RvE1) on liver infiltrating CD4$^+$ and CD8$^+$ cells. Flow cytometric analysis for hepatic lymphocytes after 12 h concanavalin A (Con A) administration. The T cells were stained with APC or PE-conjugated mAbs. (A) Shown are CD4$^+$ T cells (using anti-CD4 Ab) and CD8$^+$ T cells (using anti-CD8 Ab). (B and C) The percentage of CD4$^+$ T and CD8$^+$ T cells were counted and pretreatment of RvD1 and RvE1 significantly reduced CD4$^+$ T and CD8$^+$ T cells compared with Con A injection group. Data (n=9) are plotted as means ± SD. $^{\#}p<0.05$; $^{\#\#}p<0.01$ vs. control; $^{*}p<0.05$; $^{**}p<0.01$ vs. Con A.
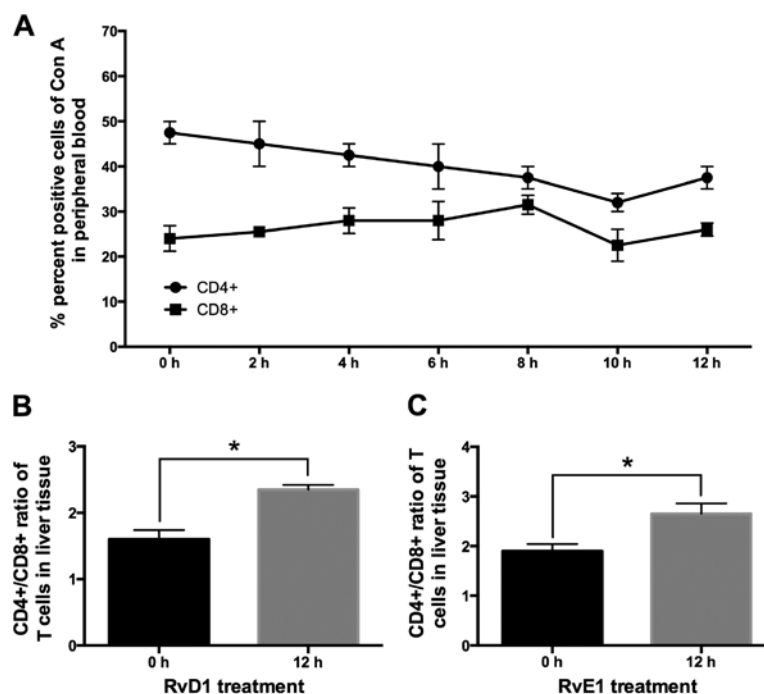

Figure 8. Effect of resolvin D1 (RvD1) and resolvin E1 (RvE1) on the ratio of liver infiltrating CD4$^+$/CD8$^+$. (A) The change of CD4$^+$ T and CD8$^+$ T cells in concanavalin A (Con A)-induced peripheral blood. (B and C) The ratio of CD4$^+$/CD8$^+$ in Con A-induced hepatitis. Data (n=9) are plotted as means ± SD. $^{*}p<0.05$ vs. Con A.
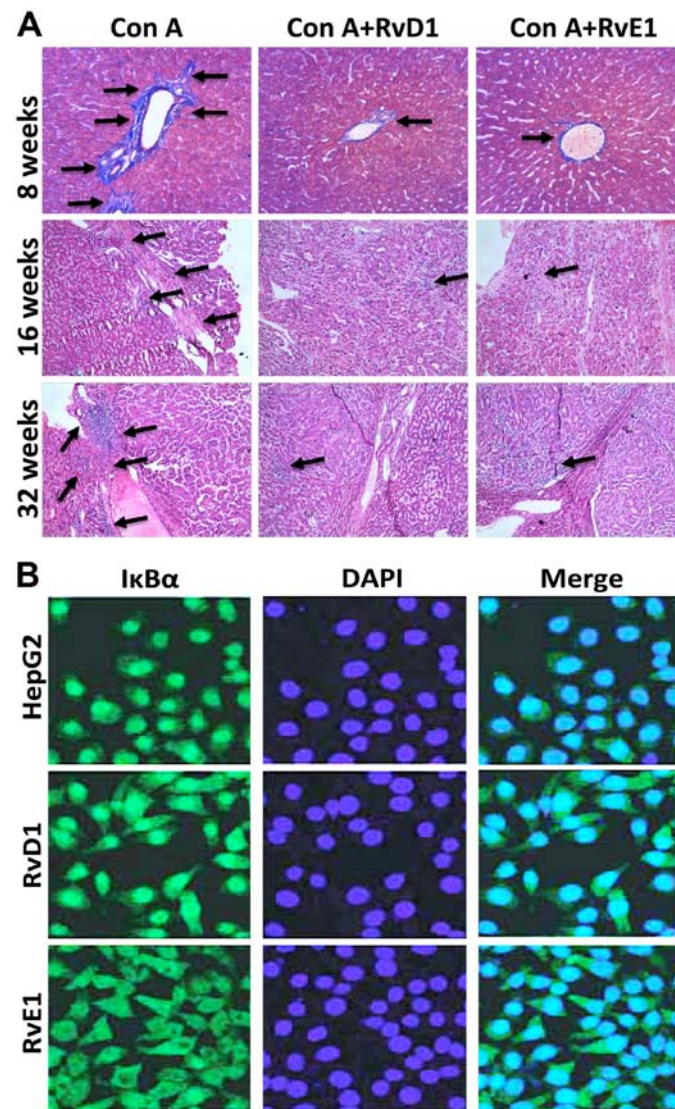
Figure 9. Resolvin D1 (RvD1) and resolvin E1 (RvE1) alleviate the progress of hepatitis toward liver cancer in long-term concanavalin A (Con A)-induced mice. (A) The changes of hepatitis toward liver cancer between 8 and 32 weeks. (B) The effect of RvD1 and RvE1 on IκBα protein expression in HepG2 cells.
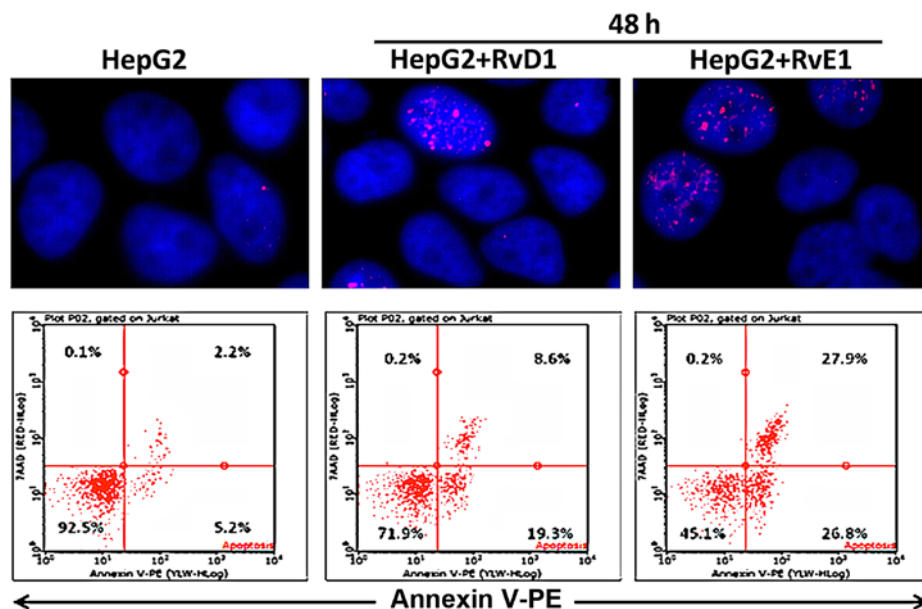

Figure 10. Resolvin D1 (RvD1) and resolvin E1 (RvE1) induce apoptosis of HepG2.

Discussion

Liver diseases are regarded as a serious threat to mankind (1-3). Various diseases are included such as acute and chronic hepatitis, alcoholic or non-alcoholic fatty liver, liver cirrhosis and hepatocellular carcinoma. Williams (13) reported that chronic diseases cause 46% of global disease and 59% of mortality. Also, the Office for National Statistics in the UK indicated that liver disease is the fifth most common cause of death after heart disease, stroke, chest disease and cancer (14). Particularly, hepatocellular carcinoma caused by hepatitis B surface antigen is among the first three causes of death from cancer in men, and a major cause of cancer in women (15-17). Substantial evidence has been collected confirming the relationship between hepatitis and hepatocellular carcinoma. As an infectious disease, hepatitis spreads rapidly and has a high mortality rate, however, no effective drugs against liver disease are available (18). Therefore, more effective drugs are needed for protect against hepatitis.

Resolvins, a family of endogenous lipid mediators derived from ω-3 polyunsaturated fatty acid, has been generated from EPA and DHA of fish oil. This family of lipid mediators include resolvin E and D, showing obvious potency in treating hepatic fibrosis in mice with infection, liver cirrhosis or neurodegenerative disorders (11), especially for the disease conditions associated with inflammation. Importantly, various studies showed that resolvins have an ability to perform potent anti-inflammatory actions in obesity-induced liver disease (19). Hence, concanavalin A (Con A), as an important and confirmed contributor to the pathological process of hepatitis caused by activate T lymphocytes *in vitro*, was used to investigate whether resolvin D1 (RvD1) and resolvin E1 (RvE1) perform potent anti-inflammatory action and the underlying mechanism. Given the fact that Con A-induced autoimmune hepatitis by releasing a number of inflammatory cytokines such as IL-2, IL-4, IL-6, TNF-α and IFN-γ (20). In our studies, RvD1 and RvE1 downregulated the expression of TNF-α, IFN-γ, IL-2, IL-1β and IL-6 in serum. After the Con A injection, the inhibition of RvD1 and RvE1 for cytokines in response to the increase of action time between 0, 2, 4, 8 and 12 h, caused dynamic changes of RvD1 and RvE1 in serum between resolvin concentrations and Con A-induced inflammatory effect. Previous studies demonstrated that decrease of TNF-α, IFN-γ or IL-6 could suppress Con A-induced liver damage in rat. Published results illustrated that resolvins family inhibited LPS-stimulated TNF-α and IL-1β release and reduced the expression of TNF-α and IFN-γ mRNA in the liver (21). Compared with the Con A, in our data, production of major inflammatory factors such as TNF-α, IFN-γ, IL-2, IL-1β, IL-6 were significantly reduced with RvD1 and RvE1 treatment in protein and mRNA levels.

Nuclear factor-κB (NF-κB) is a transcription factor playing a vital role in inflammation, immunity, cell proliferation, differentiation, and survival. Given the fact that NF-κB also has been considered as a central link in the pathogenic processes of hepatic injury, fibrosis and HCC (22), indicating its importance as a target point in treatment of liver disease. Sahin *et al* have reported that Con A-induced liver injury is a response to TLR4 upregulation as compared with the control

group (23). In our data, compared with the control, Con A significantly increased TLR4 expression; however, RvE1 and RvD1 pretreatment significantly blocked the upregulation of TLR4. Furthermore, both RvD1 and RvE1 were inhibitory on NF-κB activation and the degradation of IκBα and IKKβ. Supporting previous data, Hsieh *et al* reported that TLR4/NF-κB acts as a bridge role in inflammatory inducer and pro-inflammation production (24), showing that the inhibition of RvD1 and RvE1 effect on Con A-induced liver damage may be significantly involved in the TLR4/NF-κB signaling pathway.

The activator protein-1 (AP-1) activity was also determined as an important part in hepatic lipid metabolism and liver injury development. Notably, mitogen-activated protein kinases (MAPKs) in regulation of AP-1 activity are a common mediator of hepatitis from various stimuli. The ERK, p38 and JNK MAPK pathways, which are known to activate the AP-1 as a homo- or heterodimer, contribute to the development of liver damage and release of inflammatory factors. However, there are no related potential mechanism studies to confirm whether RvD1 and RvE1 could act as an effective inhibitor to prevent MAPK in Con A-induced hepatitis. Our preliminary study results indicated that RvD1 and RvE1 pretreatment significantly blocked ERK, p38 and JNK activation. Based on these results, it was concluded that resolvins have the ability to decrease MAPK/AP-1 activation (25).

All of the above results may provide the reason to explain the underlying mechanism of RvD1 and RvE1 inhibitory effect in liver injury. Besides, Liu *et al* (26) indicated that T lymphocytes have the key role in the development of Con A-induced liver injury. Hence, we used flow cytometry to analyze the CD4- and CD8-positive cells and the inhibitory effect of RvD1 and RvE1 on the liver. We found that the percentage of CD4+ and CD8+ cells in the liver significantly increased after Con A administration at 12 h, compared with the control. In contrast, the pretreatment of resolvin D1 and E1 group markedly decreased CD4+ and CD8+ cells. These results show that RvD1 and RvE1 pretreatment were associated with a prominent decrease of CD4+ and CD8+. Therefore, RvD1 and RvE1 recover Con A-induced injury, at least in part, by preventing CD4+ and CD8+ cell infiltration in the liver. Also, in general, CD4+/CD8+ ratio is the standard marker for the extent of liver inflammation or injury in hepatitis. Thus, we further investigated the effect of RvD1 and RvE1 on CD4+/CD8+ ratio in Con A-induced liver injury. We found that in hepatitis, there is decrease in hepatic CD4+ cells and subsequent increase in infiltration of CD8+ T cells. RRvD1 and RvE1 were able to increase the ratio of CD4+/CD8+ in Con A-induced liver injury.

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