

IL-1Ra Protects Hepatocytes from CCl₄-Induced Hepatocellular Apoptosis via Activating the ERK1/2 Pathway

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Abstract

Interleukin-1 receptor antagonist is an important acute-phase protein and an immune mediator, and its expression is associated with the development of hepatitis or acute liver failure. The aim of this study was to investigate whether recombinant human interleukin-1 receptor antagonist directly targets and improves cell survival in a carbon tetrachloride-induced hepatocyte injury model *in vitro*. A human hepatoma cell line and a mouse hepatocyte cell line were used to establish carbon tetrachloride-induced cell injury models *in vitro*, and cell viability, apoptosis, and reactive oxygen species level were determined to assess the degree of hepatocellular damage. Quantitative real-time polymerase chain reaction was used to analyze the level of interleukin-1 β , interleukin-6, and tumor necrosis factor- α mRNA in cells; extracellular regulated protein kinases 1/2 phosphorylation in hepatocytes was analyzed using western blotting. Recombinant human interleukin-1 receptor antagonist could directly target hepatocytes, improve cell survival, and decrease carbon tetrachloride-induced cell apoptosis *in vitro*. In hepatocytes, recombinant human interleukin-1 receptor antagonist remarkably downregulated expression of interleukin-1 β , interleukin-6, and tumor necrosis factor- α in hepatocytes exposed to carbon tetrachloride. It also decreased accumulation of reactive oxygen species and abrogated the suppression of extracellular regulated protein kinases 1/2 phosphorylation induced by carbon tetrachloride. However, stimulation of cells with an extracellular regulated protein kinases 1/2 inhibitor blocked the recombinant human interleukin-1 receptor antagonist-induced upregulation of extracellular regulated protein kinase 1/2 activation and abrogated the improvement in hepatocyte survival following carbon tetrachloride treatment. Collectively, these findings provide new insights into the hepatocyte-protective mechanism of recombinant human interleukin-1 receptor antagonist.

Keywords

- ▶ rhIL-1Ra
- ▶ reactive oxygen species
- ▶ CCl₄
- ▶ ERK1/2
- ▶ hepatocyte
- ▶ apoptosis
- ▶ IL-1Ra

Introduction

Interleukin-1 receptor antagonist (IL-1Ra), a structural variant of IL-1, can specifically block IL-1 receptor (IL-1R) signal transduction when IL-1 α or interleukin-1 β (IL-1 β) binds to

IL-1R. The IL-1R pathway is involved in the regulation of the immune system; it also suppresses inflammation and effectively reduces tissue damage caused by inflammation.^{1,2} It has been reported that the prognosis of patients with liver disease is positively correlated with the level of IL-1Ra.³ It is suggested that IL-1Ra exerts protective effects in acute hepatocyte injury

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and promotes liver regeneration.⁴ Interestingly, recent studies have shown that IL-1Ra can effectively attenuate hepatotoxicity in mouse models of liver injury, such as carbon tetrachloride (CCl₄) or acetaminophen-induced liver injury models.⁵⁻⁷ Hepatocyte-specific deletion of IL-1RI could depress IL-1RI-mediated inflammation, significantly attenuate liver injury, and improve hepatocyte survival in the case of *D*-galactosamine/lipopolysaccharide-induced liver injury in mice.⁸ IL-1Ra also modulated Kupffer cells to decrease inflammation in the liver in mice.⁹ However, the mechanism underlying the protective effects of IL-1Ra on hepatocytes remains unclear, and investigation of IL-1Ra directly targeting hepatocytes should be useful to further understand the function of IL-1Ra in liver injury. CCl₄ in the liver could promote the formation of free radicals by metabolism, causing peroxidation of cell and organelle membrane lipids, resulting in hepatocyte apoptosis and necrosis.¹⁰ The CCl₄-induced hepatocyte injury model is widely used to study the protective functions of compounds, natural products, or genes in hepatocytes *in vitro*.^{11,12} Hepatoblastoma cells (HepG₂ cells) present the characteristics of hepatocytes and are widely used to establish *in vitro* cell models of liver injury and for the *in vitro* evaluation of protective effects of drugs.^{13,14}

In this study, HepG₂ cells and AML-12 cells, a mouse hepatocyte cell line, were used to establish models of CCl₄-induced hepatocyte injury.¹⁵ These models were used to investigate the mechanism by which rhIL-1Ra (recombinant human IL-1Ra) directly protects hepatocytes *in vitro*. We analyzed the effects of rhIL-1Ra on cell viability or apoptosis after CCl₄ treatment and explored the regulation of intracellular reactive oxygen species (ROS) accumulation and expression of apoptosis-related cytokines, including IL-1 β , IL-6, and tumor necrosis factor- α (TNF- α) in the injured model cells. We also determined the function of the ERK (extracellular regulated protein kinases) 1/2 pathway in the mechanism by which rhIL-1Ra protects hepatocytes against CCl₄. Our data suggested that the ERK1/2 pathway played a critical role in the protective effects of rhIL-1Ra on hepatocytes, and that rhIL-1Ra could significantly regulate ROS accumulation and cell apoptosis-related cytokine expression in CCl₄-induced hepatocytes.

Materials and Methods

Cell Line and Reagents

HepG₂ and AML-12 cells were purchased from the Chinese Academy of Sciences Cell Bank. Dulbecco's minimal essential medium (DMEM), DMEM/F12, fetal bovine serum (FBS), 0.25% trypsin, 1% penicillin/streptomycin, and TRIzol reagent were obtained from Thermo Fisher (Grand Island, New York, United States). CCl₄ was purchased from Lingfeng Chemical Reagent (Shanghai, China), and rhIL-1Ra was obtained from Wuyang Biotechnology (Shanghai, China). ERK1/2 inhibitor, SCH772984, was purchased from MedChemExpress Company (Shanghai, China). Hoechst 33342 was obtained from Sigma-Aldrich (St. Louis, Missouri, United States). All other chemicals and reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China) unless specifically described.

Cell Culture and CCl₄ Treatment

HepG₂ cells were cultured in DMEM containing 10% FBS. AML-12 cells were cultured in DMEM/F12 supplemented with 10% FBS, 1% ITS liquid media supplement (Sigma, St. Louis, Michigan, United States), and 40 ng/mL dexamethasone (Sorlabo, Beijing, China). Cells were maintained in a humidified incubator supplied with 5% CO₂ at 37°C. CCl₄ solution was prepared as described previously.¹⁶ Briefly, CCl₄ was dissolved in a completed medium (DMEM + 10% FBS) and incubated overnight at 37°C, and the saturated CCl₄ culture medium was prepared. A 70% saturated CCl₄ medium (diluted with fresh medium, named CCl₄-medium) was used to induce hepatocyte injury *in vitro*. After 4 hours of CCl₄ treatment, the cells were lysed with RIPA buffer for western blot analysis, and total RNA was extracted using TRIzol reagent.

Cell Viability Assay

HepG₂ and AML-12 cells were seeded in 96-well plates and incubated overnight in a humidified incubator supplied with 5% CO₂ at 37°C. Cells were treated with CCl₄-medium alone or CCl₄-medium containing different concentrations of rhIL-1Ra (10, 20, or 50 μ g/mL). Control cells were treated with fresh medium. After 4 hours of treatment, cell viability was assessed using CCK-8 reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. To investigate the function of ERK1/2, IL-1Ra-protecting hepatocytes in the 96-well plate were treated with CCl₄-medium containing PBS, IL-1Ra (50 μ g/mL), or IL-1Ra (50 μ g/mL)/SCH772984 (3 nmol/L). Cell viability was analyzed using a CCK-8 kit after 4 hours of treatment, and data were presented as average \pm standard deviation (SD).

Flow Cytometry

HepG₂ and AML-12 cells were treated with CCl₄-medium, CCl₄-medium/rhIL-1Ra (50 μ g/mL), or fresh medium as control. Cells were harvested after 4 hours of drug treatment and washed twice with chilled PBS on ice. Cellular apoptosis was assessed using an Annexin V-FITC/PI apoptosis detection kit (Vazyme Technology, Nanjing, China). At least 10,000 cells were analyzed with a flow cytometer (CyExpert, Brea, California, United States). For analyzing intracellular ROS level, cells were harvested and washed after 15 minutes of DCFH-DA treatment.

Analysis of Intracellular ROS Level

DCFH-DA (Beyotime, Shanghai, China) was used as a probe for detecting intracellular ROS level according to manufacturer's manual book.¹⁷ Briefly, cells were incubated in the fresh medium containing 10 μ mol/L DCFH-DA for 15 minutes after 4 hours of treatment with PBS, CCl₄, or CCl₄/IL-1Ra. A flow cytometer (CyExpert) and a fluorescence microscope were used to detect the changes in fluorescence intensity, which reflect the ROS level. For fluorescence microscopy, cells in a six-well plate were washed by cold PBS and stained with Hoechst 33342 (5 μ g/mL) for 2 minutes. Cells were directly observed under the fluorescence microscope after washing twice with cold PBS.

Table 1 Oligonucleotide primers used in quantitative PCR

Gene symbol	Sense (5'–3')	Antisense (5'–3')
IL-1 β	Mouse: TGAGCACCTTCTTTTCCTC Human: TTGTTGCTCCATATCCTGTCC	Mouse: GTTCATCTCGGAGCCTGTAG Human: CACATGGGATAACGAGGCTT
IL-6	Mouse: CCACTCCAACAGACCTGTCTA Human: GGAGACTTGCCTGGTGAA	Mouse: CACAACCTTTTTCTCATTCCA Human: GCATTTGTGGTTGGGTCA
TNF- α	Mouse: CCACGTCGTAGCAAACCACC Human: CACTAAGAATTCAAACTGGGGC	Mouse: GATAGCAAATCGGCTGACGG Human: GAGGAAGGCCTAAGGTCCAC
β -Actin	Mouse: AGCCTTCCTTCTGGGTATG Human: TGTATGCCTCTGGTCTACCAC	Mouse: GTGTTGGCATAGAGGTCTTTAC Human: ACAGAGTACTTGCCTCAGGAG

Quantitative Real-Time PCR Analysis

Real-time polymerase chain reaction (RT-PCR) was performed with an Applied Biosystems StepOnePlus Real-Time PCR system (Life Technologies, United States). Cytokines associated with liver injury (IL-1 β , IL-6, and TNF- α) were selected for quantitative RT-PCR (qRT-PCR) and β -actin was used as a control. Specific primers were from literatures or designed using Primer BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>), and oligos are listed in **Table 1**.^{18,19} Total RNA from AML-12 and HepG2 cells were prepared using TRIzol reagent (Life Technologies, United States). Total RNA (2 μ g) was used as a template for synthesizing the first-strand of cDNA using reverse transcriptase reaction mixture (Takara, Japan).

For RT-PCR, PCR mix (FastStart Universal SYBR Green Master, Roche, Switzerland) was used in the reaction system and thermocycler conditions according to the manufacturer's instructions. Relative expression data were normalized with β -actin and presented as a ratio to control.

Western Blot Analysis

HepG₂ and AML-12 cells were seeded in six-well plates. Western blotting was performed as previously described.²⁰ After cells were harvested and washed with cold PBS, they were lysed with RIPA buffer (150 mmol/L NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0, protease inhibitor cocktail, phosphatase inhibitor cocktail). The antibodies used in this study, antiphosphorylated ERK1/2 and anti-ERK1/2, were purchased from Cell Signaling Technology (Danvers, Massachusetts, United States). Anti-GAPDH primary antibody was obtained from GeneTex Inc. (Irvine, California, United States). HRP-conjugated secondary antibody was purchased from Jackson Immuno Research Laboratory (Western Grove, Pennsylvania, United States). The PVDF membrane and ECL substrate were purchased from Merck Millipore (Darmstadt, Germany).

Data Analysis

All data are shown as average \pm SD. Statistical analyses were performed with Excel software (Microsoft, Washington, United States), and figures were produced with GraphPad Prism 5. In experiments, Student's *t*-test was used when comparing between two groups. All tests were two-sided, and *p* < 0.05 was considered to be statistically significant.

Results

rhIL-1Ra Attenuated CCl₄-Induced Cytotoxicity in Both HepG2 and AML-12

CCl₄ induces cytotoxicity by disturbing the redox homeostasis and the cell membrane structure in hepatocytes. Cytokines associated with proinflammatory responses (e.g., IL-1 and IL-6) are also involved in this process.⁸ CCl₄ could markedly induce changes in cell morphology in both cell lines. HepG2 and AML-12 were treated by CCl₄ with or without the endogenous inhibitor of the IL-1/IL-1R pathway, rhIL-1Ra. However, rhIL-1Ra can reduce the effects of CCl₄ on hepatocyte morphology in a dose-dependent manner (**Fig. 1A**). Hepatocytes are very sensitive to CCl₄, and the percentage of surviving cells decreased to 10.37 \pm 4.22% in the HepG2 cell line and to 11.76 \pm 2.41% in the AML-12 cell line after 4 hours of CCl₄ treatment *in vitro*. rhIL-1Ra significantly improved cell survival after HepG2 and AML-12 cells were treated with CCl₄. Our data indicate that 20 μ g/mL rhIL-1Ra could efficiently increase cell viability in HepG2 (23.05 \pm 4.13%) and AML-12 (61.62 \pm 5.12%) cells, and that the protective effects of rhIL-1Ra are dose-dependent (**Fig. 1B**).

rhIL-1Ra Downregulates ROS Accumulation and Protects Cells against Apoptosis

It is well known that ROS accumulation is one of most critical processes leading to liver injury or hepatocyte apoptosis.^{21,22} In the liver microenvironment, ROS accumulation stimulates cytokine secretion, which in turn leads to further ROS accumulation. This positive feedback loop aggravates hepatocyte necrosis and apoptosis.²³ In this study, fluorescence microscopy and flow cytometry were used to analyze the ROS levels in cells. The results from both methods were consistent. CCl₄ increased the ROS level in HepG2 or AML-12 cells, and rhIL-1Ra could significantly reduce ROS accumulation in the cells (**Fig. 2**).

CCl₄ induced hepatocyte apoptosis and necrosis in both *in vivo* and *in vitro* liver injury models. In this study, rhIL-1Ra could significantly protect HepG2 and AML-12 cells exposed to CCl₄. Compared with the control group, the proportions of necrotic and apoptotic cells were higher in the control group than in the experimental group. After the addition of rhIL-1Ra, the hepatocyte injury induced by CCl₄ was significantly reduced, and the proportion of apoptotic cells decreased (**Fig. 3**).

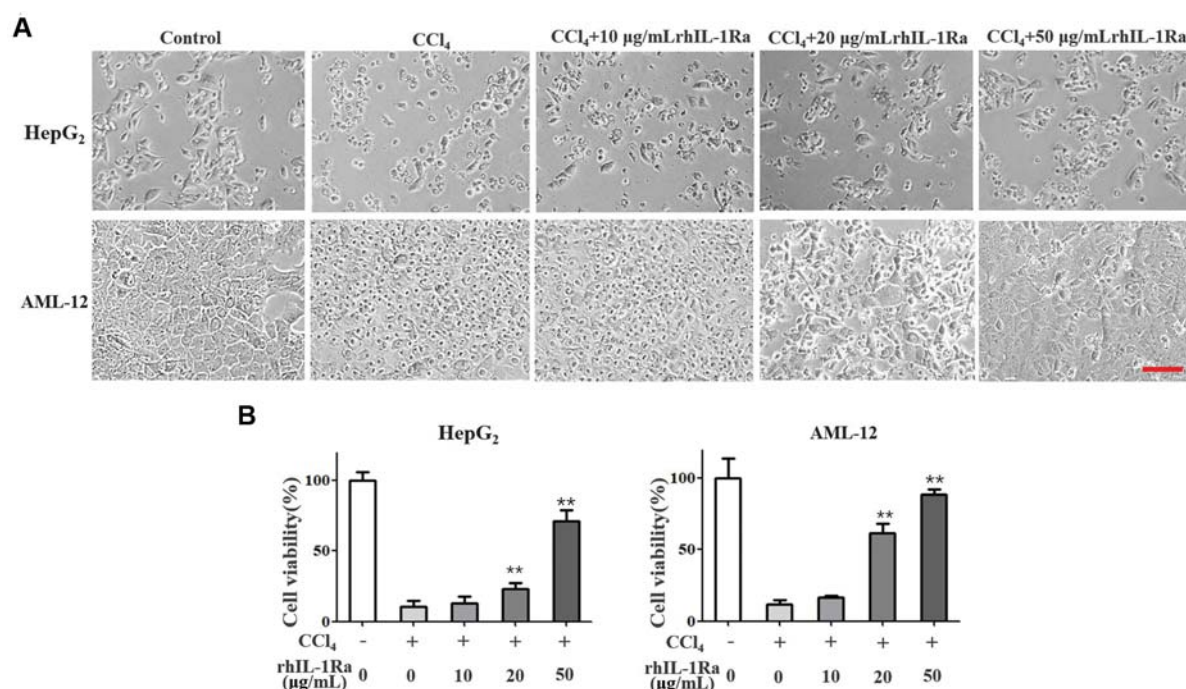


Fig. 1 rhIL-1Ra protects against CCl₄ cytotoxicity in HepG₂ and AML-12 cells. (A) Morphological changes induced by CCl₄ or CCl₄/rhIL-1Ra were observed using a light microscope (scale bar represents 100 µm). (B) Effects of rhIL-1Ra on the viability of cells treated with CCl₄. HepG₂ or AML-12 cells were plated into a 96-well plate and incubated overnight. Cells were treated with the indicated drugs for 4 hours. A CCK-8 kit was used to analyze cell viability, and data from three independent experiments were normalized with those of the nontreatment group (control group) and are presented as average ± SD (n = 5). *p < 0.05, **p < 0.01, compared with the CCl₄ group.

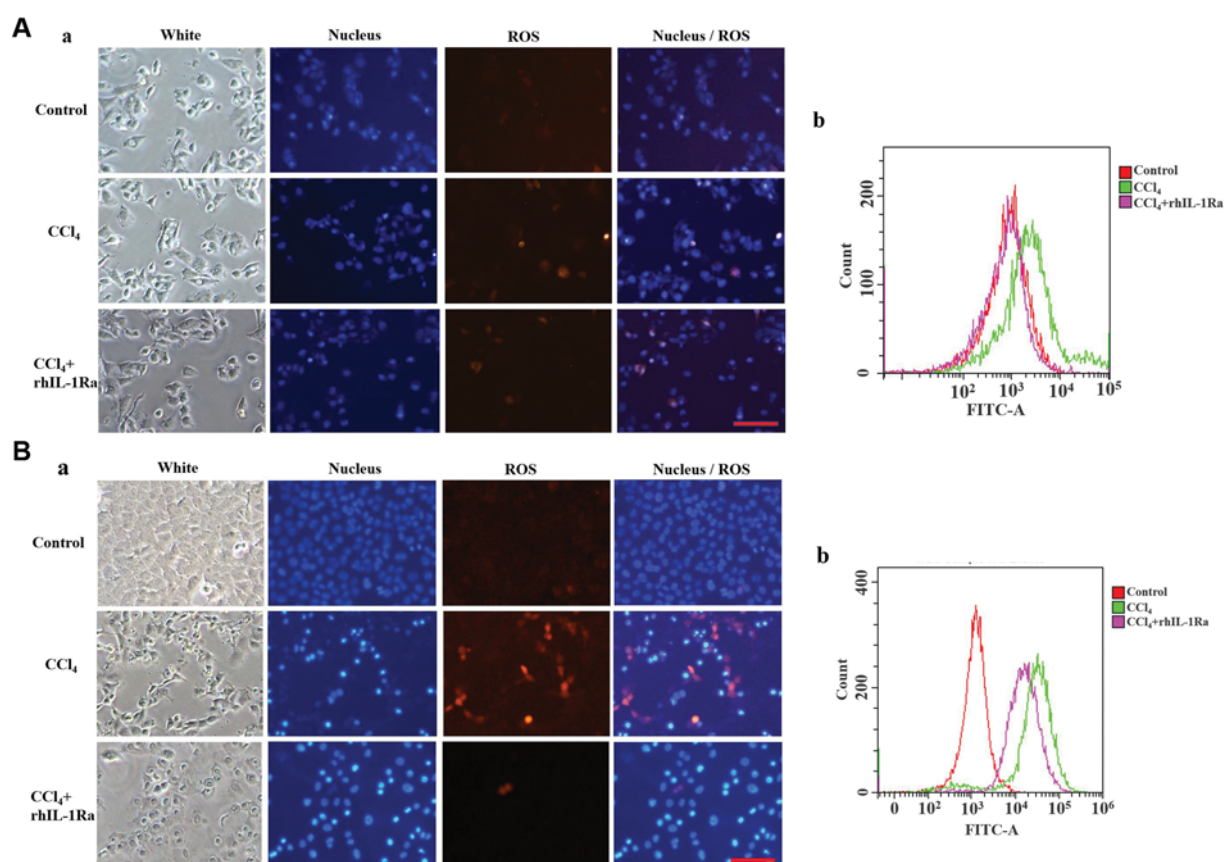


Fig. 2 rhIL-1Ra suppresses ROS accumulation induced by CCl₄ in hepatocytes. HepG₂ (A) and AML-12 (B) cells were treated with the indicated drugs, and DCFH-DA was used as a probe in ROS labeling. Fluorescence microscopy and flow cytometry were used to analyze the ROS levels in cells (scale bar represents 100 µm). The experiments were repeated three times, independently.

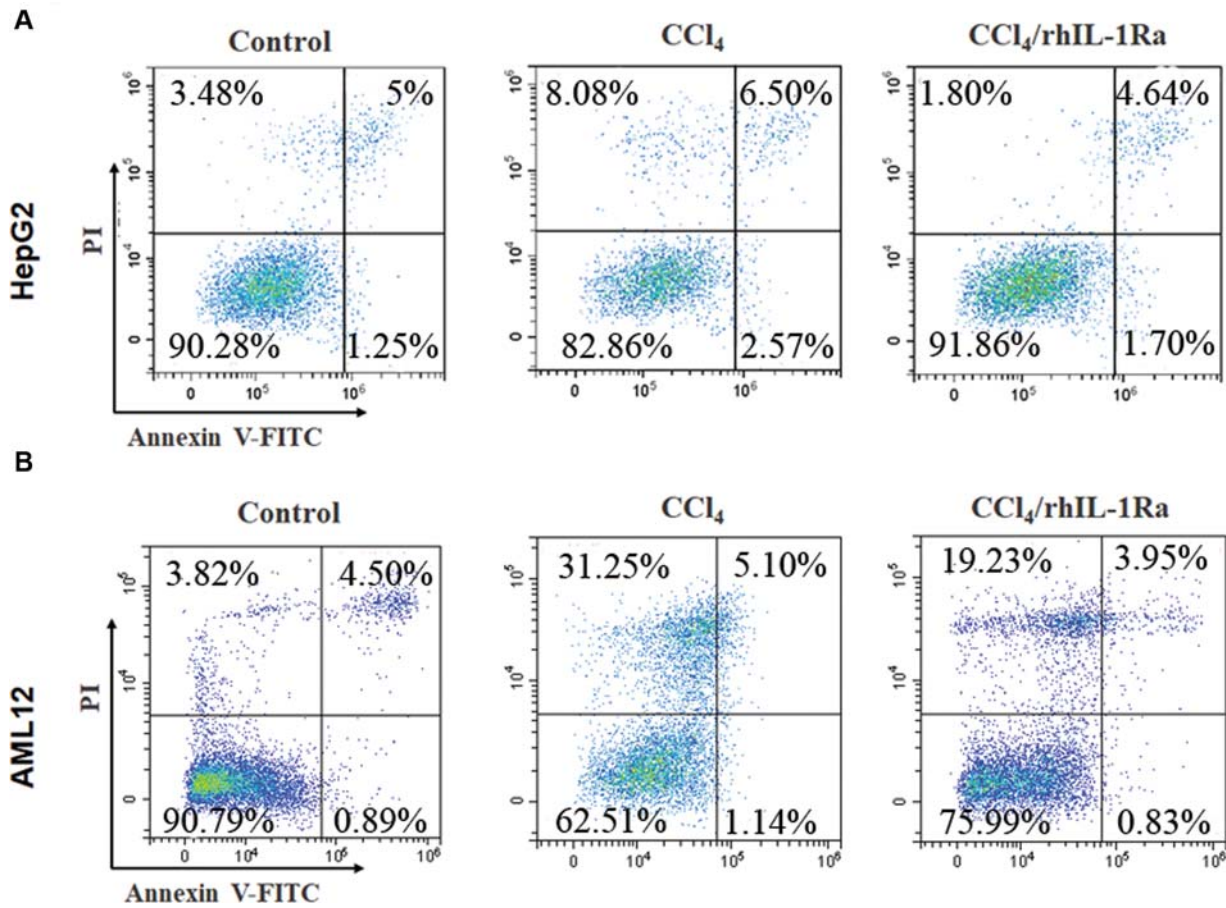


Fig. 3 rhIL-1Ra could attenuate apoptosis in CCl₄-treated hepatocytes. HepG2 (A) or AML-12 (B) cells were treated with the indicated drugs. Cells were collected and stained with PI and FITC-conjugated annexin-V, and all samples were analyzed using a flow cytometer.

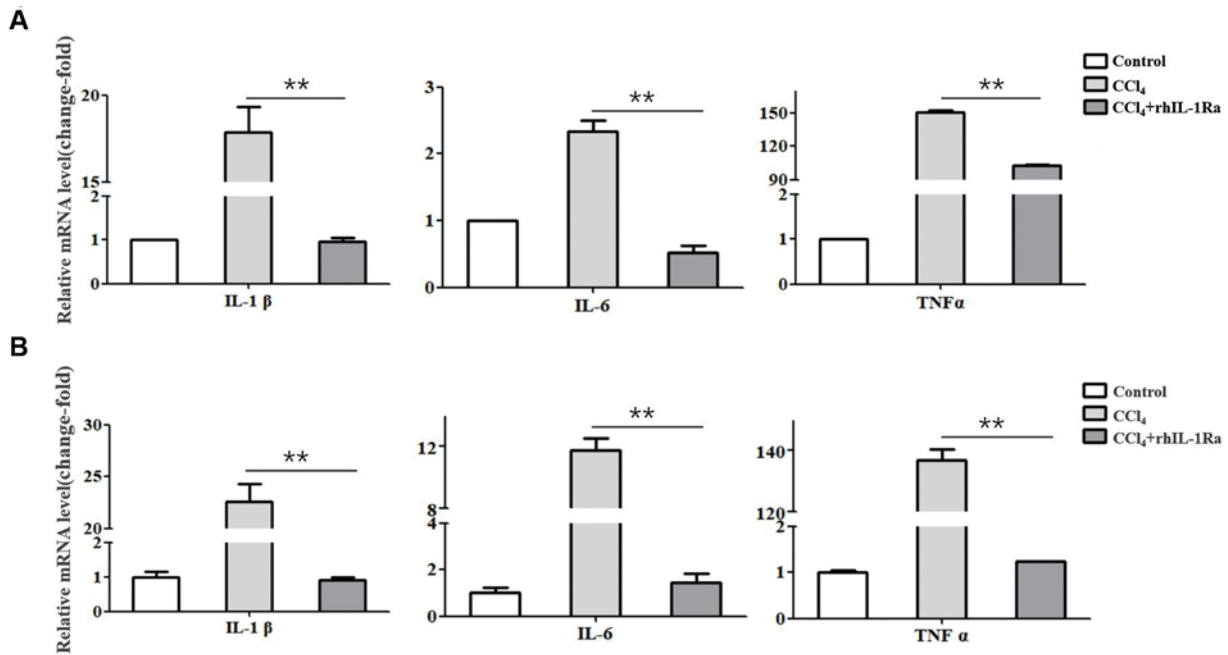


Fig. 4 rhIL-1Ra suppresses CCl₄-induced cytokine expression in hepatocytes. HepG2 (A) or AML-12 (B) cells were treated with the indicated drugs. Expression of IL-1β, IL-6, and TNF-α was analyzed using quantitative RT-PCR. ***p* < 0.01, compared with the CCl₄ group.

rhIL-1Ra Suppresses Apoptosis-Associated Cytokine Expression

Inflammatory factors play a critical role in liver injury or acute liver failure in the patients, and most of these cytokines could directly regulate apoptosis and necrosis. In liver injury, an increase in the expression of IL-1 β , IL-6, or TNF- α was always

associated with apoptosis of hepatocytes, and these cytokines were mainly derived from nonparenchymal cells in recent studies.^{24,25} Interestingly, CCl₄ could directly stimulate hepatocytes to express these cytokines (\blacktriangleright Fig. 4). rhIL-1Ra could significantly abrogate CCl₄-induced increase in the expression of these cytokines in HepG2 and AML-12 cells (\blacktriangleright Fig. 4).

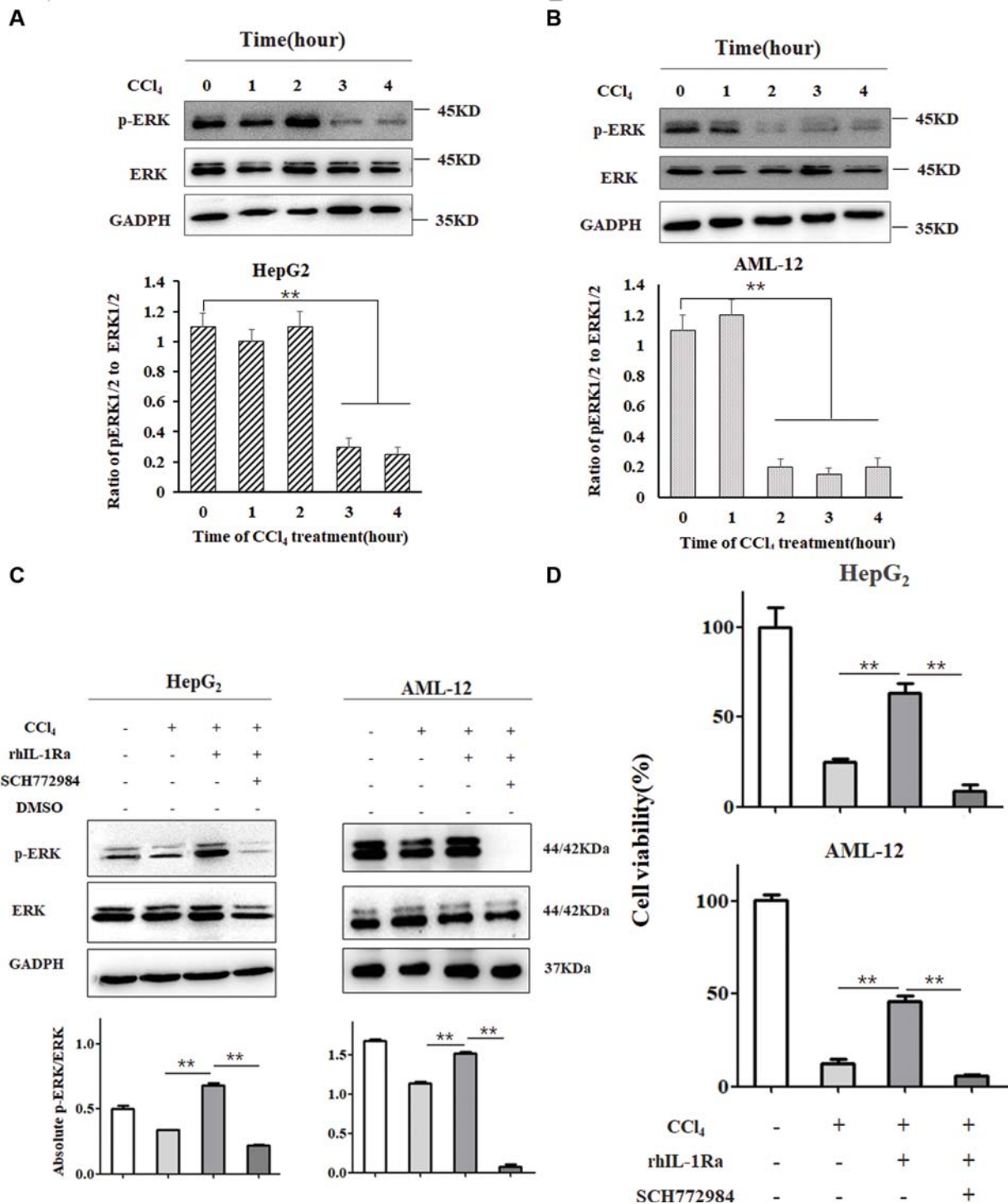


Fig. 5 ERK1/2 inhibitor blocks the protective effects of rhIL-1Ra on hepatocytes. HepG2 (A) or AML-12 (B) cells were treated with CCl₄, and samples were collected at indicated time points. Levels of phosphorylated ERK1/2 and total ERK1/2 were analyzed using western blotting, with GAPDH as the control. Cells were treated with the indicated drugs and harvested after 4 hours of treatment. The cell lysate was analyzed via western blotting by using the indicated antibodies (C). Cell viability in different treatment groups was analyzed (D). ***p* < 0.01, compared with the CCl₄ group (A) and/or indicated between two groups.

ERK1/2 Inhibitor Blocks the Protective Effect of rhIL-1Ra

CCl₄-induced oxidative stress could downregulate ERK1/2 phosphorylation, which modulates apoptosis of hepatocytes in the liver.²⁶ *In vitro* data showed that ERK1/2 phosphorylation in HepG2 or AML-12 cells was significantly decreased after 3 hours of CCl₄ treatment (► Fig. 5A, B). rhIL-1Ra could improve the ERK1/2 phosphorylation level when HepG2 and AML-12 cells were treated with CCl₄ *in vitro*, and cell viability was shown to associate with the level of ERK1/2 phosphorylation (► Fig. 5C, D). To test whether rhIL-1Ra-mediated attenuation of injury of hepatocytes induced by CCl₄ depended on ERK1/2 phosphorylation, an inhibitor of ERK phosphorylation (SCH722984) was used to block the ERK1/2 pathway. When ERK1/2 was blocked, rhIL-1Ra lost its function of protecting hepatocytes from CCl₄ toxicity and recovering ERK1/2 phosphorylation (► Fig. 5). Therefore, rhIL-1Ra-mediated decrease in CCl₄-induced apoptosis of hepatocytes is dependent on the ERK1/2 pathway.

Discussion

CCl₄ is a common hepatotoxic compound, which is widely used in the construction of hepatocyte injury models. Compared with CCl₄ dissolved in DMSO or directly added to a medium to treat cells,^{27,28} a CCl₄-saturated medium shows a better repeatability for inducing a hepatocyte injury model.¹⁶

A variety of inflammatory factors are associated with the pathogenesis of hepatocyte injury and its complications, and IL-1 β , IL-6, and TNF- α have been selected as liver injury biomarkers in both *in vitro* and *in vivo* studies.^{29–31} Our data strongly support that the IL-1RI pathway directly regulates expression and release of IL-1 β , IL-6, and TNF- α in hepatocytes, and blocks the IL-1RI signaling pathway in hepatocytes, which is a potential therapeutic strategy for treating liver failure or liver injury.⁸ The results of this study indicate that rhIL-1Ra not only downregulates secretion of inflammatory cytokines (such as IL-1 β , TNF- α , and IL-6) from liver nonparenchymal cells,³² but also targets hepatocytes to decrease the biosynthesis of cytokines.

ERK1/2 is a member of the mitogen-activated protein kinase (MAPK) family, which plays an important role in stress protection, proliferation, and death of cells.³³ ERK1/2 can be transiently or continuously activated by the regulation of MEK1/2 and upstream MAP3Ks with scaffold proteins and phosphatases.³⁴ In most cases, activation of ERK1/2 generally promotes cell survival. ERK1/2 can exert antiapoptotic effects not only by regulating the activity of survival and apoptosis signaling molecules, but also by upregulating or downregulating their protein expression.³⁴ Previous study has shown that ROS accumulation could directly inhibit the MAPK/ERK pathway through downregulation of ERK phosphorylation.³⁵ Exogenous rhIL-1Ra can block the IL-1R signaling pathway, regulate homeostasis of ROS level, and relieve inhibition of ERK phosphorylation induced by CCl₄ in hepatocytes. Therefore, IL-1Ra could protect hepatocytes against pathogens through two ways—downregulating the expression of inflammatory factors and regulating the ROS/ERK pathway in the liver cells (► Fig. 6).

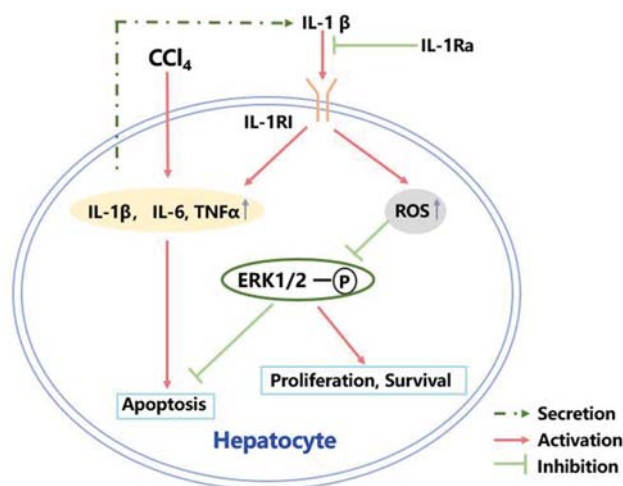


Fig. 6 IL-1Ra protects hepatocytes from CCl₄-induced apoptosis by activating ERK pathway *in vitro*.

Our study gives an insight into the mechanism of protective effect of IL-1Ra on hepatocytes.

Author Contributions

Yunsheng Yuan designed and coordinated the study; Ying Zheng, Xinyi Xiao, Zhuoyi Yang, Meiqi Zhou, Hui Chen, and Siyi Bai performed the experiments; Yunsheng Yuan, Ying Zheng, and Xinyi Xiao analyzed the data; Yunsheng Yuan wrote the manuscript. All authors read and approved the final version of this manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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