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Resveratrol attenuates hepatic inflammation and oxidative stress in collagen-induced arthritis (CIA) mice via the Nrf2/Keap1 pathway

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Summary. Background. Rheumatoid arthritis can affect extra-articular organs such as the liver, and the problem of drug-induced liver injury caused by traditional antirheumatic drugs for improving the condition cannot be ignored. This study aims to investigate the therapeutic effects of resveratrol (Res) on hepatic inflammation and oxidative stress in mice with collagen-induced arthritis (CIA) and to elucidate the relationship between the regulatory mechanisms of the nuclear factor erythroid 2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) signaling pathway.

Methods. In this study, we used chicken type II collagen in combination with complete Freund's adjuvant to induce arthritis in a mouse model, and Res was administered by tube feeding to detect the serum biochemical liver function and inflammation levels, oxidative stress, and apoptosis in the livers of mice. An *in vitro* cellular model of liver inflammation and oxidative stress was established by treating mouse primary hepatocytes (MPHs) with tumor necrosis factor-α (TNF-α) and H_2O_2. The intrinsic mechanism of Res in attenuating hepatic inflammation and oxidative stress in CIA mice was explored by treating MPHs with an Nrf2 inhibitor and Keap1 overexpression plasmid.

Results. Res significantly reduced the levels of inflammation and oxidative stress in liver tissues of CIA mice as well as in MPHs treated with TNF-α and H_2O_2 and activated the Nrf2/Keap1 signaling pathway. Inflammation and oxidative stress levels in MPHs were exacerbated by the use of Nrf2 inhibitors and Keap1 overexpression, which promoted apoptosis.

Conclusion. This study demonstrated the intrinsic mechanism of Res of attenuating hepatic inflammation and oxidative stress in CIA mice through the Nrf2/Keap1 pathway, which provides a new idea for finding hepatoprotective treatments for rheumatoid arthritis.

Key words: Rheumatoid arthritis (RA), Liver injury, Resveratrol, Nrf2/Keap1

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, which is mainly manifested by chronic inflammation and hyperplasia of the synovial membrane of the joints, encroaching on the articular cartilage, causing cartilage damage and destruction, and ultimately leading to joint deformity and loss of function (Huang et al., 2021; Radu and Bungau, 2021; Matteo et al., 2023). In addition to this, systemic organs such as the brain, heart, lungs, and digestive system can be involved, which are called extra-articular manifestations (EAMs) of RA (Conforti et al., 2021; Figus et al., 2021). EAMs are a serious disease associated with high morbidity and mortality, which may be due to the release of pro-inflammatory cytokines in the bloodstream of RA patients (Craig and Cappelli, 2018; Wang and Tsai, 2022).

A growing body of research data strongly suggests that reactive oxygen species (ROS) imbalance is an important factor in the development and progression of RA and that oxidative stress (OS) is also abundantly present in RA patients (Bassu et al., 2020; Kaur et al., 2021; Wójcik et al., 2021). OS is a state of imbalance between oxidative and antioxidant effects in the body,

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which tends to oxidize, leading to increased intracellular production of ROS, lipid peroxidation, protein oxidation, deoxyribonucleic acid (DNA) damage, and impairment of the body's antioxidant defense system, which can lead to inflammation and tissue damage and even disease (Sadasivam et al., 2022). Increased ROS formation has also been documented in the liver of collagen-induced arthritis (CIA) mice (Han et al., 2023). To circumvent the damaging effects of ROS, cells have developed a variety of defense mechanisms that range from scavenging free radicals to reducing the risk of OS-induced cellular damage. These include transcription factors that promote the expression of antioxidant response elements to neutralize oxidative therapeutic and inflammatory responses (Zheng and Sawalha, 2022; Akiyama and Ivanov, 2024; Qi et al., 2024). Among these transcription factors, we focused on nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor binding near the B-cell κ light chain gene (NF-κB) to promote cellular responses to oxidative damage and inflammation (Wang et al., 2022). The DNA sequences responsible for regulating the cellular antioxidant and cytoprotective responses have been identified as antioxidant response elements (AREs), and Nrf2 is a major regulator of the xenobiotic activated receptor (XAR) responsible for activating the ARE pathway, whereas Kelch-like ECH-associated protein 1 (Keap1) is a negative regulator of Nrf2, thus defined as the Keap1-Nrf2-ARE system (Fakhri et al., 2020; He et al., 2020; Ulasov et al., 2022). It has been shown that the Keap1-Nrf2-ARE system plays a key role in the prevention and mitigation of liver injury (Zhou et al., 2022). In addition, the interaction between the Keap1-Nrf2-ARE system and NF-κB has been further investigated in relation to inflammatory diseases (Luo et al., 2022).

Res is a naturally occurring polyphenol that is found in particularly high levels in grapes and has antioxidant, anti-inflammatory, and antibacterial properties (Berretta et al., 2020; Zhang et al., 2021). One study found that Res reduced synovial proliferation, inflammatory markers, and oxidative damage in a rat model of arthritis (Fernández-Rodríguez et al., 2021). Res has also been reported to ameliorate RA by reducing claw swelling, synovial proliferation, inflammatory cell infiltration, and cartilage degradation through activation of the silent mating-type information regulation 2 homolog-1 (SIRT1)-Nrf2 signaling pathway, and inhibiting synovial cell proliferation in synovial tissue (Wang et al., 2020). Much has been reported about the mechanism of action of Res in alleviating RA; however, whether Res is equally capable of reducing the progression of extra-articular injury in RA has been less well studied. In this study, we explored the possibility of Res as a hepatoprotective therapeutic agent for RA.

The aim of this study was to investigate the intrinsic mechanism by which Res attenuates hepatic inflammation and OS in CIA mice through the Nrf2/Keap1 pathway.

Materials and methods

Animal models

Eight-week-old male C57/BL6 mice (body weight: 20-25 g) were randomly divided into three groups of five mice each: (1) Ctrl (control group), (2) CIA (arthritis model group), and (3) CIA+Res (CIA and resveratrol-treated model group, 10 mg/kg). Male C57/BL6 mice were purchased from Jiangsu Jicui Biotechnology Co., Ltd. (Nanjing, China) and acclimatized to laboratory conditions at Anhui Medical University for at least one week prior to experimentation (approval number: NO.LLSC20210751; date: 20210301). CIA mice were treated with complete Freund's adjuvant (CFA) and chicken collagen II (CCII). Two g/L of CC II (Chondrex, Woodinville, WA, USA) was dissolved in 0.05 mol/L glacial acetic acid, stirred thoroughly, and then stored at 4 °C overnight. CFA (Chondrex, Woodinville, WA, USA) was then mixed with an equal volume of dissolved CC II and fully emulsified in a homogenizer on an ice bath and administered by tail-root injection. Animals in the CIA+Res group were administered resveratrol (Aladdin, Shanghai, China) dissolved in 0.5% sodium carboxymethylcellulose (carrier) by gavage. The final concentration was 1 mg/mL, and each animal was given 0.1 mL/10 g once daily for 10 days. The dose of type II collagen in chickens for inducing RA was determined by referring to the previous studies published by the research group and combined with the results of pre-experiments in our laboratory (Bao et al., 2022). The control group was injected with the same volume of normal saline through the trachea and tail root as a negative control. The dose of Res was based on a previous report (Bao et al., 2022). Twenty-four hours after the last Res treatment, the joint and liver tissues of mice were removed and excised for pathological analysis, and serum was taken for biochemical analysis. The remaining liver tissue and serum were immediately frozen in liquid nitrogen for subsequent bioanalysis.

Hematoxylin-Eosin (H&E) staining

After fixation with 4% neutral paraformaldehyde, mouse knee tissue specimens were placed in Ethylene Diamine Tetraacetic Acid (EDTA) decalcification solution (Boster, Wuhan, China) in a room temperature shaker for two weeks and then embedded in conventionally dehydrated paraffin. The thickness of the serial sections was about 3.5 μm, and the sections were dewaxed and washed with graded alcohol and distilled water. Hematoxylin staining was performed for 15 seconds and washed with tap water for 20 min to counterblue. After staining with 5% eosin for 3 min, the sections were rapidly dehydrated. Finally, the sections were sealed with neutral resin. The liver tissue specimens were fixed, dehydrated, paraffin-embedded, and sectioned to a thickness of approximately 3.5 μm,

and H&E stained in the same joint. The degree of osteoarthritis (OA) was evaluated with the Mankin scoring system.

Biochemical analysis

Fresh blood was taken from mice and left at room temperature for 30 min, until the blood coagulated, then centrifuged at 2000 rpm for 10 min, and the upper layer of light-yellow clarified liquid was taken as serum. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) levels were measured in the clinical laboratory of the First Affiliated Hospital of Anhui Medical University, Hefei, China, using an automated device (Monach). Hepatic tissue superoxide dismutase (SOD, A001-3-2) activity, reduced glutathione (GSH, A006-2-1), malondialdehyde (MDA, A003-1-2), and ROS (69-86537) levels were determined using a commercial kit (Nanjing Institute of Construction Bioengineering, Nanjing, China).

Cell culture

MPHs were isolated as previously reported and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin) (Yuan et al., 2024). The cells were incubated in a 37°C incubator containing 5% CO_2. To induce a cellular model of CIA-associated liver injury, recombinant TNF-α (PeproTech, New Jersey, USA) and H_2O_2 (Merck, #7722-84-1, 3%) were added to the cell culture medium for 24h. The Nrf2 inhibitor, ML385, was purchased from MedChemExpress. The Keap1 overexpression plasmid was purchased from Prime Biotech.

Cell Counting Kit-8 (CCK-8) cell viability assay

MPH cells were inoculated in 96-well plates at a density of 3×10^3 cells per well. After 6-12h, the cells were induced using TNF-α and H_2O_2 for 24h. Then, after being treated with Res for 24h, the cells were incubated for 1-4h after adding 10 uL of CCK-8 solution (Biosharp, Beijing, China) to each well. The absorbance of the cells at 450 nm was measured using an enzyme marker to determine the effect of Res on their viability.

Annexin V-Allophycocyanin (APC)/Propidium Iodide (PI) double staining combined with flow cytometry for apoptosis detection

Cells were inoculated into six-well plates at a density of 1×10^6/well and cultured for 24h. Recombinant TNF-α, H_2O_2 was added to the cell culture medium for 24h and then treated with Res for 24h. The cells were then treated with PI. Cells were digested by adding 0.25% trypsin solution (without EDTA), rinsed with phosphate-buffered saline (PBS), centrifuged, and resuspended by adding 100 μL of buffer. Then 5 μL Annexin V-APC and 5 μL PI (Bestbio, Shanghai, China) were added and incubated at room temperature and protected from light for 15 min. Subsequently, 400 μL of buffer was added, and apoptosis was detected by flow cytometry (BD Celesta, USA).

Detection of cellular ROS levels

Cellular ROS levels were detected by a 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) green fluorescent probe (Beyotime Biotechnology, Shanghai, China). Cell culture and treatment were as described above. DCFH-DA was diluted 1000-fold using RPMI-1640 to formulate the working solution; 1~2 mL of the working solution was added to fully cover the growing cells, incubated at 37°C for 20 min away from light, and the cells were washed with PBS buffer three times, and the ROS changes in MPH cells were analyzed by using an orthogonal fluorescence microscope for photomicrographs and ImageJ software.

Western blot

Total proteins were extracted from model mouse liver tissues and MPH cells, treated as described above, using a prepared sodium dodecyl sulfate (SDS) lysis buffer for protein blotting analysis. Proteins were separated using SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (PVDF, Merck Millipore, Germany). The membranes were then sealed in 5% skimmed milk for two hours. Western blot was performed using specific antibodies: rabbit anti-B-cell lymphoma-2 (Bcl-2), anti-Bcl-2-associated X protein (Bax), anti-phospho-NF-kappa-B p65 (p-p65), and anti-Interleukin-1beta (IL-1β) antibodies were obtained from Wanlei Biologicals, Wuhan, China; anti-β-actin, anti-Nrf2, anti-Keap1, anti-SOD2, anti-NF-kappa-B inhibitor alpha (p-κBα), anti-Heme oxygenase 1 (HO-1), anti-Histone H3, anti-p65, and anti-Nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) antibodies were purchased from Abmart, Shanghai, China; and TNF-α was purchased from Proteintech, Wuhan, China, and assayed according to their manufacturers' instructions.

Immunofluorescence

Paraffin sections were prepared from liver specimens and deparaffinized using the method described above. Sections were placed in citric acid repair solution (pH 6.0) for antigen repair and then washed three times for 5 min each with PBS (pH 7.4). Endogenous peroxidase was blocked with hydrogen peroxide solution (3%), and serum was blocked with bovine serum albumin (BSA, 3%). Then, the sections were incubated overnight at 4°C with primary antibodies against SOD2 and TNF-α. After PBS with Tween 20 (PBST) washing, the samples were incubated with

secondary antibodies coupled with goat anti-rabbit antibody (Proteintech) for an hour at room temperature, followed by incubation of the cells with 4',6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, Shanghai, China) for 10 min in the dark. The samples were sealed with an anti-fluorescence quencher (Beyotime Biotechnology, Shanghai, China). Finally, the sections were observed under a fluorescence microscope (Leica, Germany).

MPH cells were inoculated in 24-well plates at a density of 1×10^4/well, and the cells were treated as above, then fixed with 4% paraformaldehyde for 30 min at room temperature and then washed with PBS. Subsequently, cells were treated with 0.1% Triton X for 20 min and then washed again. Afterward, the cells were closed with 5% BSA for 60 min at room temperature and then incubated with antibodies against Nrf2, HO-1, etc. (1:200 dilution) at 4°C overnight. Next, the cells were washed again and incubated with secondary antibodies protected from light for 1h. The nuclei were stained with DAPI, and the samples were sealed with an anti-fluorescence quencher and observed using an orthogonal fluorescence microscope.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Cellular ribonucleic acid (RNA) was extracted using an RNA extraction kit (Accurate Biology, Hunan, China). The concentration of RNA was determined by ultraviolet and visible spectrophotometry and stored at -80°C. The expression of Nrf2, Keap1, and HO-1 was detected by the Synergetic Binding Reagent (SYBR) Green qPCR master mix (Beyotime Biotechnology, Shanghai, China) in the Roche LightCycler 480 real-time fluorescence quantitative PCR system. After completion of the reaction, the cycle threshold (Ct) values were determined, and the relative RNA levels of each sample were calculated using the $2^{-\Delta\Delta Ct}$ method, with normalization to β-actin levels. The primer sequences are shown in Table 1.

Cell transfection and processing

Keap1 overexpression plasmids were designed and synthesized by DynaPro. Cells were transfected with the plasmid at a concentration of 1 μg/mL using Lipo2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The cells were then used for experiments.

Statistical analyses

Each experiment was repeated at least three times. The data were analyzed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) and are expressed as the mean ± standard deviation. The Brown-Forsythe test was used to evaluate the homogeneity of variance between multiple groups. Since the variances were homogeneous ($p>0.05$), a one-way analysis of variance followed by Tukey's *post-hoc* test was applied to create statistically homogeneous groups. $p<0.05$ was considered statistically significant.

Results

Res inhibits the progression of liver injury associated with CIA in mice

Previous studies have reported that Res can alleviate the progression of RA (Yang et al., 2018). However, whether Res has the same effect in RA-associated liver injury is rarely reported. Here we established a mouse model of CIA treated with Res, and H&E staining showed that Res attenuated inflammation in the joints and mitigated liver injury in mice (Fig. 1A, B). Serum ALT ($p=0.0005$), AST ($p=0.0105$), ALP ($p=0.0433$), and LDH ($p=0.021$) tests also showed that Res reduced liver function damage in CIA mice (Fig. 1C). In addition, western blot results showed that Res not only inhibited the expression of Bax but also promoted the expression of Bcl-2 (Fig. 1D). The above results indicated that Res could inhibit the progression of liver injury associated with CIA mice.

Res attenuates inflammation and oxidative stress in the liver of CIA mice.

To investigate whether Res attenuated inflammation and OS in the liver of CIA mice, we detected the content of GSH, SOD, MDA, and ROS in liver tissues as well as the expression of oxidative-related proteins, such as HO-1, SOD2, etc., by western blot, and the results indicated that Res attenuated the level of OS in the liver of CIA mice (Fig. 2A,B). In addition, Western blot detected the expression of inflammation-related proteins, such as

Table 1. Sequences of qRT-PCR primers.

Gene	Primer sequence 5'-3'
Nrf2 (Mouse)	Forward CAGAGGTGGCTGCTCTTGTT Reverse GGTTACAACGTGGGGATGGT
Keap1 (Mouse)	Forward CGCGTGCCCTTCTCCAA Reverse GACCCCTACCTCTACTCGCC
HO-1 (Mouse)	Forward ATGGCGTCACTTCGTCAGAG Reverse CTGATCTGGGGTTTCCCTCG
SOD2 (Mouse)	Forward GGAGCAAGGTCGCTTACAGA Reverse TGTTCCTTGCAATGGGTCCT
NLRP3 (Mouse)	Forward CCCCTTTATTTGTACCCAAGGC Reverse CAGACGTATGTCCTGAGCCA
TNF-α (Mouse)	Forward TGTCTACTCCTCAGAGCCCC Reverse ACCCTGAGCCATAATCCCCT
IL-1β (Mouse)	Forward AAGGGGACATTAGGCAGCAC Reverse ATGAAAGACCTCAGTGCGGG
β-actin (Mouse)	Forward CTCCAGAACGCAAGTACTCT Reverse CCAGCTTCGTCGTATTCCTG

NLRP3, TNF-α, IL-1β, etc., and the results indicated that Res attenuated the level of inflammation in the liver of CIA mice (Fig. 2C). Meanwhile, our tissue immunofluorescence results also showed that Res attenuated inflammation and OS in the liver of CIA mice (Fig. 2D).

Establishment of an in vitro cellular model of CIA mouse-associated liver injury

To investigate the specific mechanism by which Res reduces liver inflammation and OS in CIA mice, we used recombinant TNF-α and H_2O_2 to stimulate MPHs and establish an *in vitro* model of liver injury, followed by the addition of Res for treatment. As shown below, we used different concentrations of TNF-α to stimulate MPHs, and cell viability was detected by CCK-8 24h later. The results showed a gradual decrease in cell viability with increasing concentration, and we selected the lowest effective concentration (5 μg/L) as the subsequent experimental concentration (Fig. 3 A). Based on the addition of TNF-α, the most appropriate H_2O_2 stimulation concentration (5 μM) and Res treatment concentration (20 μM) were selected using the same method, and the *in vitro* hepatocyte damage and treatment model was constructed (Fig. 3B,C). To further validate the cell model, we detected the expression of HO-1, Bax, and Bcl-2 in the cell model by western blot (Fig. 3D-H) and detected apoptosis by flow cytometry (Fig. 3I). The results showed that we successfully constructed an *in vitro* cellular model of liver injury in CIA mice.

Res attenuates inflammation and oxidative stress in MPH cells and modulates the Nrf2/Keap1 pathway

To investigate whether Res reduced inflammation and OS in MPH cells treated with TNF-α and H_2O_2, we detected the expression of Nrf2, Keap1, HO-1, SOD2, and other proteins and RNA expression by western blot and qRT-PCR (Fig. 4A,C), as well as the immunofluorescence detection of Nrf2 and HO-1 protein expression (Fig. 4E). The results showed that Res alleviated the OS in damaged MPH cells and could regulate the Nrf2/Keap1 pathway. In addition, western blot and qRT-PCR detected the expression of inflammation-related proteins such as NLRP3, TNF-α, IL-1β, and RNA expression, and the results showed that Res reduced the inflammation level of damaged MPH cells (Fig. 4B,D).

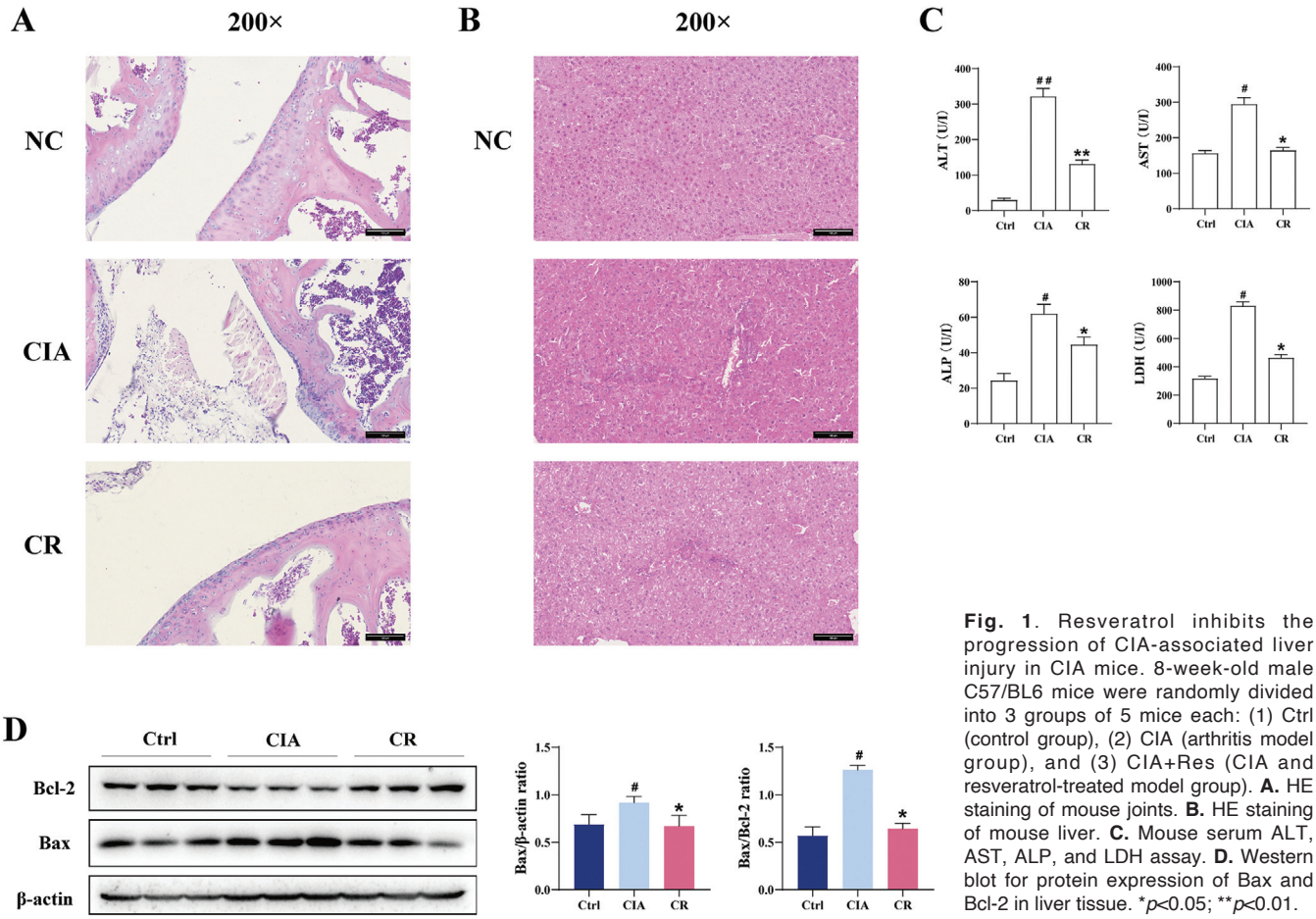

Fig. 1. Resveratrol inhibits the progression of CIA-associated liver injury in CIA mice. 8-week-old male C57/BL6 mice were randomly divided into 3 groups of 5 mice each: (1) Ctrl (control group), (2) CIA (arthritis model group), and (3) CIA+Res (CIA and resveratrol-treated model group). **A.** HE staining of mouse joints. **B.** HE staining of mouse liver. **C.** Mouse serum ALT, AST, ALP, and LDH assay. **D.** Western blot for protein expression of Bax and Bcl-2 in liver tissue. *p<0.05; **p<0.01.

Inhibition of Nrf2 promotes inflammation and oxidative stress in MPH cells

To further investigate whether Res could alleviate MPH cell inflammation and OS by regulating the expression of Nrf2, we treated the cells with the Nrf2 inhibitor ML385 and detected the protein expression of Nrf2, HO-1, and SOD2 by western blot (Fig. 5A-C), and the protein expression of HO-1 by immuno-fluorescence (Fig. 5G). The results showed that the level of OS was significantly increased in the Nrf2 inhibition group. We then detected the protein and RNA expression of NLRP3, TNF-α, and IL-1β by western blot and qRT-PCR. The results showed that the level of inflammation was also significantly increased in the Nrf2 inhibition group (Fig. 5D-F). The above results suggest that Nrf2 inhibition promotes inflammation and OS in MPH cells and may also indicate that Res plays an anti-inflammatory and antioxidant role by promoting Nrf2 expression in MPH cells.

Overexpression of Keap1 promotes inflammation and oxidative stress in MPHs

It has been reported that Keap1 is a negative regulator of Nrf2 (Crisman et al., 2023), so we constructed a Keap1 overexpression plasmid to transfect MPH cells. The expression of Nrf2 in the nucleus of MPH cells was detected by western blot and immunofluorescence; the protein and RNA expression of cellular Keap1, HO-1, and SOD2 were detected by western blot and qRT-PCR, and the results showed that the expression of Nrf2, HO-1, and SOD2 in the nucleus of cells overexpressing Keap1 was significantly reduced (Fig. 6A,B). Then we detected the intracellular expression of NLRP3, TNF-α, IL-1β, and other proteins

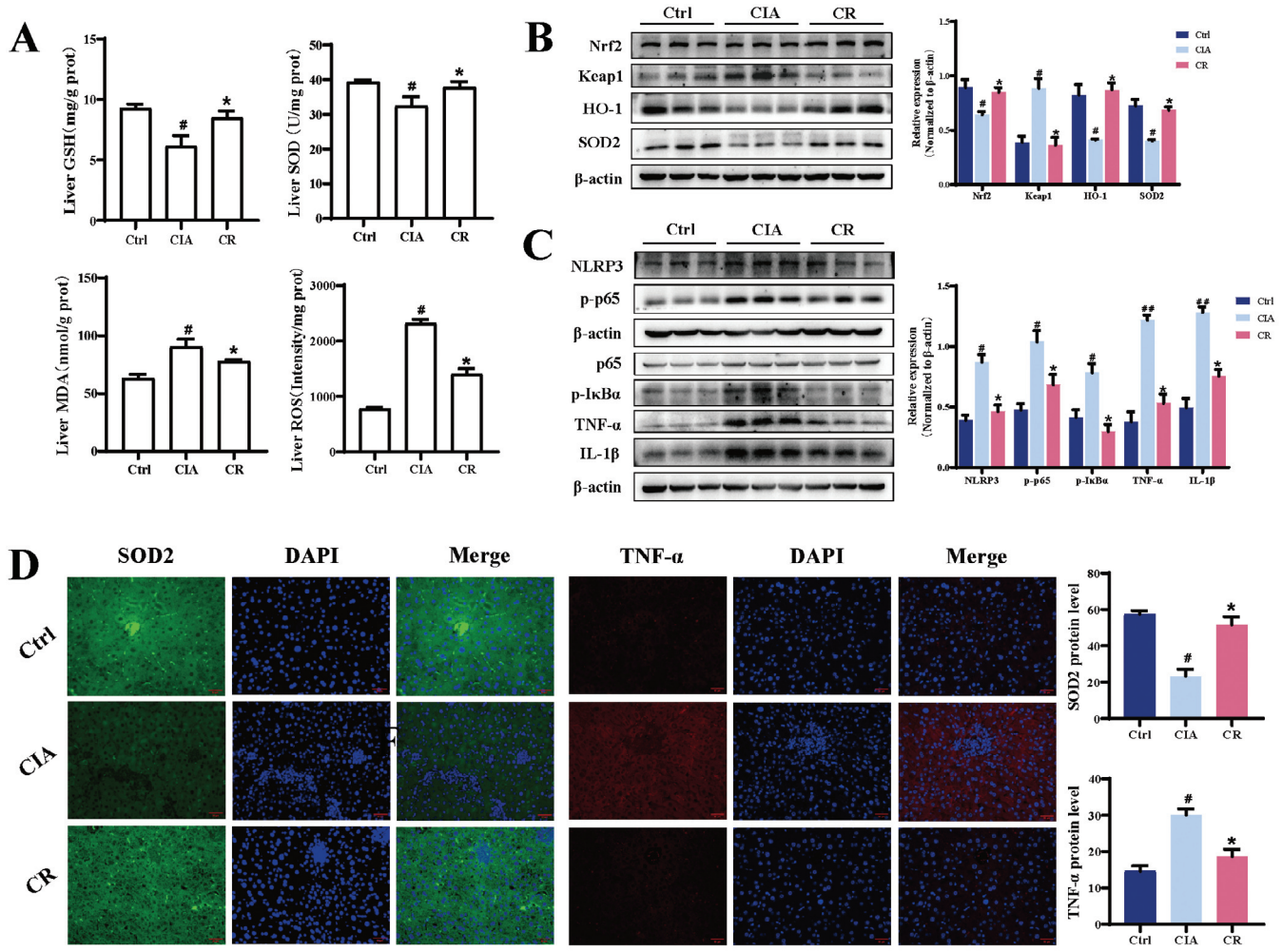

Fig. 2. Res attenuates inflammation and oxidative stress in the liver of CIA mice. **A.** The kit detects mouse liver GSH, SOD, MDA, and ROS levels. **B.** Western blot detection of Nrf2, Keap1, HO-1, and SOD2 protein expression in mouse liver. **C.** Western blot detection of mouse liver NLRP3, p-p65, p65, p-IκBα, TNF-α, and IL-1β protein expression. **D.** Tissue immunofluorescence to detect protein expression of hepatic SOD2 and TNF-α. x 400. **p*<0.05; ***p*<0.01.

by western blot and qRT-PCR, and the results showed that the inflammation level of MPHs cells was significantly increased in the Keap1 overexpression group (Fig. 6C). In summary, Keap1 can act as a negative regulator of Nrf2 expression, thus modulating the anti-inflammatory and antioxidant capacity of cells.

Discussion

Here, we explored the relationship between CIA mouse-associated liver injury and OS as well as the intrinsic mechanism by which Res restores antioxidant capacity through the Nrf2/Keap1 pathway. Our results showed that Res increased the entry of Nrf2 into the nucleus by inhibiting the expression of Keap1, which activated the nuclear antioxidant components, restored the antioxidant capacity of mouse liver, and attenuated inflammation and OS levels in the liver of CIA mice. In this study, we used a mouse model of arthritis induced by the combination of chicken type II collagen and Freunds' complete adjuvant, and the experimental results of the relevant study showed that Keap1, inflammation,

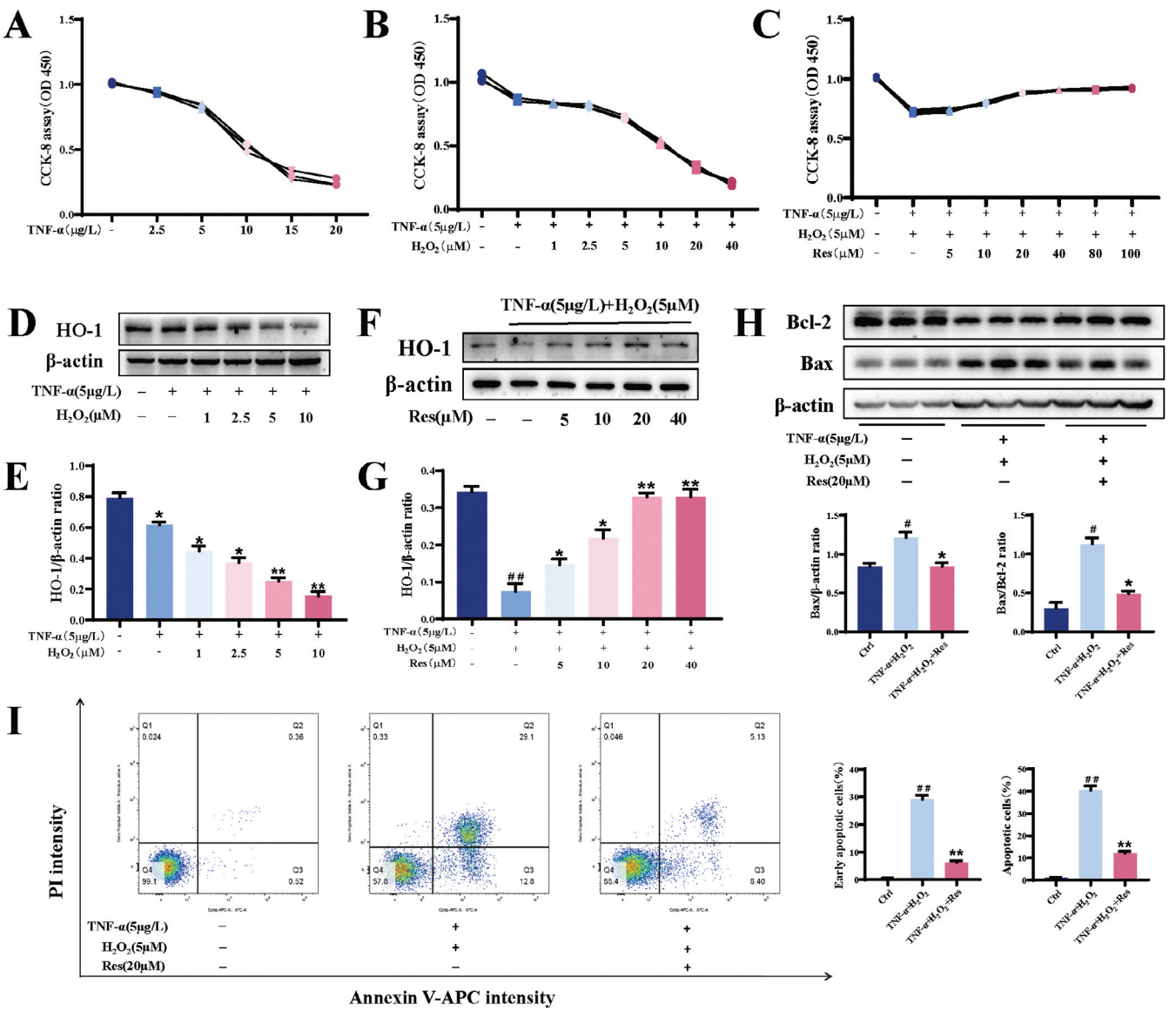

Fig. 3. Establishment of an in vitro cellular model of CIA mouse-associated liver injury. **A.** MPHs were stimulated with different concentrations of TNF-α, and the cell viability was measured by CCK-8 after 24h. The cell viability of MPHs was measured by CCK-8. **B.** TNF-α (5 μg/L) was added to MPHs, followed by different concentrations of H_2O_2, and the cell viability was measured by CCK-8 after 24h. The cell viability of MPHs was measured by CCK-8 after 24h. **C.** TNF-α (5 μg/L) and H_2O_2 (5 μM) were added to MPHs, followed by different concentrations of Res, and cell viability was detected by CCK-8 after 24h. **D-G.** Western blot for HO-1 protein expression. **H.** Western blot for Bax and Bcl-2 protein expression. **I.** Flow cytometry to detect apoptosis. *p<0.05; **p<0.01.

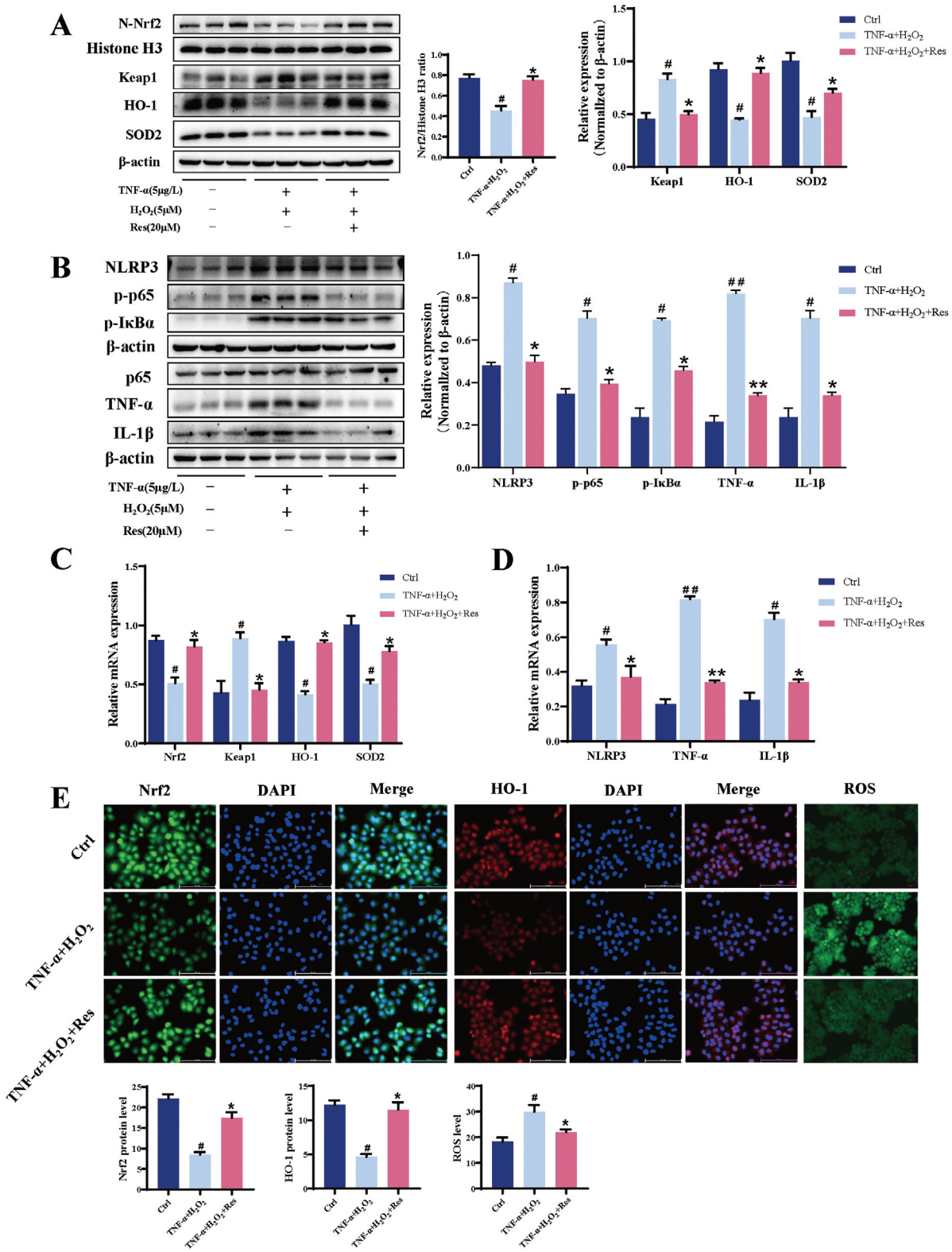

Fig. 4. Res attenuated inflammation and oxidative stress levels in MPH cells and modulated the Nrf2/Keap1 pathway. **A.** Western blot was performed to detect the protein expression of Nrf2 in the nucleus and the protein expression of Keap1, HO-1 and SOD2 in MPH cells. **B.** Western blot detection of NLRP3, p-p65, p65, p-IκBα, TNF-α, and IL-1β protein expression in MPH cells. **C.** qRT-PCR to detect RNA expression of Nrf2, Keap1, HO-1, and SOD2 in MPH cells. **D.** qRT-PCR to detect RNA expression of NLRP3, TNF-α, and IL-1β in MPH cells. **E.** Immunofluorescence detection of protein expression of Nrf2 and HO-1 in MPH cells. x 400. *p<0.05; **p<0.01.

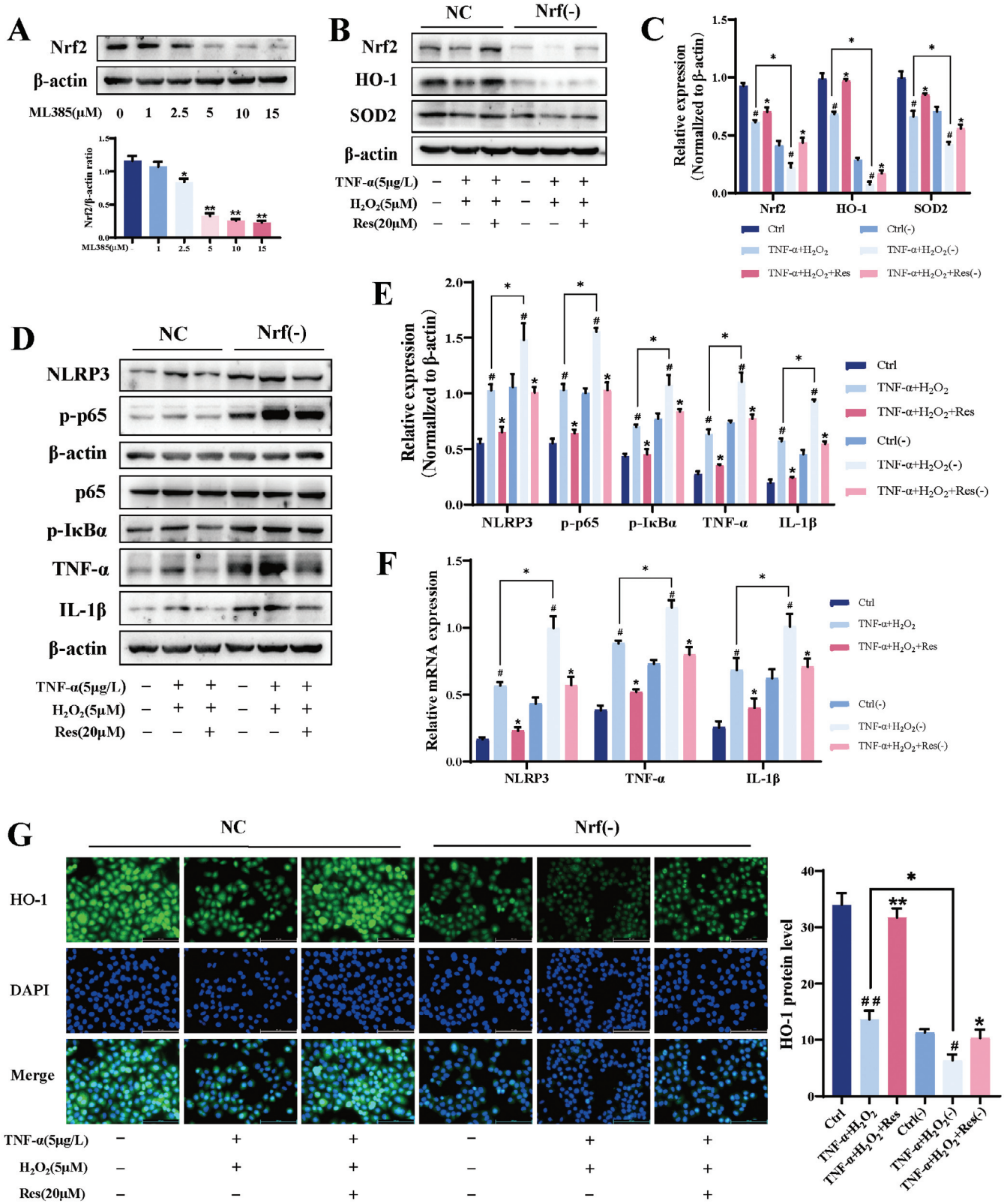

Fig. 5. Inhibition of Nrf2 promotes inflammation and oxidative stress in MPH cells. **A.** Cells were treated with different concentrations of ML385, and changes in Nrf2 protein expression were detected by Western blot. **B, C.** Western blot detection of protein expression of Nrf2, HO-1, and SOD2. **D, E.** Western blot detection of protein expression of NLRP3, p-p65, p65, p-IκBα, TNF-α, and IL-1β. **F.** qRT-PCR to detect RNA expression of NLRP3, TNF-α, and IL-1β. **G.** Immunofluorescence to detect the protein expression of HO-1. x 400. *p<0.05; **p<0.01.

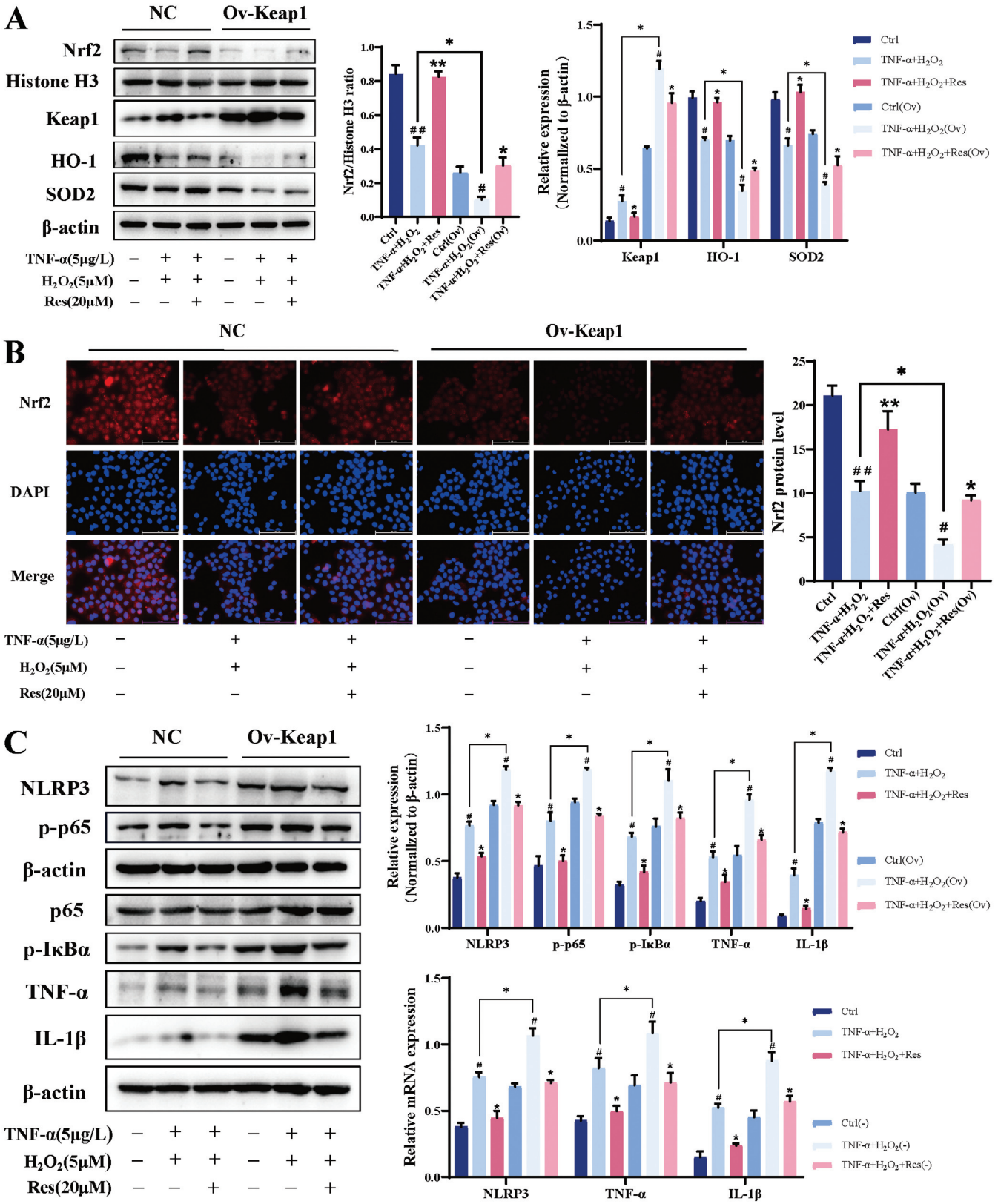

Fig. 6. Overexpression of Keap1 promotes inflammation and oxidative stress in MPHs. **A.** Western blot detection of protein expression of Nrf2, Keap1, HO-1, and SOD2. **B.** Immunofluorescence detection of Nrf2 protein expression . x 400. **C.** Western blot to detect NLRP3, p-p65, p65, p-IκBα, TNF-α, and IL-1β protein expression; qRT-PCR to detect RNA expression of NLRP3, TNF-α, and IL-1β. *p<0.05; **p<0.01.

OS, and apoptosis were increased in the model group, while Nrf2 was decreased, which was alleviated by Res treatment. We used TNF-α+H_2O_2 to stimulate MPH cells to construct an *in vitro* cellular model; the experimental results showed that TNF-α, as well as H_2O_2 treatment promoted Keap1 expression, leading to Nrf2 degradation, a decrease in nucleated Nrf2, and a blockage of the cellular antioxidant pathway, which was reversed by Res treatment. In summary, we conclude that Res alleviated hepatic inflammation and OS in CIA mice by restoring antioxidant capacity through the Nrf2/Keap1 pathway.

RA-EAMs are serious diseases associated with high morbidity and mortality (Samhouri et al., 2022; Mitrović et al., 2023); furthermore, it is important to note that EAMs are often underestimated and undertreated, despite their severity, due to the lack of specific guidelines. The liver is the largest substantial organ in the abdominal cavity and carries out important physiological functions in the body; patients with RA often also have liver involvement, but its exact etiology is unknown. Some studies have suggested that it may be due to the release of pro-inflammatory cytokines and increased production of ROS in the bloodstream of RA patients (Craig and Cappelli, 2018; Kamal et al., 2021). In this study, we showed that CIA mice had elevated liver function indexes, such as serum AST and ALT, increased protein levels of NLRP3, TNF-α, IL-β, and Bax, and decreased protein levels of HO-1, SOD2, and Bcl-2 in the liver. These results suggest that CIA mice have impaired liver function and elevated levels of hepatic inflammation and OS.

Res occurs naturally in plants, such as grapes and peanuts, and it belongs to a group of phytonutrients known as flavonoids. Res is considered a natural antioxidant that may help fight oxygen radical damage and reduce OS and inflammatory responses (Ahmadi and Ebrahimzadeh, 2020; Ashrafizadeh et al., 2020; Tian and Liu, 2020). Some studies have suggested that Res may be beneficial for cardiovascular health, helping to lower cholesterol and blood pressure and preventing cardiovascular diseases such as atherosclerosis (Yu et al., 2021). In our study, we found that Res reduced serum AST and ALT levels, decreased the expression of proteins such as NLRP3, TNF-α, SOD2, and Bax in the liver of CIA mice to attenuate the level of inflammation, and increased the expression of proteins such as HO-1, SOD2, and Bcl-2, which enhanced the antioxidant capacity of the liver. These results suggest that Res reduced the level of inflammation and OS in the liver and alleviated liver injury by restoring the antioxidant capacity of the liver in CIA mice.

OS is a state of imbalance between oxidative and antioxidant effects in the body, which tends to oxidize, leading to increased intracellular production of ROS, lipid peroxidation, protein oxidation, DNA damage, and impairment of the body's antioxidant defense system, which can lead to inflammation and tissue damage and even disease (Georgiou and Margaritis, 2021; Xiao, 2021). Increased formation of ROS has also been documented in the liver of CIA mice (Comar et al., 2013). Nrf2/Keap1-ARE is an important element in the liver of CIA mice (Georgiou and Margaritis, 2021; Xiao, 2021). The response element (ARE) signaling pathway is one of the most important defense mechanisms against OS in the organism, which maintains intracellular redox homeostasis and metabolic and protein homeostasis by regulating the transcription of several downstream cytoprotective genes and exerts biological functions, such as anti-inflammatory, anticancer, and anti-aging (Adelusi et al., 2020; Sykiotis, 2021). In our study, we found that GSH and SOD contents were decreased and MDA and ROS were increased in the livers of CIA mice, in addition to the decrease in the expression of Nrf2 and the increase in the expression of Keap1 in the livers of CIA mice and MPH cells treated with TNF-α+H_2O_2. These results suggest that Res is able to attenuate OS in the liver of CIA mice by regulating the Nrf2/Keap1 pathway.

However, we only studied the effect of Res on mouse hepatocytes and did not investigate the effect of Res on non-substantive cells such as hepatic stellate cells. Further and deeper studies are needed to investigate the mechanism underlying the Res regulation of the Nrf2/Keap1 pathway.

Conclusion

Our findings suggest that Res acts as a hepato-protective agent by attenuating OS and inflammation levels. This suggests a possibility of Res as an effective drug for the treatment of RA-related liver injury. In addition, we clarified the intrinsic mechanism of Res in alleviating RA-associated liver injury by restoring hepatic antioxidant capacity through the Nrf2/Keap1 pathway, which provides a theoretical basisto enhance the search for therapeutic targets for liver injury.

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Data availability. Data will be made available upon request.

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References

Adelusi T.I., Du L., Hao M., Zhou X.Y., Xuan Q., Apu C., Sun Y., Lu Q. and Yin X.X. (2020). Keap1/Nrf2/ARE signaling unfolds therapeutic targets for redox imbalanced-mediated diseases and diabetic nephropathy. Biomed. Pharmacother. 123, 109732.

Ahmadi R. and Ebrahimzadeh M.A. (2020). Resveratrol - A comprehensive review of recent advances in anticancer drug design and development. Eur. J. Med. Chem. 200, 112356.

Akiyama Y. and Ivanov P. (2024). Oxidative stress, transfer RNA metabolism, and protein synthesis. Antioxid. Redox Signal. 40, 715-735.

Ashrafizadeh M., Zarrabi A., Najafi M. and Samarghandian S. (2020). Resveratrol targeting tau proteins, amyloid-beta aggregations, and their adverse effects: An updated review. Phytother. Res. 34, 2867-2888.

Bao L.X., Ye J., Liu N.N., Shao Y.B., Li W.H., Fan X.F., Zhao D.H., Wang H.Z. and Chen X.Y. (2022). Resveratrol ameliorates fibrosis in rheumatoid arthritis-associated interstitial lung disease via the autophagy-lysosome pathway. Molecules 27, 8475.

Bassu S., Zinellu A., Sotgia S., Mangoni A.A., Floris A., Farina G., Passiu G., Carru C. and Erre G.L. (2020). Oxidative stress biomarkers and peripheral endothelial dysfunction in rheumatoid arthritis: A monocentric cross-sectional case-control study. Molecules 25, 3855.

Berretta M., Bignucolo A., Francia R.D., Comello F., Facchini G., Ceccarelli M., Iaffaioli R.V., Quagliariello V. and Maurea V. (2020). Resveratrol in cancer patients: From bench to bedside. Int. J. Mol. Sci. 21, 2945.

Comar J.F., Sá-Nakanishi A.B., Oliveira A.L., Wendt M.M.N., Amado C.A.B., Luiza Ishii Iwamoto E., Peralta R.M. and Bracht A. (2013). Oxidative state of the liver of rats with adjuvant-induced arthritis. Free Radic. Biol. Med. 58, 144-153.

Conforti A., Di Cola I., Pavlych V., Ruscitti P., Berardicurti O., Ursini Francesco., Giacomelli R. and Cipriani P. (2021). Beyond the joints, the extra-articular manifestations in rheumatoid arthritis. Autoimmun. Rev. 20, 102735.

Craig E. and Cappelli L.C. (2018). Gastrointestinal and hepatic disease in rheumatoid arthritis. Rheum. Dis. Clin. North Am. 44, 89-111.

Crisman E., Duarte P., Dauden E., Cuadrado A., Rodríguez-Franco M.I., López M.G. and León R. (2023). KEAP1-NRF2 protein-protein interaction inhibitors: Design, pharmacological properties and therapeutic potential. Med. Res. Rev. 43, 237-287.

Fakhri S., Pesce M., Patruno A., Moradi S.Z., Iranpanah A., Farzaei M.H. and Sobarzo-Sánchez E. (2020). Attenuation of Nrf2/Keap1/ARE in Alzheimer's disease by plant secondary metabolites: A mechanistic review. Molecules 25, 4926.

Fernández-Rodríguez J.A., Almonte-Becerril M., Ramil-Gómez O., Hermida-Carballo L., Viñas-Diz S., Vela-Anero Á., Concha Á., Camacho-Encina M., Blanco F.J. and López-Armada M.J. (2021). Autophagy activation by resveratrol reduces severity of experimental rheumatoid arthritis. Mol. Nutr. Food Res. 65, e2000377.

Figus F.A., Piga M., Azzolin I., McConnell R. and Iagnocco A. (2021). Rheumatoid arthritis: Extra-articular manifestations and comorbidities. Autoimmun. Rev. 20, 102776.

Georgiou C.D. and Margaritis L.H. (2021). Oxidative stress and NADPH oxidase: Connecting electromagnetic fields, cation channels and biological effects. Int. J. Mol. Sci. 22, 10041.

Han Z.H., Gao X., Wang Y.J., Cheng S.N., Zhong X.Y., Xu Y., Zhou X.Z., Zhang Z.L., Liu Z. and Cheng L. (2023). Ultrasmall iron-quercetin metal natural product nanocomplex with antioxidant and macrophage regulation in rheumatoid arthritis. Acta Pharm. Sin. B. 13, 1726-1739.

He F., Ru X.L. and Wen T. (2020). NRF2, a transcription factor for stress response and beyond. Int. J. Mol. Sci. 21, 4777.

Huang J., Fu X.K., Chen X.X., Li Z., Huang Y.H. and Liang C. (2021). Promising therapeutic targets for treatment of rheumatoid arthritis. Front. Immunol. 12, 686155.

Kamal E., Kaddam L.A., Alagib A. and Saeed A. (2021). Dietary fibers (Gum Arabic) supplementation modulates hepatic and renal profile among rheumatoid arthritis patients, Phase II trial. Front. Nutr. 8, 552049.

Kaur G., Sharma A. and Bhatnagar A. (2021). Role of oxidative stress in pathophysiology of rheumatoid arthritis: insights into NRF2-KEAP1 signalling. Autoimmunity 54, 385-397.

Luo J., Wang J., Zhang J., Sang A.M., Ye X.J., Cheng Z.S. and Li X.Y. (2022). Nrf2 deficiency exacerbated CLP-Induced pulmonary injury and inflammation through autophagy- and NF-κB/PPARγ-mediated macrophage polarization. Cells 11, 3727.

Matteo A., Bathon J.M. and Emery P. (2023). Rheumatoid arthritis. Lancet 402, 2019-2033.

Mitrović J., Hrkač S., Tečer J., Golob M., Posavec A.L., Mitrović H.K. and Grgurević L. (2023). Pathogenesis of extraarticular manifestations in rheumatoid arthritis-A comprehensive review. Biomedicines 11,1262.

Qi F., Li Z.W., Ai Y.F., Shangguan K.K., Li P., Lin F.C. and Liang Y. (2024). DGK5β-derived phosphatidic acid regulates ROS production in plant immunity by stabilizing NADPH oxidase. Cell Host Microbe 32, 425-440.e7.

Radu A.F. and Bungau S.G. (2021). Management of rheumatoid arthritis: An overview. Cells 10, 2857.

Sadasivam N., Kim Y.J., Radhakrishnan K. and Kim D.K. (2022). Oxidative stress, genomic integrity, and liver diseases. Molecules 27, 3159.

Samhouri B.F., Vassallo R., Achenbach S.J., Kronzer V.L., Davis 3rd J.M., Myasoedova E. and Crowson C.S. (2022). Incidence, risk factors, and mortality of clinical and subclinical rheumatoid arthritis-associated interstitial lung disease: A population-based cohort. Arthritis Care Res. (Hoboken). 74, 2042-2049.

Sykiotis G.P. (2021). Keap1/Nrf2 signaling pathway. Antioxidants (Basel). 10, 828.

Tian B.R. and Liu J.Y. (2020). Resveratrol: A review of plant sources, synthesis, stability, modification and food application. J. Sci. Food Agric. 100, 1392-1404.

Ulasov A.V., Rosenkranz A.A., Georgiev G.P. and Sobolev A.S. (2022). Nrf2/Keap1/ARE signaling: Towards specific regulation. Life Sci. 291, 120111.

Wang C.R. and Tsai H.W. (2022). Autoimmune liver diseases in systemic rheumatic diseases. World J. Gastroenterol. 28, 2527-2545.

Wang G.Y., Xie X.X., Yuan L.L., Qiu J., Duan W.C., Xu B. and Chen X.Y. (2020). Resveratrol ameliorates rheumatoid arthritis via activation of SIRT1-Nrf2 signaling pathway. Biofactors 46, 441-453.

Wang L., Zhang X., Xiong X.X., Zhu H., Chen R., Zhang S.D., Chen G. and Jian Z.H. (2022). Nrf2 Regulates oxidative stress and its role in cerebral ischemic stroke. Antioxidants 11, 2237.

Wójcik P., Gęgotek A., Žarković N. and Skrzydlewska E. (2021). Oxidative stress and lipid mediators modulate immune cell functions

in autoimmune diseases. Int. J. Mol. Sci. 22, 723.
Xiao H.H. (2021). The role of oxidative stress and natural products in maintaining human health. Nutrients 16, 1268.
Yang G.L., Chang C.C., Yang Y.W., Yuan L., Xu L.S.Y., Ho C.T. and Li S.M. (2018). Resveratrol alleviates rheumatoid arthritis via reducing ROS and inflammation, inhibiting MAPK signaling pathways, and suppressing angiogenesis. J. Agric. Food Chem. 66, 12953-12960.
Yu D.M., Tang Z.X., Li B., Yu J.J., Li W.T., Liu Z.Y. and Tian C.N. (2021). Resveratrol against cardiac fibrosis: Research progress in experimental animal models. Molecules 26, 6860.
Yuan Q.Q., Zhang X.M., Yang X.N., Zhang Q., Wei X., Ding Z.M., Chen J.J., Hua H.Y., Huang D.K., Xu Y.X., Wang X.Y., Gao C.B., Liu S.X. and Zhang H.B. (2024). Knockdown of ACOT4 alleviates gluconeogenesis and lipid accumulation in hepatocytes. Heliyon 10, e27618.
Zhang L.X., Li C.X., Kakar M.U., Khan M.S., Wu P.F., Amir R.M., Dai D.F., Naveed M., Li Q.Y., Saeed M., Shen J.Q., Rajput S.A. and Li J.H. (2021). Resveratrol (RV): A pharmacological review and call for further research. Biomed. Pharmacother. 143, 112164.
Zheng X. and Sawalha A.H. (2022). The role of oxidative stress in epigenetic changes underlying autoimmunity. Antioxid. Redox Signal. 36, 423-440.
Zhou J., Zheng Q. and Chen Z. (2022). The Nrf2 pathway in liver diseases. Front. Cell Dev. Biol. 10, 826204.

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