



Cordycepin Attenuates IFN- γ -Induced Macrophage IP-10 and Mig Expressions by Inhibiting STAT1 Activity in CFA-Induced Inflammation Mice Model

Rirong Yang ^{1,5}, Xiaoli Wang,¹ Deshuang Xi,¹ Jian Mo,¹ Ke Wang,² Shunrong Luo,¹ Jiao Wei,² Zhenghua Ren,³ Hui Pang,² and Yu Luo^{4,5}

Abstract— Cordycepin, a natural derivative of adenosine, has been shown to exert pharmacological properties including anti-oxidation, antitumor, and immune regulation. It is reported that cordycepin is involved in the regulation of macrophage function. However, the effect of cordycepin on inflammatory cell infiltration in inflammation remains ambiguous. In this study, we investigated the potential role of cordycepin playing in macrophage function in CFA-induced inflammation mice model. In this model, we found that cordycepin prevented against macrophage infiltration in paw tissue and reduced interferon- γ (IFN- γ) production in both serum and paw tissue. Using luciferase reporter assay, we found that cordycepin suppressed IFN- γ -induced activators of transcription-1 (STAT1) transcriptional activity in a

Rirong Yang and Xiaoli Wang contributed equally to this work.

¹ Department of Immunology, School of Preclinical Medicine, Center for Genomic and Personalized Medicine, Guangxi Medical University, 22 Shuangyong Road, Nanning, Guangxi 530021, People's Republic of China

² Department of Physiology, School of Preclinical Medicine, Guangxi Medical University, 22 Shuangyong Road, Nanning, Guangxi 530021, People's Republic of China

³ State Key Laboratory of Biocontrol, Guangdong Province Key Laboratory of Pharmaceutical Functional Genes, National Engineering Research Center of South China Sea Marine Biotechnology, Department of Biochemistry, College of Life Sciences, Sun Yat-sen University, Guangzhou, 510275, People's Republic of China

⁴ Department of Clinical Laboratory, Peoples's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi 530021, People's Republic of China

⁵ To whom correspondence should be addressed to Rirong Yang at Department of Immunology, School of Preclinical Medicine, Center for Genomic and Personalized Medicine, Guangxi Medical University, 22 Shuangyong Road, Nanning, Guangxi 530021, People's Republic of China. E-mail: 520yrr@163.com; and Yu Luo at Department of Clinical Laboratory, Peoples's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi 530021, People's Republic of China. E-mail: 275851348@qq.com

dose-dependent manner. Moreover, western blotting data demonstrated that cordycepin inhibited IFN- γ -induced STAT1 activation through attenuating STAT1 phosphorylation. Further investigations revealed that cordycepin inhibited the expressions of IFN- γ -inducible protein 10 (IP-10) and monokine induced by IFN- γ (Mig), which were the effector genes in IFN- γ -induced STAT1 signaling. Meanwhile, the excessive inflammatory cell infiltration in paw tissue was reduced by cordycepin. These findings demonstrate that cordycepin alleviates excessive inflammatory cell infiltration through down-regulation of macrophage IP-10 and Mig expressions *via* suppressing STAT1 phosphorylation. Thus, cordycepin may be a potential therapeutic approach to prevent and treat inflammation-associated diseases.

KEY WORDS: cordycepin; macrophage; IP-10; mig; IFN- γ .

INTRODUCTION

Cordyceps militaris, which is well-known as a species of fungal genus *Cordyceps* in traditional Chinese medicine [1], has been reported to possess the capacity to antimicrobial [2] and anti-angiogenic [3] and decrease blood glucose [4]. Cordycepin (3'-deoxyadenosine), one of the main bioactive compounds extracted from *Cordyceps militaris* [5], has been reported to exhibit a great variety of biological and pharmacological activities, such as antitumor [6], anti-fungi [7], and antiviral [8]. Additionally, cordycepin was found to possess anti-inflammatory [9] and immune modulatory properties [10]. In the past few years, some reports have demonstrated that cordycepin is involved in the inhibition of macrophage activation and infiltration by regulating mitogen-activated protein kinase (MAPK) [11] and NF- κ B signaling pathway [11, 12]. For instance, cordycepin could effectively suppress lipopolysaccharide (LPS)-stimulated inflammatory response in RAW 264.7 macrophage through inactivation of the MAPK and NF- κ B pathways by inhibiting TLR4-mediated signaling [11]. It has also been shown that cordycepin could exert a protective effect against LPS-induced macrophage infiltration by inhibiting the activation of the NF- κ B pathway [12]. Furthermore, we proposed a detailed molecular mechanism that cordycepin regulating NF- κ B signaling pathway [13]. However, it is not clear whether the suppression of macrophage function by cordycepin is mediated *via* the Janus kinase (JAK)-signal transducer and STAT1 pathway.

JAK-STAT1 signaling pathway is triggered once IFN- γ binds to its receptor on the surface of immune cells, leading to a rapid increase in tyrosine phosphorylation of JAK1 and JAK2. Subsequently, STAT1 is phosphorylated and activated in response to upstream tyrosine kinases (JAK1 and JAK2) and form a dimer, and then STAT1

dimer travels to the nuclei and binds to the IFN-stimulated response element (ISRE) and the gamma-interferon activation site (GAS), leading to the transcription of responsive genes, such as IP-10, Mig [14, 15]. Increased release of pro-inflammatory chemokines (IP-10 and Mig) in turn results in the immune cell infiltration to the infection site, which plays important role in the occurrence and development of inflammation [16].

Complete Freund's Adjuvant (CFA)-induced paw edema or arthritis in mice shared with common pathological features with human arthritis has been a popular inflammatory model for evaluating the anti-inflammatory effect of compounds [17–19]. Under inflammatory agent stimulation, it is observed that macrophage was activated, and activated macrophage infiltrated into infection site. Aberrant activation and infiltration of macrophage often leads to inflammation development [20–22]. IFN- γ is one of the major factors in the induction of classically activated macrophages in inflamed tissue or lesions, while macrophage classical activation induced by IFN- γ requires sustaining STAT1 activation signal [23–25]. It has a therapeutic potential on inflammatory diseases with excessive macrophages activation and infiltration by targeting JAK/STAT1 signaling pathway. For example, a study reported that JAK inhibitors can suppress the STAT1 activation and downstream inflammatory target gene expression in JAK-STAT signaling pathway [26]. However, there is no report about that how cordycepin exerts its anti-inflammatory effect *via* interfering with IFN- γ -induced JAK-STAT1 signaling pathway.

In the present study, a CFA-induced inflammation mice model was established to investigate the anti-inflammatory effect of cordycepin. Both macrophage infiltration and IFN- γ expression in mouse paw were detected. We used IFN- γ as a STAT1 signaling inducer to further explore the mechanism for the interaction of cordycepin

with STAT1 in RAW264.7 macrophage, and the expressions of pro-inflammatory chemokines IP-10 and Mig were determined. Our findings suggested that cordycepin effectively exhibited anti-inflammatory effect on macrophage by inhibiting IFN- γ -induced IP-10 and Mig expressions in CFA-induced inflammation mice model.

MATERIALS AND METHODS

Preparation of Cordycepin, Plasmids, and Reagents

Cordycepin (purity 99.7%) was purchased from Guangzhou Trojan Pharmatec Ltd., P.R. China. Dexamethasone (hexadecadrol, specifications: 1 ml:5 mg, catalog number: 1501407-B12) was obtained from the First Affiliated Hospital of Guangxi Medical University, China.

Renilla, GAS (GAS Luc), and ISRE (ISRE Luc) luciferase reporter plasmids were kept by Dr. Yang, and both GAS and ISRE Luc plasmids contained STAT1 recognition site [27]. Dual luciferase reporter assay system was from Promega.

Antibodies for F4/80, IFN- γ , IP-10, and Mig as well as ELISA kits were purchased from Bioss Inc. GAPDH antibody was purchased from KangChen Bio-tech Inc. Anti-STAT1 (phospho Y701) antibody [M135] (ab29045) was from Abcam, Cambridge, UK. HRP-conjugated secondary antibody was purchased from Santa Cruz Biotechnology Inc. Radio-Immunoprecipitation Assay (RIPA) lysis buffer was obtained from Beyotime Institute of Biotechnology, Haimen, China. Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories, Richmond, CA. IFN- γ was obtained from GenScript. CFA (1 mg/ml) was purchased from Sigma. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Thermo Fisher Scientific).

Cell Culture and Stimulation

The mouse macrophage line RAW264.7, a product of ATCC (American Type Culture Collection, Manassas, VA, USA), was cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidity-saturated atmosphere with 5% CO₂. The cells were grown as a monolayer on 6-well plates at 5×10^5 cells/well in 2 ml of complete medium each well. Complete medium containing cordycepin (0, 25, 50, 100 μ g/ml, final concentration) was added to each well in a final volume of 3 ml. After 2 h, IFN- γ was added to each well at a final concentration of 100 ng/ml and incubated for 30 min. Then, the culture medium was removed. After

washing with PBS for twice, the cells were harvested for western blotting. For ELISA, the cells were treated with cordycepin for 2 h and then stimulated by IFN- γ for 24 h. Then the medium was collected for ELISA.

Animal Model

Experiments involving animals were approved by the Institutional Animal Care and Use Committee at Guangxi Medical University. Female KM mice purchased from the Experimental Animal Center of Guangxi Medical University were kept under a relative humidity of 54–56% and ambient temperature (23–26 °C) in a 12 h light-dark cycle with access to water and chow freely. All mice were allowed to adapt to environmental for 5 days before the experiments started.

Mice weighting about $35 \text{ g} \pm 3 \text{ g}$ were randomly assigned to six groups ($n = 10$ each). Mice were intraperitoneally injected daily for 7 days with saline solution, Dexamethasone (Dex: 2 mg/kg), and different doses of cordycepin (low-dose cordycepin, L-Cor: 2 mg/kg; moderate-dose cordycepin, M-Cor: 10 mg/kg; high-dose cordycepin, H-Cor: 20 mg/kg). Then, mice were administered with intradermal injection of CFA (20 μ l each) into the right hind paw for 72 h to induce inflammation. For control group, mice pretreated with saline solution were without CFA injection. Seventy-two hours later, mice were weighed and sacrificed.

Immunohistochemistry Analysis and Hematoxylin-Eosin Staining

After mice sacrifice, the animal paw tissues were collected and fixed in 4% paraformaldehyde fixing solution at 4 °C. After fixation, the paw tissues were decalcified, dehydrated, embedded in paraffin, and cut into about 4 μ m pieces. The sections were deparaffinized, rehydrated, and incubated with primary antibody (F4/80, IFN- γ , IP-10, and Mig) at 4 °C overnight. Then the sections were incubated with HRP-labeled secondary antibody and developed with DAB kit (ZSGB-BIO, China). Sections were photographed under microscope ($\times 400$), and 5–8 fields of view were selected on each section. The protein expression was quantified by software Image-Pro Plus v6.0 (Media Cybernetics, Inc., USA), and image analyses were performed as described [28, 29]. Data were presented as mean (\pm SD) optical density with DAB staining. For the observation of CFA-induced inflammatory cell infiltration in mouse paw, hematoxylin-eosin staining was performed, and sections were photographed under microscope ($\times 400$).

ELISA

Mice were treated with cordycepin for 7 days and then treated with CFA or vehicle control for 72 h. The mouse blood was taken by removalling eyeballs and collected in sterile 1.5 ml EP tube. The whole blood samples were placed in the refrigerator at 4 °C for 30–45 min and centrifuged at 3000 rpm for 10 min to obtain serum. Supernatant was collected and stored at –80 °C until analysis. All measurements were performed in duplicate. In brief, the blood samples or standards were added with 100 µl per well in 96-well plates. All samples were incubated at 37 °C for 2 h. After the plates was washed five times, substrates A (50 µl) and B (50 µl) were added into each well and were incubated for 30 min at 37 °C in the dark. Then, stop solution (50 µl) was added into each well. The optical density (O.D.) of each well was determined using a microplate reader (Bio-Rad, Thermo, USA) with an absorbency maximum at 450 nm. The concentrations of IFN- γ , IP-10, and Mig were calculated according to standard curves. Final results were reported as pg/ml.

Luciferase Reporter Assay

For the STAT1 transcriptional activity assay, RAW264.7 cells were seeded to 48-well plate and transiently cotransfected with 50 ng ISRE Luc reporter plasmid (or GAS Luc reporter plasmid) together with 5 ng pRL-TK, which expresses *Renilla* luciferase and was used to normalize transfection efficiency, using lipofectamine2000 reagent (Thermo Fisher Scientific). Twenty-four hours after transfection, cells were treated with the indicated dose of cordycepin and stimulated by IFN- γ (100 ng/ml) at the same time. After 24 h, cells were collected and the luciferase activity was determined using Dual-Glo™ Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. *Renilla* luciferase activity was used as a control, and ISRE Luc or GAS Luc activity was presented as the fold stimulation. The experiment was conducted in triplicate and repeated at least three times. Data were expressed as 'relative induction folds' (mean \pm SD).

Western Blotting Analysis

RAW264.7 cells were treated with cordycepin for 2 h, then stimulated with IFN- γ for 30 min, and harvested as described previously. Total cells were lysed with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, and 1% sodium dodecyl sulfate) containing

protease inhibitor and phosphatase inhibitor to obtain extracts of cells proteins. The protein concentration of cell lysates was tested using Bio-Rad protein assay kit. Subsequently, the samples were separated by 10% SDS-PAGE and were transferred onto nitrocellulose transfer membrane (Millipore) by electro-blotting. Following blocking with 10% non-fat milk solution for 2 h and washing with TBST three times, the membranes were incubated at 4 °C overnight with primary antibody for GAPDH (1:1000 dilution) or p-STAT1 (1:1000 dilution). After washing with TBST three times, the membranes were incubated with HRP-conjugated secondary antibody (1:2000 dilution) in TBST at 37 °C for 1 h. After washing, the membranes were reacted with Clarity western ECL substrate (Bio-Rad) and visualized by an imaging system (Image Station 4000MM, Carestream).

Statistical Analysis

The data are expressed as the mean \pm SD. The differences among groups were compared using one-way analysis of variance (ANOVA). All analyses were performed using the computer program SPSS 22.0 (SPSS Inc.) with a statistical significance of $p < 0.05$, and $p < 0.01$ was considered to be statistically very significant.

RESULTS

Cordycepin Reduces CFA-Induced Macrophage Infiltration in Mice

We established a CFA-induced inflammation mice model for examining the effect of cordycepin on macrophage infiltration (Fig. 1a). Mice were treated by intraperitoneal injection of cordycepin for 7 days, followed by paw injection of CFA for 72 h (Fig. 1a). Using F4/80 antibody, macrophages in paw tissue were detected by immunohistochemistry analysis. Results showed that an increased number of macrophages were found in Saline + CFA-treated group than that in control group (Fig. 1b and c, Saline + CFA). Dexamethasone was used as a positive control because of its anti-inflammatory action, and it exerted a preventive effect on CFA-induced macrophage infiltration (Fig. 1b and c, Dexa + CFA). Although there was no statistically significant difference in the number of macrophages between low-dose cordycepin group (L-Cor + CFA) and Saline + CFA group, a lower number of macrophages were observed with moderate-dose and high-dose cordycepin treatments (M-Cor + CFA and H-Cor + CFA) when compared to Saline + CFA group (Fig. 1b and c).

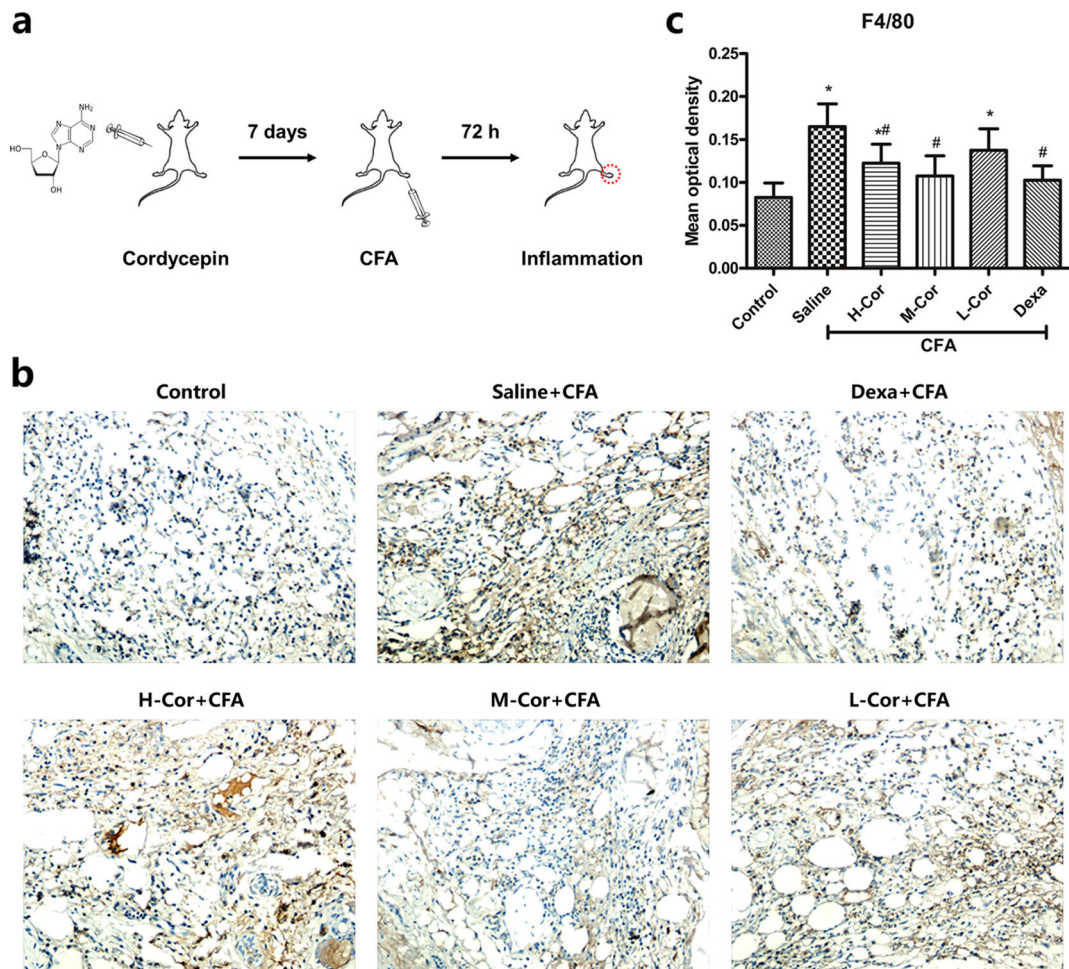


Fig. 1. The inhibitory effect of cordycepin on CFA-induced macrophage infiltration. (a) Mice were treated with cordycepin for 7 days, followed by paw injection of CFA for 72 h. (b) Immunohistochemistry analysis of F4/80 in mouse paw. Moderate (10 mg/kg) and high dose (20 mg/kg) of cordycepin decreased macrophage infiltration in mouse paw by comparison of Saline + CFA group. Magnification $\times 400$. (c) The mean optical density values for F4/80 expression in mouse paw were presented from CFA-induced inflammation mice model. F4/80 expression decreased with moderate and high doses of cordycepin treatment. Data were presented as mean \pm SD. *, compared with control, $p < 0.05$. #, compared with Saline + CFA group, $p < 0.05$.

Especially in moderate-dose cordycepin treatment group (Fig. 1b and c, M-Cor + CFA), the effectiveness on decreasing macrophage infiltration was similar to that in dexamethasone treatment group (Fig. 1b and c, Dexa + CFA). These data indicated that cordycepin inhibited CFA-induced inflammation through suppressing macrophage infiltration.

Cordycepin Reduces IFN- γ Production in CFA-Induced Inflammation

IFN- γ is one of the major factors in induction of classically activated macrophages in inflamed tissue or

lesions [24], and the activated macrophages secreted abundant IFN- γ [30]. Therefore, we investigated the effect of cordycepin on IFN- γ production in both paw tissue and serum in CFA-induced inflammation mice model by immunohistochemistry and ELISA analysis. Compared with control group, IFN- γ production in Saline + CFA group increased obviously in paw tissue (Fig. 2a and b, Saline + CFA). By comparison with Saline + CFA group, treatment with cordycepin at moderate and high doses (M-Cor + CFA and H-Cor + CFA) decreased CFA-induced IFN- γ release in paw tissue (Fig. 2a and b). The repressive effect of cordycepin on CFA-induced IFN- γ production was also observed in serum samples (Fig. 2c). Especially, moderate

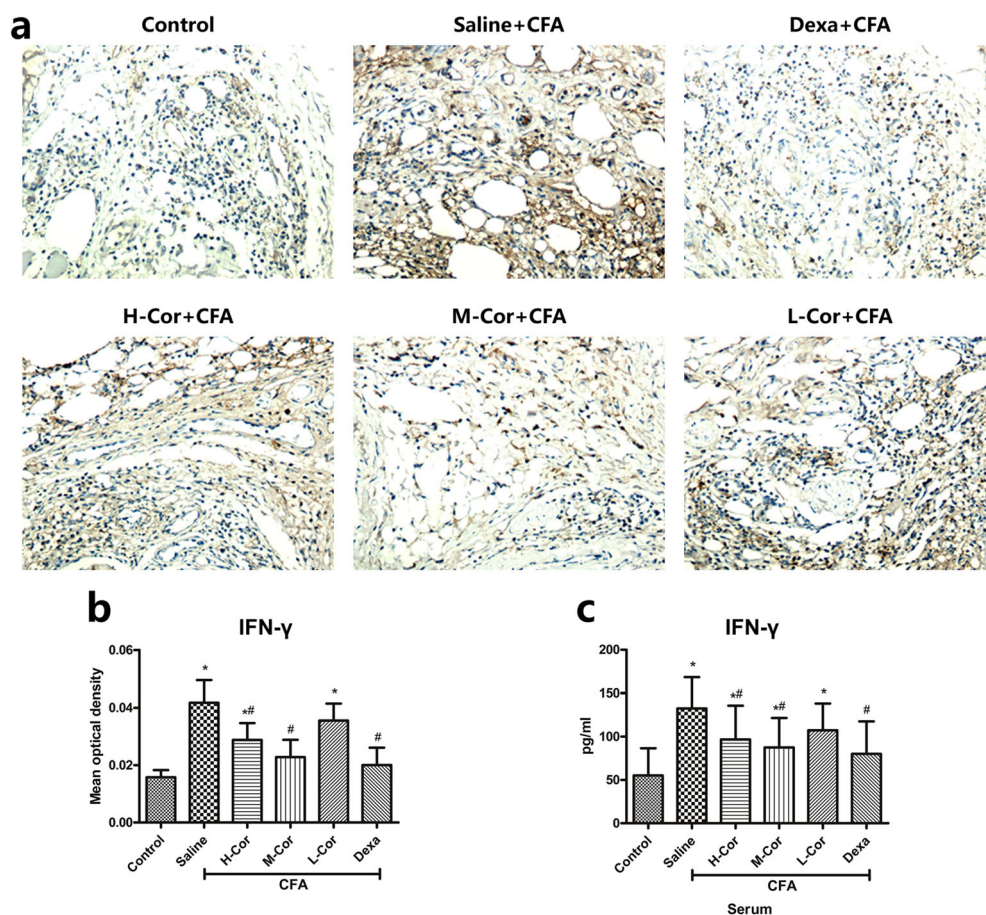


Fig. 2. The inhibitory effect of cordycepin on IFN- γ production in CFA-induced inflammation. (a) IFN- γ level in mouse paw was analyzed by immunohistochemistry. (b) The mean optical density values for IFN- γ expression in mouse paw were presented from CFA-induced inflammation. (c) IFN- γ level in mouse serum was determined by ELISA. Data were presented as mean \pm SD. *, compared with control, $p < 0.05$. #, compared with Saline + CFA group, $p < 0.05$.

dose of cordycepin showed similar efficacy to dexamethasone treatment (Fig. 2, M-Cor + CFA and Dexa + CFA). These results implied that cordycepin reduced IFN- γ production to regulate macrophage classical activation in CFA-induced inflammation.

Cordycepin Inhibits IFN- γ -Induced ISRE and GAS Activations

IFN- γ can induce macrophage activation by regulating STAT1 signaling [31]. To further explore whether the repressive effect of cordycepin on macrophage infiltration and IFN- γ secretion was mediated *via* IFN- γ -induced STAT1 transcriptional activation, effect of cordycepin on STAT1 transcriptional activity was measured by luciferase reporter assay. Without IFN- γ induction, we could not

detect both ISRE and GAS Luc activities. With only IFN- γ stimulation, both ISRE and GAS Luc activities were highly induced (Fig. 3a and b). Treatment with cordycepin markedly inhibited the IFN- γ -induced transcriptional activations of both ISRE and GAS (Fig. 3a and b) by comparison of the group stimulated only with IFN- γ and exhibited a dose-dependent manner. The data implied that cordycepin down-regulated IFN- γ -induced STAT1 transcriptional activity.

Cordycepin Suppresses IFN- γ -Induced STAT1 Phosphorylation

STAT1 phosphorylation leads to form STAT1 dimer, which binds to the IFN-stimulated response elements (ISRE and GAS), resulting in transcription of target genes,

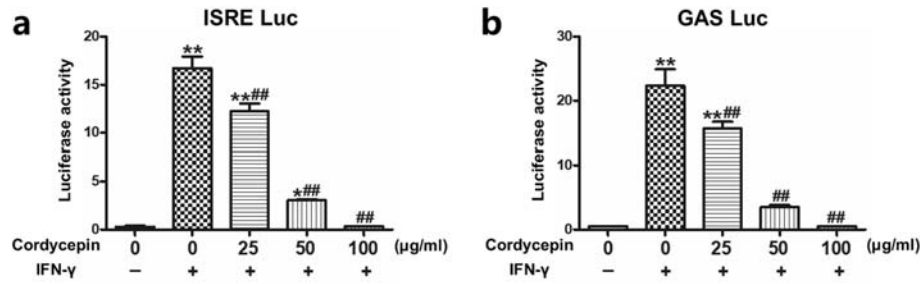


Fig. 3. The suppressive effect of cordycepin on IFN- γ -induced ISRE and GAS Luc activations. Increasing amounts of cordycepin resulted in a dose-dependent reduction in both ISRE (a) and GAS (b) luciferase activities. Bars indicate the mean of three independent measurements (\pm SD). Compared with blank (without cordycepin treatment and IFN- γ stimulation), *, $p < 0.05$, **, $p < 0.01$. Compared with only IFN- γ stimulated group, #, $p < 0.05$, ##, $p < 0.01$.

including IP-10 and Mig [26]. To distinguish whether the reduced ISRE and GAS Luc activation was attributable to decreased STAT1 phosphorylation, western blot assay was performed. Without IFN- γ induction, p-STAT1 was not observed. After IFN- γ stimulation, abundant p-STAT1 was detected, and cordycepin decreased phosphorylated STAT1 protein level in a dose-dependent manner (Fig. 4). The result suggested that cordycepin inhibited STAT1 phosphorylation to decrease STAT1 transcriptional activity. This prompted us to further investigate the effect of cordycepin on the expression of IP-10 and Mig, which were the downstream target genes of STAT1 signal [32].

Cordycepin Blocks IFN- γ -Induced IP-10 Expression

To analyze the effect of cordycepin on IP-10 production, ELISA analysis was conducted. IP-10 protein expression levels in IFN- γ -induced RAW264.7 macrophages were significantly higher than that without IFN- γ stimulation (Fig. 5a). Compared with only IFN- γ -stimulated cells, cordycepin treatment at dose of 100 μ g/ml repressed IFN- γ -induced IP-10 expression (Fig. 5a). We further examined the effect of cordycepin on IP-10 expression in CFA-induced inflammation mice model. The IP-10 expression induced by CFA in serum was suppressed by cordycepin at moderate and high doses (M-Cor + CFA and H-Cor +

CFA) as compared with Saline + CFA group (Fig. 5b). In paw tissue, cordycepin treatment shared the similar outcome as dexamethasone treatment on IP-10 expression (Fig. 5c and d).

Cordycepin Blocks IFN- γ -Induced Mig Expression

We further evaluated the inhibitory effect of cordycepin on Mig production, another effector gene in STAT1 signaling. Similar to the effect on IP-10, cordycepin treatment at dose of 100 μ g/ml markedly inhibited IFN- γ -induced Mig expression in RAW264.7 cells (Fig. 6a). In mice model, cordycepin treatment at moderate and high doses (M-Cor + CFA and H-Cor + CFA) reduced Mig production in both serum (Fig. 6b) and paw tissue (Fig. 6c and d), which shared the similar outcome as dexamethasone treatment on Mig expression (Fig. 6b–d, Dexa + CFA). These data demonstrated that cordycepin was able to suppress STAT1 signaling to reduce IP-10 and Mig expressions.

Cordycepin Protects against Recruitments of Inflammatory Cells

To further observe CFA-induced inflammatory cell infiltration in mouse paw, hematoxylin-eosin staining was performed. As shown in Fig. 7, compared with

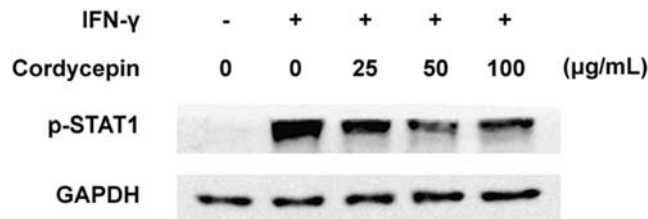


Fig. 4. Cordycepin suppressed IFN- γ -induced STAT1 phosphorylation in RAW264.7 cells. Phosphorylated STAT1 (p-STAT1) was not detected without IFN- γ stimulation. After IFN- γ stimulation, abundant p-STAT1 was detected, and cordycepin decreased p-STAT1 protein level in a dose-dependent manner.

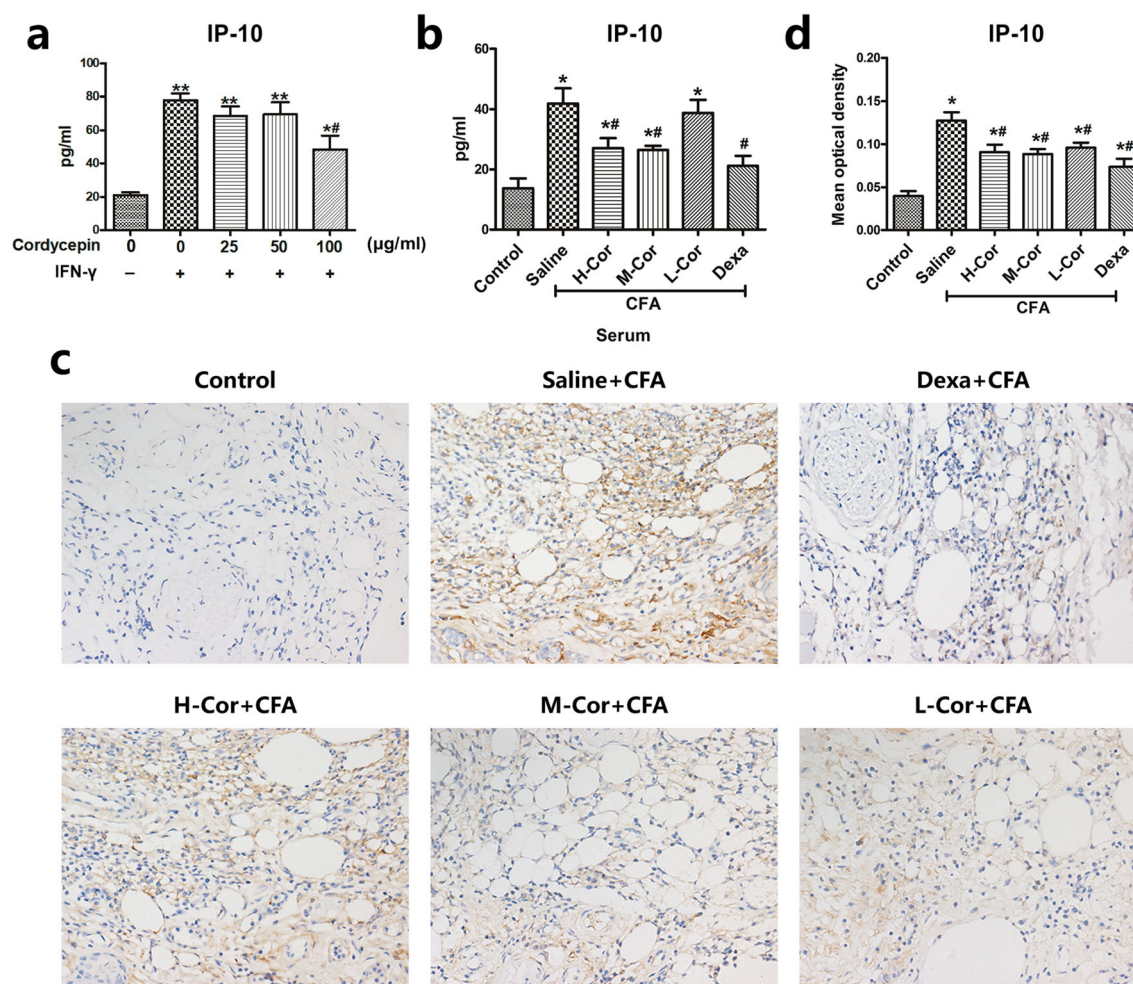


Fig. 5. Cordycepin inhibited IFN- γ -induced IP-10 expression. (a) Cordycepin treatment at dose of 100 μ g/ml inhibited IFN- γ -induced IP-10 protein production in RAW264.7 cells. Compared with blank, *, $p < 0.05$, **, $p < 0.01$. Compared with only IFN- γ stimulation, #, $p < 0.05$. (b) Effect of cordycepin on IP-10 production in mouse serum in CFA-induced inflammation. Cordycepin treatment at moderate and high doses decreased IP-10 production in mouse serum by comparison of Saline + CFA group. (c) IP-10 production in mouse paw was analyzed by immunohistochemistry in CFA-induced inflammation. Magnification $\times 400$. (d) The mean optical density values for IP-10 expression in mouse paw. (b and d), Compared with control, *, $p < 0.05$. Compared with Saline + CFA group, #, $p < 0.05$.

control group, an increased number of inflammatory cells infiltrating in mouse paw were found in CFA-treated group (Fig. 7, Saline + CFA), and it was effective in reducing CFA-induced inflammatory cell infiltration with low and high doses of cordycepin after CFA treatment (Fig. 7, L-Cor + CFA and H-Cor + CFA). Furthermore, the mice received moderate dose of cordycepin or dexamethasone treatment exhibited a remarkable reduction in CFA-induced inflammatory cell infiltration by comparison with the Saline + CFA group.

DISCUSSION

Macrophage plays an important role in the initiation and propagation of inflammatory response as well as restoring cellular homeostasis *via* modulating the production of pro-inflammatory or anti-inflammatory mediators [20]. In inflamed tissue or lesions, macrophages infiltrate to protect host from damages [33–35]. However, excessive macrophage activation has been described to produce excessive pro-inflammatory cytokines and chemokines, promoting inflammation and autoimmune disorders [36, 37].

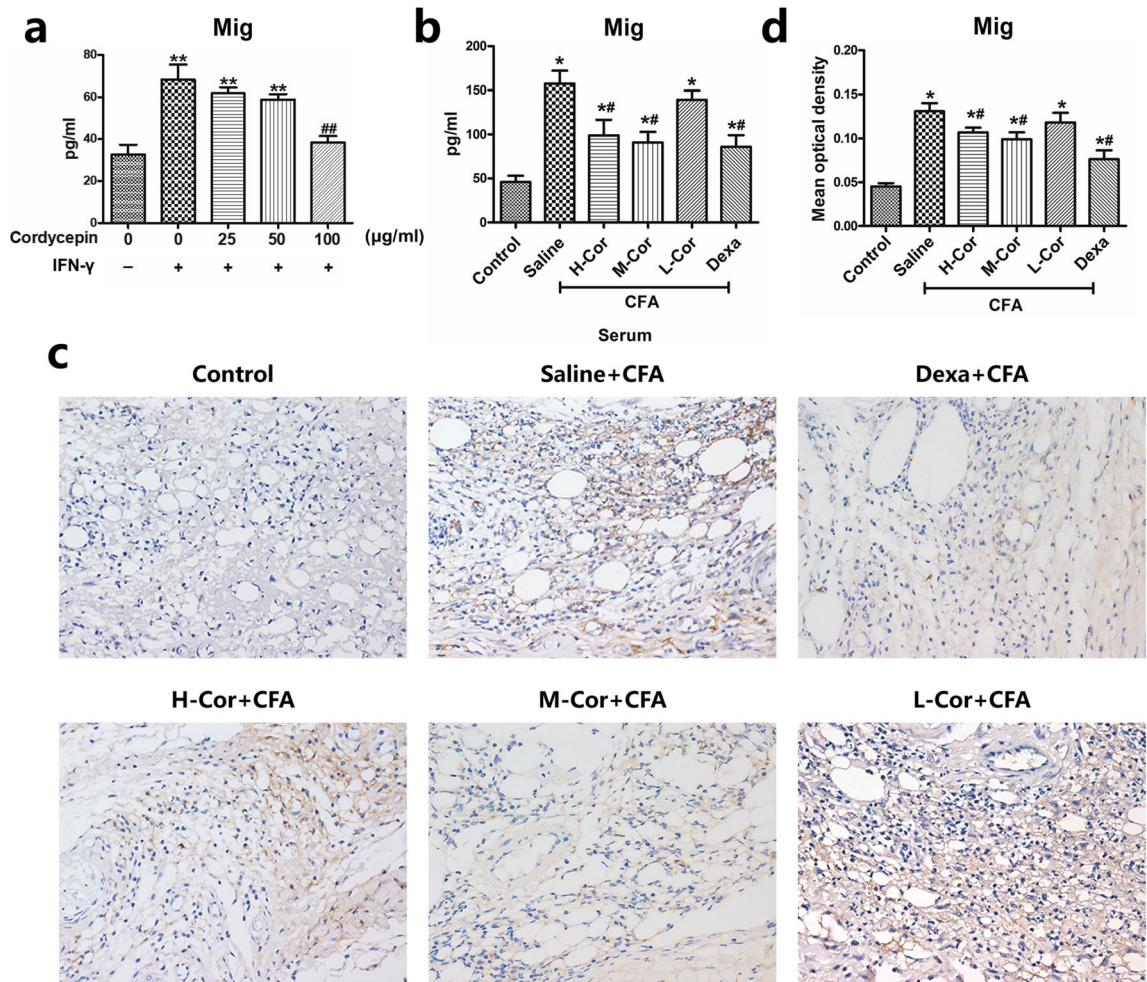


Fig. 6. Cordycepin inhibited IFN- γ -induced Mig expression. (a) Cordycepin treatment at dose of 100 μ g/ml significantly inhibited IFN- γ -induced Mig protein production in RAW264.7 cells. Compared with blank, **, $p < 0.01$. Compared with only IFN- γ stimulation, ##, $p < 0.01$. (b) Effect of cordycepin on Mig production in mouse serum in CFA-induced inflammation. The Mig level in mouse serum was decreased by cordycepin at moderate and high doses compared with Saline + CFA group. (c) Mig production in mouse paw was detected by immunohistochemistry. Magnification $\times 400$. (d) The mean optical density values for Mig expression in mouse paw. (b and d) Compared with control, *, $p < 0.05$. Compared with Saline + CFA group, #, $p < 0.05$.

Our data reveal that cordycepin attenuates macrophage infiltration, which is consistent with a previous study that cordycepin attenuates the increase of local neutrophils and macrophages on D-galactosamine/lipopolysaccharide-induced acute liver injury [38]. Cordycepin has also been reported to down-regulate the LPS-induced macrophage migration in the intervertebral disc [12]. Moreover, cordycepin could inhibit the mononuclear cell infiltration in the portal areas in inflammation-induced osteoporosis model [39]. In addition, cordycepin significantly inhibited LPS-induced activities of MPO and MDA, showing that cordycepin decreased neutrophil and macrophage infiltration in acute lung injury [40]. These studies demonstrate

that cordycepin attenuates the injuries in organs caused by inflammatory agents through negatively regulating macrophage infiltration.

IFN- γ is one of the major factors in induction of classically activated macrophages in inflamed tissue or lesions [23–25]. Classically activated macrophages dominantly secrete pro-inflammatory cytokines and chemokines, such as IFN- γ , IL-1 β , IL-6, and TNF- α , which will further incur damage in the infected tissue [30, 41]. Overproduction of IFN- γ served as a predictor of inflammation progression, and suppression of IFN- γ production might have promising therapeutic strategy for treating and preventing excessive inflammatory response

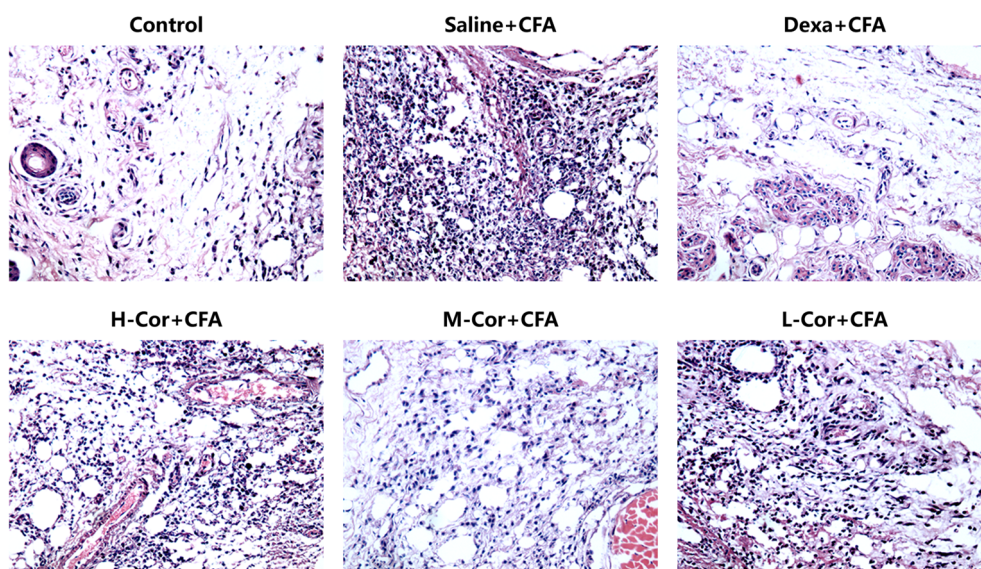


Fig. 7. The inhibitory effect of cordycepin on inflammatory cell infiltration in mouse paw in CFA-induced inflammation. Hematoxylin-eosin staining analysis of inflammatory cell infiltration in mouse paw tissue. Magnification $\times 400$. The inflammatory cells significantly decreased with middle dose of cordycepin treatment (10 mg/kg).

and disease. In the present study, we demonstrate that cordycepin significantly reduces IFN- γ expression in both paw tissue and serum in CFA-induced inflammation mice model (Fig. 2). On the one hand, cordycepin inhibits the activation of macrophages to suppress IFN- γ production.

On the other hand, cordycepin suppresses macrophage infiltration (Fig. 1) in inflamed tissue so as to reduce pro-inflammatory cytokine productions such as IFN- γ , IP-10, and Mig (Figs. 2, 5, and 6). A similar research, which to some extent supported our results, reported that LPS-

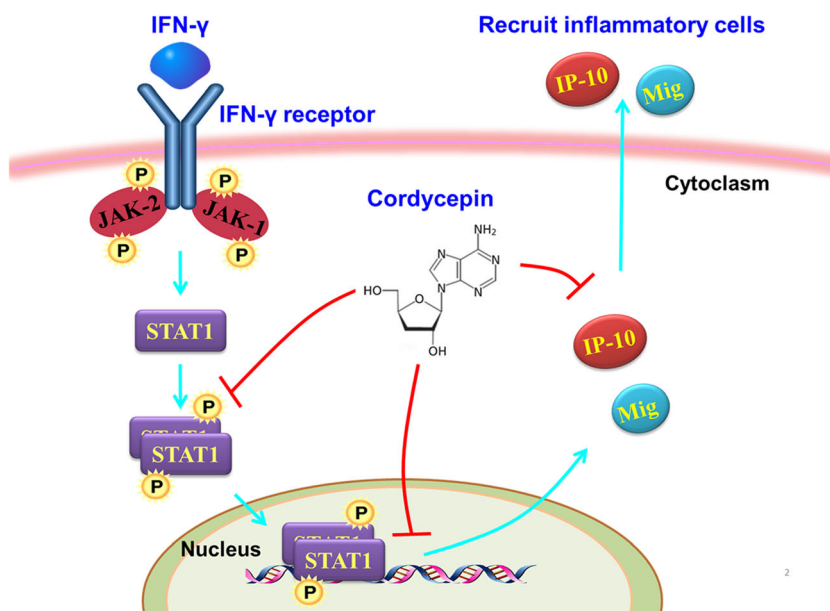


Fig. 8. A proposed mechanism that cordycepin attenuates IFN- γ -induced IP-10 and Mig expressions in macrophage in CFA-induced inflammation mice model. Cordycepin inhibits IFN- γ -induced STAT1 phosphorylation and transcriptional activation, leading to the reduction of chemokine IP-10 and Mig and decrease of inflammatory cell infiltration. The symbol ‘ \rightarrow ’ represents signal transition, and the red symbol T-shape represents inhibitory effectiveness.

activated macrophages returned to the original inactivated state after cordycepin treatment [41]. These observations indicate that cordycepin reduces macrophage infiltration to decrease IFN- γ production.

Because transcription factor STAT1 has an important role in IFN- γ -induced macrophage activation in human and mice [31], we attempted to further investigate the possible mechanism of anti-inflammatory effect of cordycepin on macrophage. The activity and phosphorylation level of transcriptional factor STAT1 were examined in the present study. The current data demonstrated that cordycepin decreased the activities of ISRE and GAS luciferase reporter induced by IFN- γ (Fig. 3). Further study indicated that cordycepin significantly inhibited STAT1 phosphorylation in IFN- γ -induced RAW264.7 cells (Fig. 4). Nevertheless, other research group reported that cordycepin could markedly enhance p-STAT1 expression in a murine oral cancer model [17]. These contradictory results may be attributed to the difference between IFN- γ signaling and IL-17RA signaling or the different cell lines used in the experiments.

Studies have revealed that the classical STAT1 target genes (including IP-10 and Mig) are predominantly expressed by activated macrophages in inflamed tissue or lesions [20]. Increased release of chemokine IP-10 and Mig by activated macrophages in turn selectively recruits peripheral blood mononuclear cells such as monocytes/macrophages and T cells to the inflammatory site, which plays important roles in the occurrence and development of inflammation [16]. In our research, CFA up-regulates the expressions of IP-10 and Mig in macrophage, thereby promotes the migration of macrophage and other inflammatory cells in CFA-induced inflammation mice model (Fig. 7). However, cordycepin suppresses IP-10 and Mig expressions in this model (Figs. 5 and 6), thus reduces the inflammation in mouse paw. We also demonstrated that cordycepin could inhibit IP-10 and Mig gene expressions at protein levels in IFN- γ -induced RAW264.7 cells (Figs. 5 and 6), suggesting that cordycepin exerting its protective function against inflammatory cell infiltration is mediated by down-regulating IP-10 and Mig expressions.

Our findings demonstrate that cordycepin suppresses STAT1 activity to inhibit chemokine IP-10 and Mig expressions in IFN- γ -induced macrophage activation, leading to decreasing inflammatory cell infiltration in inflammation (Fig. 8). The inhibitory effect of cordycepin on macrophage might alleviate inflammation-induced tissue damage and organ failure

with marked inflammatory cell infiltration, thus cordycepin may be a potential new agent for treating and preventing inflammatory diseases.

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