WITHDRAWN: Qihuzha Granule Alleviated Cyclophosphamide-induced Immune Deficiency in Mice via IL-6 Related Signaling Pathways

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Research

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The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Background

Qihuzha granule (QHZG) is a Chinese patent medicine, composed of 11 kinds of edible medicinal plants, which is used to treat dyspepsia and anorexia in children caused by spleen and stomach deficiency syndrome. However, its role and mechanism in immunosuppression induced by cyclophosphamide remained unclear.

The purpose of this study is to investigate the effect of QHZG on immunosuppression induced by cyclophosphamide in mice and its possible mechanism.

Methods

The immunosuppression injury model was induced by intraperitoneal injection of cyclophosphamide (100 mg/kg); the mRNA level of cytokines (IL-2/4/6, IFN-γ) and critical targets of signaling pathways related to immune regulation (JNK, ERK, P38, JAK2, SRC and STAT3) were tested by QPCR; related protein levels were detected by western blotting; hematoxylin-eosin (HE) staining was employed to observe the histological alterations; macrophages and neutrophils in the mouse spleen were examined by immunofluorescence analysis.

Results

QHZG significantly increased the spleen index and thymus index of mice with immunodeficiency induced by cyclophosphamide and up-regulated the mRNA expression of cytokines (IL-2/4/6, IFN-γ) and critical targets of signaling pathways related to immune regulation (JNK, ERK, P38, JAK2, SRC and STAT3), which were decreased by cyclophosphamide treatment. The results of immunofluorescence staining and histological analysis showed that QHZG could also protect mice from immunosuppressive injury caused by cyclophosphamide via keeping structural integrity of spleen, and partially restoring the production levels of macrophages and monocytes in the spleen. Further studies indicated that QHZG could significantly counter the decline of phosphorylated protein levels of JAK2/SRC-STAT3 axes (P-JAK2, P-SRC and P-STAT3), and MAPK pathways (P-JNK, P-ERK and P-P38) induced by cyclophosphamide, suggesting that the protective effects of QHZG on immunosuppressive injury triggered by cyclophosphamide were involved in JAK2/SRC-STAT3 axes, and MAPK pathways. Meanwhile, we also found that QHZG could partially restore the vital phosphorylated proteins of PI3K/Akt/mTOR signaling pathway (P-Akt, P-mTOR), which were reduced by cyclophosphamide. The data implied that PI3K signaling pathway was also responsible for the protection of QHZG against the immunosuppression induced by cyclophosphamide in mice.

Conclusions

Our study demonstrated that QHZG protected mice from cyclophosphamide-triggered immunosuppressive injury via IL-6 and its downstream signaling pathways including PI3K/Akt/mTOR

signal pathway and JAK2-SRC/MAPK/STAT3 axes. These results suggested that QHZG might serve as a new drug for the treatment of the immunosuppression caused by cyclophosphamide therapy.

Introduction

The immune system plays a prominent role in resisting microorganisms and foreign antigens, and involves in the recovery from diseases determined by the host immune status [1]. Cyclophosphamide (CTX) is a broad-spectrum antitumor agent that is effective against both leukemia and many solid tumors. However, CTX has been found to inhibit humoral and cellular immune responses of animals and resulted in immunosuppression of animals [2]. Hence, as an immunosuppressant, it has been widely used in the establishment of immunosuppressive animal models. In this study, the immunosuppressive model of BALB/c mice was established by cyclophosphamide to observe the immunoregulation effect of QHZG on cyclophosphamide-induced immunosuppressive model mice.

Over the years, the discovery of novel immunomodulators has attracted researchers' great attentions and interests [3], and a series of breakthroughs have been made. However, serious adverse reactions of clinical drugs, such as general discomfort, neurotoxicity, etc. [4], were still not enough to meet the treatment requirements of immunosuppressive patients. QHZG is a kind of Chinese patent medicine derived from natural products and mainly composed of 11 traditional edible and medicinal plants [5], such as *Dioscorea zingiberensis, Astragalus membranaceus, Poria cocos, roasted licorice, melon, Dendrobium, Atractylodes macrocephala, hawthorn, Codonopsis pilosula, malt, papaya*, etc. It was mainly used to treat anorexia and dyspepsia in children with spleen and stomach deficiency syndrome [6]. Due to its good health care effect and high safety, it was also widely used in the adjuvant treatment of children with recurrent respiratory tract infections in clinical [5, 7, 8]. Moreover, modern pharmacological studies have shown that *polysaccharides* from *Dendrobium, astragalus, Codonopsis pilosula* and other major traditional Chinese medicines in QHZG could inhibit the inflammatory reaction caused by a variety of infections, and then have immunomodulatory effect [9, 10, 11, 12]. In addition, some compounds identified in QHZG, including *Atractyloside II* and *Atractyloside III* in *Atractylodes lancea*, *pachymic acid* in *Poria cocos* and *lobeliol* in *Codonopsis pilosula* [13], showed a certain degree of immune enhancing activity. These results suggested that QHZG might has multiple immunomodulatory effects. However, its regulatory effects and the potential mechanisms on immunosuppression have not been reported.

IL-6, a pleiotropic cytokine that acts on a variety of cell types and affects various biological activities, is a key mediator in various inflammatory processes, through which tissues respond to injury and infection, either in acute phase or chronic inflammatory diseases[14], In addition, IL-6 also plays crucial role on the host innate immunity and leads to the transition of acquired immune response [15]. Previous study had shown that IL-6 might contribute to reconstitution of immune system function in immunocompromised mice caused by cyclophosphamide[16]. In summary, IL-6 signaling pathway might serve as potential targets for development of immune modulators.

IL-6 utilize gp130 to conduct intracellular signal transduction [17] and gp130 can form complexes with the Janus kinases (JAKs), including JAK2. JAKs were activated through auto-phosphorylation and then phosphorylated gp130, STAT and the tyrosine phosphatase SHP-2. Phosphorylated STAT3 dimerizes is translocated to the nucleus where it acts as a transcription factor. Additionally, phosphorylated SHP-2 and activated gp130 lead to activation of the PI3k/AKT pathway [18]. In the present study, we intended to investigate the immunomodulatory effects of QHZG on cyclophosphamide-induced immunocompromised mice and examined the effects of QHZG on crucial components of IL-6 related pathway to elucidate the possible mechanism.

Material And Methods

Materials

Qihuzha granules and cyclophosphamide were provided by Sunflower Pharmaceutical Group (Guizhou) Hongqi Co., Ltd, (Guizhou, China) and Innochem Co., Ltd (INA100012, Beijing, China).

Establishment of the mice models

Balb/c mice, 16±1g, were purchased from Liaoning Changsheng Biotechnology Co., Ltd, (Liaoning, China). All animal experiments were carried out according to the requirements of animal protection and use Committee of Guizhou Medical University. Briefly, animals were randomly divided into three groups (10 BALB/c mice in each group) and given different treatments: control group: normal saline; Model group: cyclophosphamide 100 mg/kg/d; QHZG group: mice were given QHZG 100 mg/kg/d by gavage. Except for the control group, CTX (100 mg/kg/d) was injected subcutaneously on the 1 st, 3 rd, 5 th, 7 th, 9 th, 16 th, 18 th, 20 th and 22 nd day. The mice in each group was weighed from the first day of feeding, and the body weight was recorded every 3 days, a total of 10 times. Thymus index and spleen index were calculated by thymus or spleen weight/body weight.

Histological analysis

In short, the spleen tissue was fixed with 4 % paraformaldehyde and stained with hematoxylin eosin. The changes of spleen tissue was observed under inverted microscope (DMi8, Leica, Germany).

Immunofluorescence staining of spleen tissue

After dewaxing, rehydration and antigen repair under high temperature and high pressure, embedded splenic sections were treated with F4/80 (1:500, Abcam) or Ly-6G (1:500, Cell signaling technology) in 4 $^{\circ}$ C incubated overnight. At room temperature, anti rabbit IgG labeled with Alexa flour 568 (Thermo Fisher Scientific, Waltham, Ma, USA) was incubated for 1 hour, then washed three times with TBST and then cultured with DAPI 5 minutes. Finally, slices were rinsed with TBST for 3 times and the image was obtained by microscope (DMi8, Leica, Germany).

Western blot analysis

BCA protein analysis kit (Thermo Scientific, Rockford, IL, USA) was used to determine the protein concentration in fully dissolved spleen tissue. Protein samples separated from 10% SDS polyacrylamide gel electrophoresis were transferred to the PVDF membrane and 4 ℃ incubated with Phospho-JNK (CST, #4685), JNK (CST, #9252), Phospho-SRC (CST, #12432), Phospho-STAT3 (CST, #9145), Phospho-ERK (CST, #4370), Phospho-p38 (CST, #4511), SRC (Proteintech, 11097-1-AP), STAT3 (Proteintech, 10253-2-AP), ERK (Proteintech,16443-1-AP), P38 (CST, #9212S), GAPDH (Proteintech, 10494-1-AP). After washing with TBST buffer for three times, goat anti-Rabbit lgG-HRP Abs (As007) antibody was incubated at room temperature for 1 h. Electrochemiluminescence (ECL) detection kit (Bio Rad, USA) and chemodoc TM MP imaging system (Bio Rad, USA) were used to image the imprint.

Real-time PCR

1ml Trizol reagent was added into the EP tube of RNA free in bladder tissue, grind it with a grinder for 20 s without grinding, rest for 10 s until there was no residue at the bottom of the EP tube, and let it stand at room temperature for 5 min. 12000 rpm centrifuged at 4 ℃ for 5 min, the supernatant was drawn to another EP tube, 200 μl chloroform was added, and the centrifuge tube was tightly covered, shake violently for 25 s. Let it stand at room temperature for 5 minutes. After centrifugation at 4 ℃, 12000 rpm for 15 minutes, transfer the upper colorless aqueous phase containing RNA to another EP tube, add 0.5 ml isopropanol, turn it upside down and mix well. After centrifugation, leave it in refrigerator at 4 ℃ for 15 min. After centrifugation at 4 ℃ and 12000 rpm for 10 min, transparent precipitates appeared at the bottom. Discard the supernatant, add 1 ml of precooled 75 % ethanol, and turn it upside down until the precipitate floats in 75 % ethanol. After centrifugation at 4 ℃, 7500 rpm for 5 min, the supernatant was discarded, dried and precipitated at room temperature for 1-2 min, then 20-50 ul RNase free water was added to dissolve and precipitate. The extracted RNA was reverse transcribed according to the instructions of primescript RT reagent kit (TAKALA, Japan). The primers in **Table 1** were diluted according to the manufacturer's requirements. QPCR 20 μL system: primer I 0.5 μL, primer II 0.5 μL, SY Green 10 μL, cDNA 1 μL. Gene expression level was calculated by $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Results were presented as mean±SD. Data analysis was achieved by using One-way analysis of variance (ANOVA) followed by Tukey post-hoc test (GraphPad Software, San Diego, CA). $P < 0.05$ was considered of great significance.

Results

Immune improvement of QHZG in CTX-induced immunocompromised mice

Thymus and spleen are vital immune organs and are accountable for initiating immune reactions in the body [19, 20, 21]. Thymus and spleen will atrophy when the immune function of the body declines. Therefore, thymus index and spleen index are often used to evaluate the immunity of

immunocompromised mice. As shown in Fig. 1, the effects of cyclophosphamide and QHZG on the immune organ weight were tested. Compared to the control group, lower thymus index and spleen index were observed in the CTX treated group, which suggested the mouse immunodeficiency model was successfully established by intraperitoneal injection of cyclophosphamide. Whereas QHZG (100 mg/kg) significantly raised the thymus index and spleen index of CTX treated mice, suggesting their partial recovery of immune function. Collectively, QHZG might partly contribute to reconstitution of immune system function in CTX induced immunocompromised mice.

Histological analysis of spleen

Since the spleen is the body's largest immune organ [19, 20], we further examined the effects of QHZG on the alteration of histomorphology of spleen in CTX treated mice. As shown in Fig. 2, in the control group, the red and white pulp were clearly demarcated and lymphocytes in the white pulp were more densely distributed. However, the spleen tissue structure of the CTX treated group, the boundary between red and white pulp was not clear, the structure of white pulp was more scattered and lymphocytes were sparsely distributed. On the contrary, the white intramedullary lymphocytes were increased and the lymphocytes became dense in the spleen of QHZG treated group, compared with that in the CTX treated group. These results certified that QHZG at least protected the spleen from CTX induced immunosuppression injury to a certain extent, thus improving the immune function of immunocompromised mice.

Effects of QHZ on neutrophils in CTX-treated mice

Neutrophils, the first line of defense to protect the body from infection or inflammation, are key participants in innate immune responses and commonly used to assess nonspecific immune status [21]. Therefore, Ly-6G immunostaining analysis was used to disclose neutrophil infiltration in cyclophosphamide-induced immunosuppression injury. As shown in Fig. 3, the number of ly-6G positive cells in the cyclophosphamide treated group was markedly decreased compared to that in the control group. Importantly, QHZG treatment led to an increase in the number of ly-6G-positive cells. In a conclusion, in vivo results implied that QHZG treatment could partly restore impaired neutrophils.

Effects of QHZ on macrophage

Macrophage activation plays an important role in innate and adaptive immune responses and is believed to be involved in the pathogenesis of immunosuppression respond [22]. To further explore the possible protective mechanism of QHZG on cyclophosphamide-induced immunosuppressive injury, F4/80 (macrophage surface marker)-positive cells in the spleen were examined. As shown Fig. 4, compared with the control group, cyclophosphamide treatment decreased the number of F4/80-positive cells. While, QHZG-treated group dramatically increased the number of F4/80-positive cells in the spleen. Collectively, these results demonstrated QHZG has protective effect on immunosuppression induced by cyclophosphamide via increasing spleen macrophages.

Effects of QHZG on the mRNA of cytokine

Immune stimulation is one of the important strategies to improve the body's defense mechanism [23].Activated splenic lymphocytes plays an important role in innate and adaptive immune responses by producing cytokines, such as IL-2, IL-4, IL-6, IFN-γ and other inflammatory mediators. As illustrated in Fig. 5, compared with control group, mRNA levels of IL-2/4/6 and IFN-γ in cyclophosphamide treated group were significantly decreased. In contrast, QHZG treatment at 100 mg/kg markedly upregulated the mRNA levels of these cytokines mentioned above, which were reduced by CTX treatment, suggesting that QHZG markedly improved splenocytes function of CTX-treated mice and contributed to reconstitution of immune system function in immunocompromised mice caused by CTX. As is known to all, interleukin-6, a cytokine critical to proinflammatory and immune regulatory cascades, has a broad effect on cells of the immune system and those not of the immune system [24, 25] Hence, therapeutic agents targeting the IL-6 axis was effective in immunodeficiency disease.

QHZ inhibited PI3k/Akt/mTOR signaling pathway in CTX-treated mice

PI3K/Akt signaling pathway is one of crucial downstream events of IL-6 signaling [26]. To further investigate the potential mechanism that QHZG improved splenocytes function and contributed to reconstitution of immune system function in immunocompromised mice caused by CTX, we firstly evaluated the effects of QHZG on PI3K/Akt signaling pathway in CTX-treated mice. As shown in Fig. 6A-6B, the phosphorylation of AKT and mTOR in spleen tissue of CTX treated mice was remarkably decreased without affecting total protein, compared with that in normal group. While, QHZG obviously reversed the alteration of phosphorylated proteins triggered by cyclophosphamide in spleen tissue of mice. Together, QHZG might attenuate immunosuppression induced by cyclophosphamide via the regulation of PI3k/Akt/mTOR signaling pathway.

QHZG prevented mice from CTX-induced immunosuppression injury

through JAK2-SRC/MAPK/STAT3 signaling pathway

STAT3 is also one of the most important components of the IL-6 signaling pathway. We next investigated whether STAT3 was involved in the splenic protective activities of QHZG. As expected, we observed decreased protein and mRNA levels of STAT3, as well as the p-STAT3 in CTX treated group. Whereas, QHZG treatment significantly alleviated the effect of CTX on STAT3 (Fig. 7A and 7D). These findings suggested that QHZG might exhibit splenic protective activities by regulating the transcription and translation of STAT3.

The activation and expression of cytokines depend on the signal transduction including MAPK [27, 28]. In addition, MAPK signaling is also one of the major regulators of the transcription and translation of STAT3 [29]. Based on these findings, we speculated that QHZG might improve the immunologic function of CTX treated mice through MAPK signaling pathway. To clarify whether QHZG abated cyclophosphamide-triggered immunosuppression via MAPK signaling pathways, we analyzed the alteration of related protein levels of MAPKs pathway. As shown in Fig. 8A-8C, cyclophosphamide treatment decreased the mRNA expression of JNK, ERK and P38 and QHZG could partly reverse the mRNA changes induced by cyclophosphamide (Fig. 8A-8C). Meanwhile, the trend of the phosphorylation of JNK, ERK and P38 in spleen tissue after cyclophosphamide treatment was prominently consistent with the alteration of their total proteins (Fig. 8D).

Additionally, SRC has been shown to mediate the MAPK activation and regulate the STAT3 transcriptional activity through in direct or indirect way, which in turn mediated the production of inflammatory cytokines [30, 31]. Compared to the control group, substantially lower levels of the mRNA levels and the protein phosphorylation of SRC in CTX treated group, but not reduced that much in the QHZG co-treatment group. JAK2 is another important mediator of STAT3 and cytokine signal transduction pathway [32]. Therefore, we further investigated the effects of QHZG on the phosphorylation and protein levels of JAK2 in CTX treated mice. Similar to the effects of QHZG on STAT3, CTX treatment significantly decreased the mRNA expression and the protein phosphorylation of JAK2 in spleen tissue (Fig. 8A-8D). However, these effects caused by cyclocoagulamide could be significantly reversed by pretreatment with QHZG (Fig. 8D). In a conclusion, QHZG might ameliorate immunosuppression caused by CTX treatment via JAK2-SRC/MAPK/STAT3 signaling pathway.

Table.1. QPCR primer sequences

Gens	Sequences (5− 3)
IL-4 F	TACCAGGAGCCATATCCACGGATG
IL-4 R	TGTGGTGTTCTTCGTTGCTGTGAG
IL-2 F	GTAAAACTAAAGGGCTCTGAC
IL-2 R	TTGAGGGCTTGTTGAGAT
INF-γ F	CTTGAAAGACAATCAGGCCATC
INF-γ R	CTTGGCAATACTCATGAATGCA
IL-6 F	CTCCCAACAGACCTGTCTATAC
IL-6 R	CCATTGCACAACTCTTTTCTCA
ERK F	CAGCTCAACCACATTCTAGGTA
ERK R	TCAAGAGCTTTGGAGTCAGATT
P38 F	AGGAATTCAATGACGTGTACCT
P38 R	AGGTCCCTGTGAATTATGTCAG
JNK F	TTGAAAACAGGCCTAAATACGC
JNK R	GTTTGTTATGCTCTGAGTCAGC
SRC F	CTATGTGGAGCGGATGAACTAT
SRC R	ATTCGTTGTCTTCTATGAGCCG
STAT3 F	TGTCAGATCACATGGGCTAAAT
STAT3 R	GGTCGATGATATTGTCTAGCCA
GAPDH F	GGTTGTCTCCTGCGACTTCA
GAPDH R	TGGTCCAGGGTTTCTTACTCC

Discussion

CTX is a alkylating agent, which is widely used in the treatment of tumor and immune diseases, due to its good medicinal effect and low price. However, it is also highly cytotoxic and causes a variety of side effects, in particular, reducing the body's normal cellular and humoral immune responses and then leading to immune-deficiency [33, 34]. Spleen is the body's most important immune organ, where the immune cells developed and maturated. CTX treatment will lead to atrophy of the spleen and a decline in the spleen index. Therefore, it is of great significance to develop a safe and effective immunomodulator to reduce the immunosuppression induced by CTX therapy.

Qihuzha granule (QHZG) is rich in trace elements such as zinc, iron, selenium and numerious bioactive components and mainly composed of 11 kinds of traditional edible and medicinal plants. Because of its effectiveness and safety, it is considered to be one of the best-selling drugs for children in China. Modern pharmacological studies have elucidated that many components of QHZG prescription could significantly participate in immunostimulant activities [35]. Accumulating evidence displayed major active compounds from the main medicinal materials of QHZG, such as *liquiritigenin, isoliquiritigenin and flavonoids* isolated from *Glycyrrhiza uralensis*, and *astragalosides* from *Astragalus*, have great immunoregulatory activity [8]. Besides, compounds such as *Atractyloside II* and *Atractyloside III* in *Atractylodes macrocephala*, *pachymic acid* in *Poria cocos* and *Luteolin* in *Codonopsis pilosula* were also found in QHZG and all of these compounds have immune-enhancing effects [13]. Collectively, QHZG might be developed as a promising immunomodulator against CTX-induced immunosuppression. However, the potential molecular mechanisms of QHZG against CTX induced immunosuppressive injury were still unclear.

In the present study, a CTX-induced immunosuppressive mice model was established to investigated the protective effects of QHZG on CTX-induced immunosuppression [36]. The spleen index and immune function of CTX treated mice decreased, compared with that in the untreated mice (Fig. 1). Given the spleen was one of the most important immune organs, the spleen index is often used to evaluate immunity of the tested mice. As shown in Fig. 1, lower thymus index and spleen index were observed in the CTX treated group, whereas QHZG (100 mg/kg) significantly raised the thymus index and spleen index of CTX treated mice, suggesting their partial recovery of immune function.

In the immune response, activated splenic lymphocytes produce cytokines including IL-2/4/6 and other inflammatory mediators. IL-2 is an important immune factor secreted by helper T lymphocytes, which has a variety of immune enhancing effects. Among proinflammatory cytokines, IL-6 is one of the most important immune and inflammatory mediators, regulating a variety of cell functions, including the proliferation and differentiation of B cells and T cells [14, 15]. IFN-γ is one of the main immune regulatory molecules in inducing immune response to exogenous infectious agents. Compared to the CTX treated group, QHZG significantly increased the mRNA levels of IL-2/4/6 and IFN-γ. The data showed that QHZG could significantly improve the splenocyte function of the spleen in CTX treated mice.

Macrophages and neutrophils play an important role in resisting microbial infection and maintaining tissue homeostasis, and are activated or inhibited immediately during disease [37]. Consistent with pervious findings, the monocytes and neutrophils in spleen were lower in immunocompromised mice in the CTX treated group. However, QHZG could significantly increase the spleen macrophages and neutrophils, suggesting QHZG could reverse the immunosuppression induced by cyclophosphamide and improve the immune function by promoting the production of macrophages and neutrophils.

PI3K/Akt signaling pathway, one of crucial downstream events of IL-6 signaling, is a central pathway downstream of many cell surface receptors including T cell receptor (TCR), which was involved in autoimmunity [38]. Our results showed that the protein expression of P-Akt and P-mTOR in the model

group decreased without affecting the total protein, while QHZG treatment significantly increased the protein expression of P-Akt and P-mTOR. These results suggested that PI3K/Akt/mTOR pathway played an important role in QHZG's improvement of immunosuppressive diseases caused by cyclophosphamide.

Extracellular stimulation, such as pathogen infection or chemokines, can rapidly activate MAPKs signaling pathway, and then participate in the synthesis and release of inflammatory factors, and ultimately regulate the immune response [29]. Western blot and qPCR analysis showed cyclophosphamide decreased the phosphorylation of P38, ERK and JNK, and these changes caused by CTX in related protein levels of MAPKs were reversed by QHZG pretreatment. Src is widely believed to have multiple functions in macrophage mediated innate immunity, such as phagocytosis and release of inflammatory cytokines. In addition, Src is also a regulator of MAPK and STAT3 mediated signal transduction, which can activate and stimulate macrophages to produce inflammatory cytokines [30]. Moreover, JAK2 is important for cytokine receptor signal transduction. Once activated, JAK2 kinase phosphorylates STAT3 [30, 31, 32]. In addition, it has been reported that IL-6 signal interacted with many regulatory factors, such as JAK2/STAT3 [38]. QHZG significantly restored the mRNA and phosphorylated protein levels of SRC, JAK2 and STAT3 inhibited by cyclophosphamide, which suggested that QHZG might restore the immune system function in CTX induced immunocompromised mice by regulating JAK2-SRC/MAPK/STAT3 crosstalk signaling pathway (Fig. 9). These results were of great significance to understand the molecular mechanism of QHZG in CTX induced immunosuppression. In general, these findings might provide a basis for the use of QHZG as an effective immunoenhancement therapy or as an alternative strategy to reduce chemotherapy-induced immunosuppression.

Conclusions

In conclusion, QHZG could reverse CTX induced immunosuppression in mice for the first time. Although the detailed immune enhancement mechanisms of QHZG still needed further exploration, our findings suggested that QHZG might enhance the innate immune function of mice via IL-6 and its downstream signaling pathways including PI3K/Akt/mTOR signal pathways and JAK2-SRC/MAPK/STAT3 axes. These results suggested that QHZG might serve as a new drug for the treatment of the immunosuppression caused by cyclophosphamide therapy.

Abbreviations

ERK
Mitogen-activated protein kinase
P38
Mitogen-activated protein kinase
JNK
stress-activated protein kinase
MAPK
Mitogen-activated protein kinase

CTX
Cyclophosphamide
BCA
bicinchoninic acid
AKT
Protein kinase B
JAK2
Janus kinase 2
STAT3
signal transducer and activator of transcription 3
SRC
Sparse representation-based classifier
BSA
Bovine Serum Albumin
H&E staining
Hematoxylin-eosin staining
PI3K
Phosphatidylinositol 3 kinase
mTOR
mammalian target of rapamycin
IL-6
Interleukin- 6
QHZG
Qihuzha granule
IL-2
Interleukin- 2
IL-4
Interleukin- 4
IFN-γ
Interferon-gamma
TCR
T cell receptor.

Declarations

Acknowledgments

Not applicable

Author contributions

The authors declared that they had no conflict of interest. YH Fan contributed conception, designed of the study, reviewed & edited the manuscript. XS Yang reviewed the manuscript and provided advices for revision. T Zhong and M Feng performed the study and drafted the manuscript. MZ Su contributed to manuscript revision. DP wang, HB Wang, Q Li, F Luo, SQ Jia and EM Hu assisted in carrying out the relevant experiments.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from

the corresponding author on reasonable request.

Ethics approval and consent to participate

The experimental procedures were approved by animal protection and use Committee of Guizhou Medical University.

Consent for publication

Not applicable

Competing interest

The authors declared no conflicts of interest.

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References

1. Dhabhar FS. Efects of stress on immune function: the good, the bad, and the beautiful. Immunol Res. 2014; 58:193–210.

2. Gao HY , Li GY , Huang J, Han Y , Sun FZ, Du XW , An LJ, Wang HY and Wang JH. Protective effects of Zhuyeqing liquor on the immune function of normal and immunosuppressed mice in vivo. BMC Complement Altern Med. 2013; 13: 252.

1. Hayley Barnes, Anne E H, Glen PW, Nicole SG, Ian NG. Cyclophosphamide for connective tissue disease-associated interstitial lung disease. Cochrane Database Syst Rev. 2018; 1(1):CD010908.CD010908.
2. Płonka PM. Hair pigmentation disorders or 50 years of German Polish alliance for study on a severe side effect of chemo-therapy: Kostanecki's legacy. Exp Dermatol. 2015; 24: 10-11.
3. Hu XJ, Tan LL.Clinical efficacy of qihuzha granules. JIANGXIJOURNAL OF TRADITIONAL CHINESE MEDICINE. 2015.
4. De BFM, Bush A, 2018. Recurrent lower respiratory tract infections in children. BMJ. 2018; 362:k2698.
5. Shen XF, Cao K, Jiang JP, Guan WX, Du JF. Neutrophil dysregulation during sepsis: an overview and update. J Cell Mol Med. 2017;1687-1697.
6. Liang YH, Ye LH, Luo XH. Clinical observation of qihuzha granule in the treatment of malnutrition complicated with recurrent respiratory tract infection in children. Sichuan Medical Journal. 2012; 33, 6.
7. Adesso S, Russo R, Quaroni A, Autore G, Marzocco S. Astragalus membranaceus Extract Attenuates Inflammation and Oxidative Stress in Intestinal Epithelial Cells via NF-κB Activation and Nrf2 Response. Int J Mol Sci. 2018; 19(3):800.
8. Tian CC, Zha XQ, Luo JP. A polysaccharide from Dendrobium huoshanense prevents hepatic inflammatory response caused by carbon tetrachloride. Biotechnol Biotechnol Equip. 2015; 29(1):132-138.
9. Hiam E, Wang HP, Adel S, Aya M. Astragalus membranaceus nanoparticles markedly improve immune and anti-oxidative responses; and protection against Aeromonas veronii in Nile tilapia Oreochromis niloticus. Fish Shellfish Immunol. 2020; 97: 248- 256.
10. Liang YX , Zhang QY, Zhang LJ, Wang RF, Xu XY, Hu XH. Astragalus Membranaceus Treatment Protects Raw264.7 Cells from Influenza Virus by Regulating G1 Phase and the TLR3-Mediated Signaling Pathway. Evid Based Complement Alternat Med. 2019; 2971604.
11. Li XL, He YL, Zeng PJ, Liu Y, Zhang M, Hao C, Wang H, Lv ZH, Zhang LJ. Molecular basis for Poria cocos mushroom polysaccharide used as an antitumour drug in China. J Cell Mol Med. 2018; 1-17.
12. Rose-John S, Scheller J, Elson G, Jones SA. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol. 2006; 80:227–36.
13. Stanley CJ, Jua C, Irene K, Gordon W, Mieko T, Bonga S, Ashley V. Interleukin-6, A Cytokine Critical to Mediation of Inflammation, Autoimmunity and Allograft Rejection: Therapeutic Implications of IL-6 Receptor Blockade.Transplantation. 2017; 101(1):32-44.
14. Artym J, Zimecki M, Kruzel ML. Effects of lactoferrin on IL-6 production by peritoneal and alveolar cells in cyclophosphamide-treated mice. J Chemother. 2004; 16(2):187-92.

15. Jones SA. Directing transition from innate to acquired immunity: defining a role for IL-6. J Immunol. 2005; 175:3463–8.
16. Stéphanie K, Peter JP. Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. J Cell Biol. 2008; 182(5):855-63.
17. Lewis SM, Williams A, Eisenbarth SC. Structure and function of the immune system in the spleen. Sci Immunol. 2019; 4(33):eaau6085.
18. McKenzie CV, Colonne CK, Yeo JH, Fraser ST. Splenomegaly: Pathophysiological bases and therapeutic options. Int J Biochem Cell Biol. 2018; 94:40-43.
19. Yao J, Du XR, Chen SX, Shao Y, Deng KL, Jiang MZ, Liu JN, Shen ZY, Chen XL, Feng GZ. Rv2346c enhances mycobacterial survival within macrophages by inhibiting TNF-α and IL-6 production via the p38/miRNA/NF-κB pathway. Emerg Microbes Infect. 2018; 7(1):158.
20. Chu Q, Zhang YR, Chen W, Jia RY, Yu X, Wang YX, Li YL, Liu YY, Ye X, Yu LS, Zheng XD. Apios americana Medik flowers polysaccharide (AFP) alleviate Cyclophosphamide-induced immunosuppression in ICR mice. Int J Biol Macromol. 2020; 144:829-836.
21. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol. 2014; 6: a016295.
22. Kang S., Tanaka T., Narazaki M., Kishimoto T.. Targeting Interleukin-6 Signaling in Clinic. Immunity. 2019; 50 (4), 1007-1023.
23. Mulugeta M Z, Madelene L, Knut F, Ashok KK, Geena P, Magnus G, Allan S, Liza UL. Activation of the JAK/STAT3 and PI3K/AKT pathways are crucial for IL-6 trans-signaling-mediated pro-inflammatory response in human vascular endothelial cells. Cell Commun Signal. 2018; 16(1):55.
24. Bode JG, Ehlting C, Häussinger D. The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. Cell Signal. 2012; 24(6):1185-94.
25. Arthur J.S. and Ley S.C.. Mitogen-activated protein kinases in innate immunity. Nature Reviews. Immunology. 2013; 13 (9): 679–692.
26. Anandita B, Anindhya SD, Pallab KB, Raj KD, Rupak M. Biochanin A impedes STAT3 activation by upregulating p38δ MAPK phosphorylation in IL-6-stimulated macrophages Inflamm Res. 2020; 69(11):1143-1156.
27. Zhu JY, Luo L, Tian LX, Yin SQ, Ma XY, Cheng SW, Tang WQ, Yu J, Ma W, Zhou XY, Fan X, Yang X, Yan J, Xu X, Lv CZ, Liang HP. Aryl Hydrocarbon Receptor Promotes IL-10 Expression in Inflammatory Macrophages Through Src-STAT3 Signaling Pathway. Front Immunol. 2018; 9:2033.
28. Kim MJ, Nam HJ, Kim HP, et al.. OPB-31121, a novel small molecular inhibitor, disrupts the JAK2/STAT3 pathway and exhibits an antitumor activity in gastric cancer cells. Cancer Lett. 2013; 335(1):145e152.
29. Wu KJ, Huang JM, Zhong HJ, et al. A natural product-like JAK2/STAT3 inhibitor induces apoptosis of malignant melanoma cells. PLoS One. 2017; 12(6). e0177123.

30. Han M, Cheng H, Wang J, Yu Y, Wang F, Zhu R, Wang W, Yang S, Li H. Abnormal aggregation of myeloid-derived suppressor cells in a mouse model of cyclophosphamide-induced premature ovarian failure. Gynecol Endocrinol. 2019; 35(11):985-990.
31. Choi DW, Jung SY, Kang J, Nam YD, Lim SI, Kim KT, Shin HS. Immune-Enhancing Effect of Nanometric Lactobacillus plantarum nF1 (nLp-nF1) in a Mouse Model of Cyclophosphamide-Induced Immunosuppression. J Microbiol Biotechnol. 2018; 28(2):218-226.
32. Bai M, Zhu SD. Clinical observation of qihuzha granule in the treatment of infantile diarrhea of spleen deficiency type. Tianjin Pharmacy. 2007; 4,19.
33. Ding Y, Yan Y, Chen D, Ran L, Mi J, Lu L, Jing B, Li X, Zeng X, Cao Y. Modulating effects of polysaccharides from the fruits of Lycium barbarum on the immune response and gut microbiota in cyclophosphamide-treated mice. Food Funct. 2019; 10(6):3671-3683.
34. Iba T , Levy JH. Inflammation and thrombosis: roles of neutrophils, platelets and endothelial cells and their interactions in thrombus formation during sepsis.J Thromb Haemost. 2018; 16(2):231-241.
35. Völkl S, et al. Hyperactive mTOR pathway promotes lymphoproliferation and abnormal differentiation in autoimmune lymphoproliferative syndrome. Blood. 2016; 128(2):227–238.
36. Piao XH, Zou YP, Sui XD, Liu BH, Meng FJ, Li SD, Zhang QY, Ma C, Wu T. Hydrostatin-SN10 Ameliorates Pancreatitis-Induced Lung Injury by Affecting IL-6-Induced JAK2/STAT3-Associated Inflammation and Oxidative Stress. Oxid Med Cell Longev. 2019; 9659757.

Figures

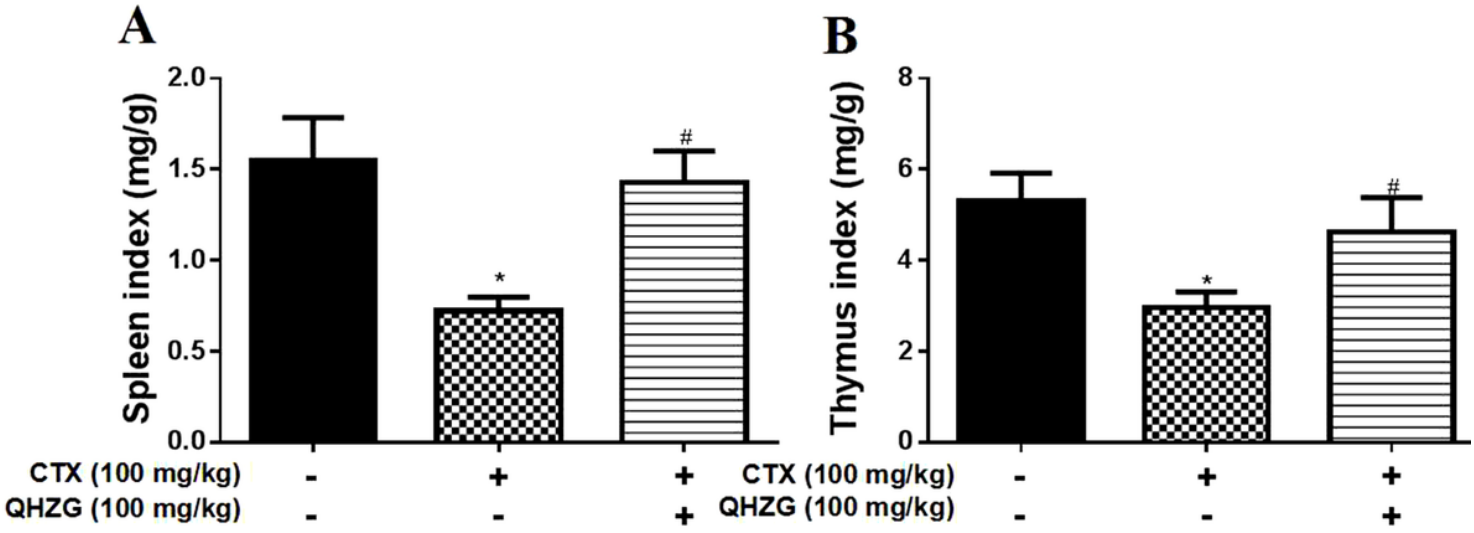

Figure 1

Immune improvement of QHZG in CTX-induced immunosuppression mice. (A) Effect of QHZG on spleen index. (B) Effect of QHZG on thymus index. Data were presented as the mean ± SD of five independent experiments. *p < 0.05, **p < 0.01 compared with indicated groups.

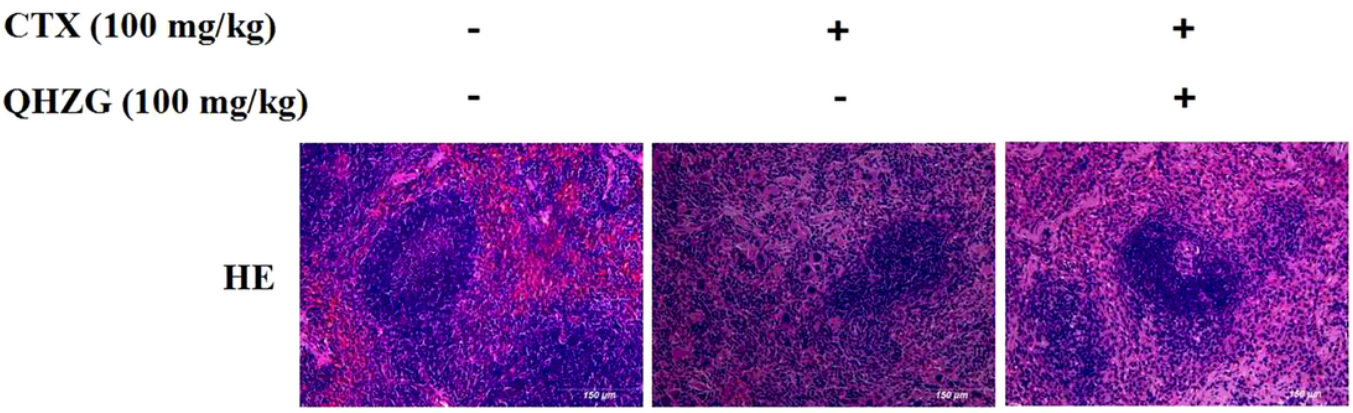

Figure 2

Effect of QHZG on morphological alterations of spleen tissues in mice. The mouse spleens was harvested and fixed in 4 % buffered formalin. Hematoxylin and eosin staining of spleen sections from the mice was shown. Scale bar, 200 μm.

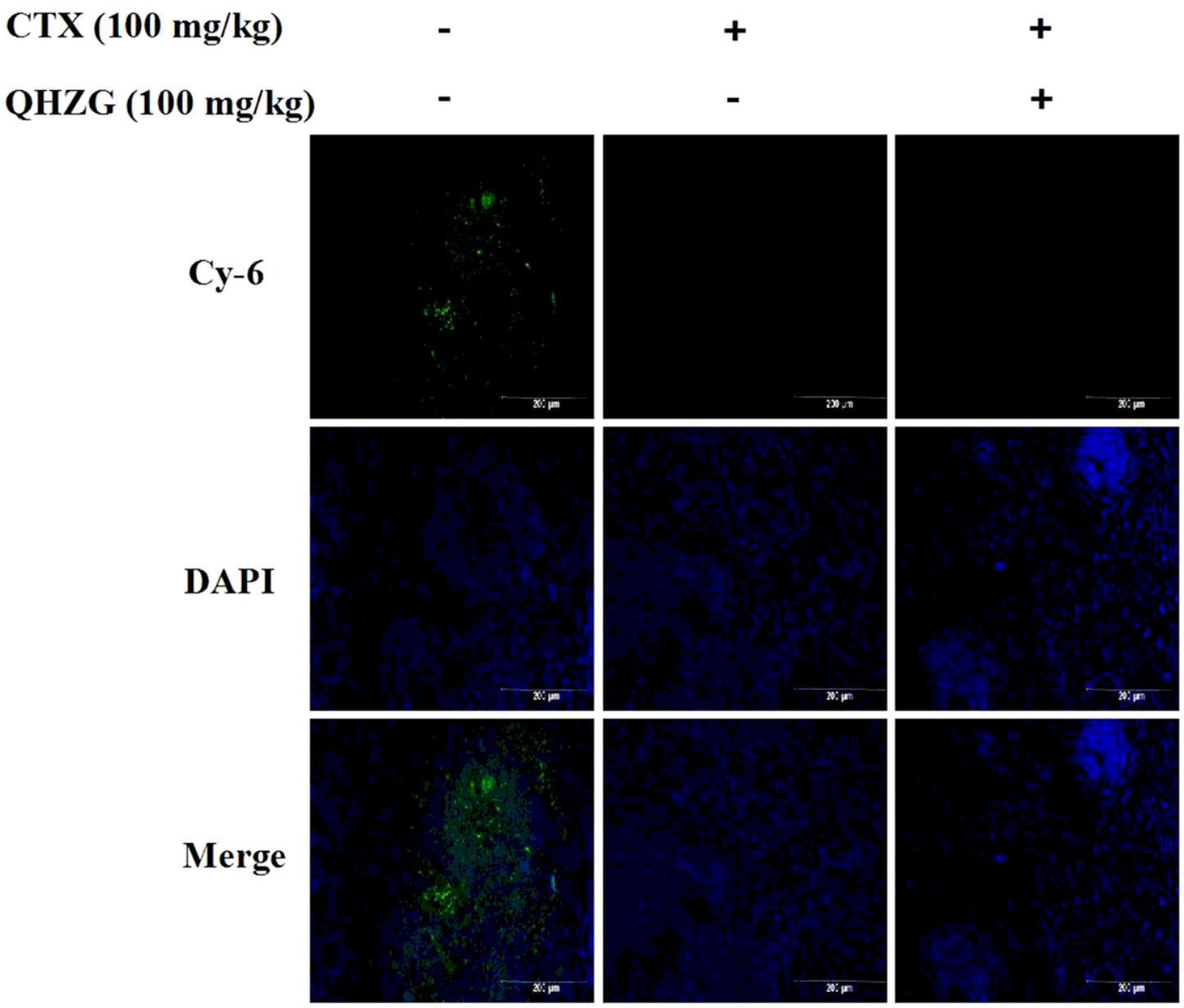

Figure 3

Effects of QHZ on neutrophils in CTX-treated mice. Mouse spleens were harvested and fixed in 4% buffered formalin. Sections were stained with anti-Ly-6G and DAP in spleen tissues. Nuclei were stained with DAPI (blue). Scale bar, 200 μm.

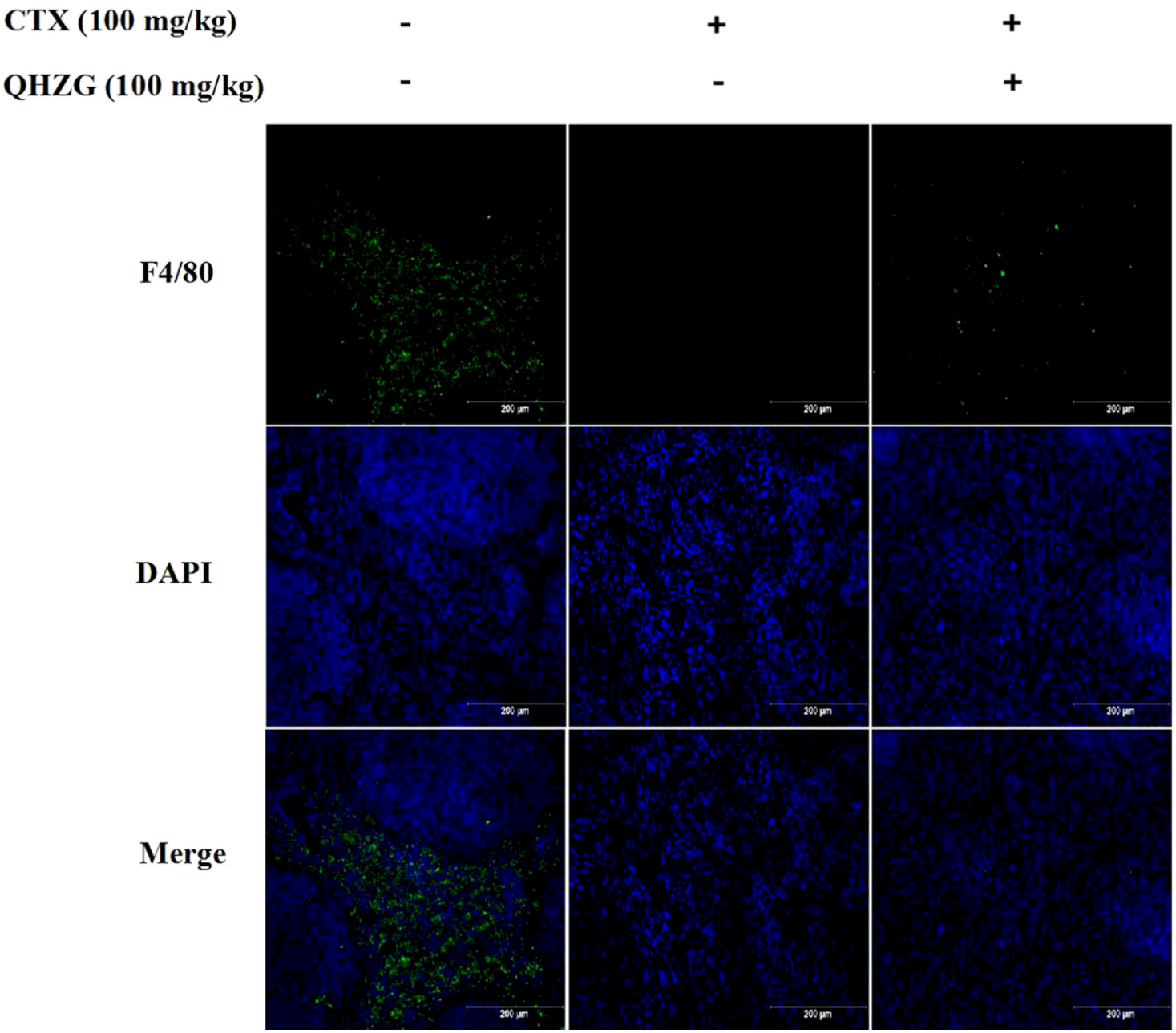

Figure 4

Effects of QHZ on macrophage. Mouse spleens were harvested and fixed in 4 % buffered formalin. Sections were immunostained with the indicated antibodies and analyzed by fluorescence microscopy. Nuclei were stained with DAPI (blue) Scale bar, 200μm.

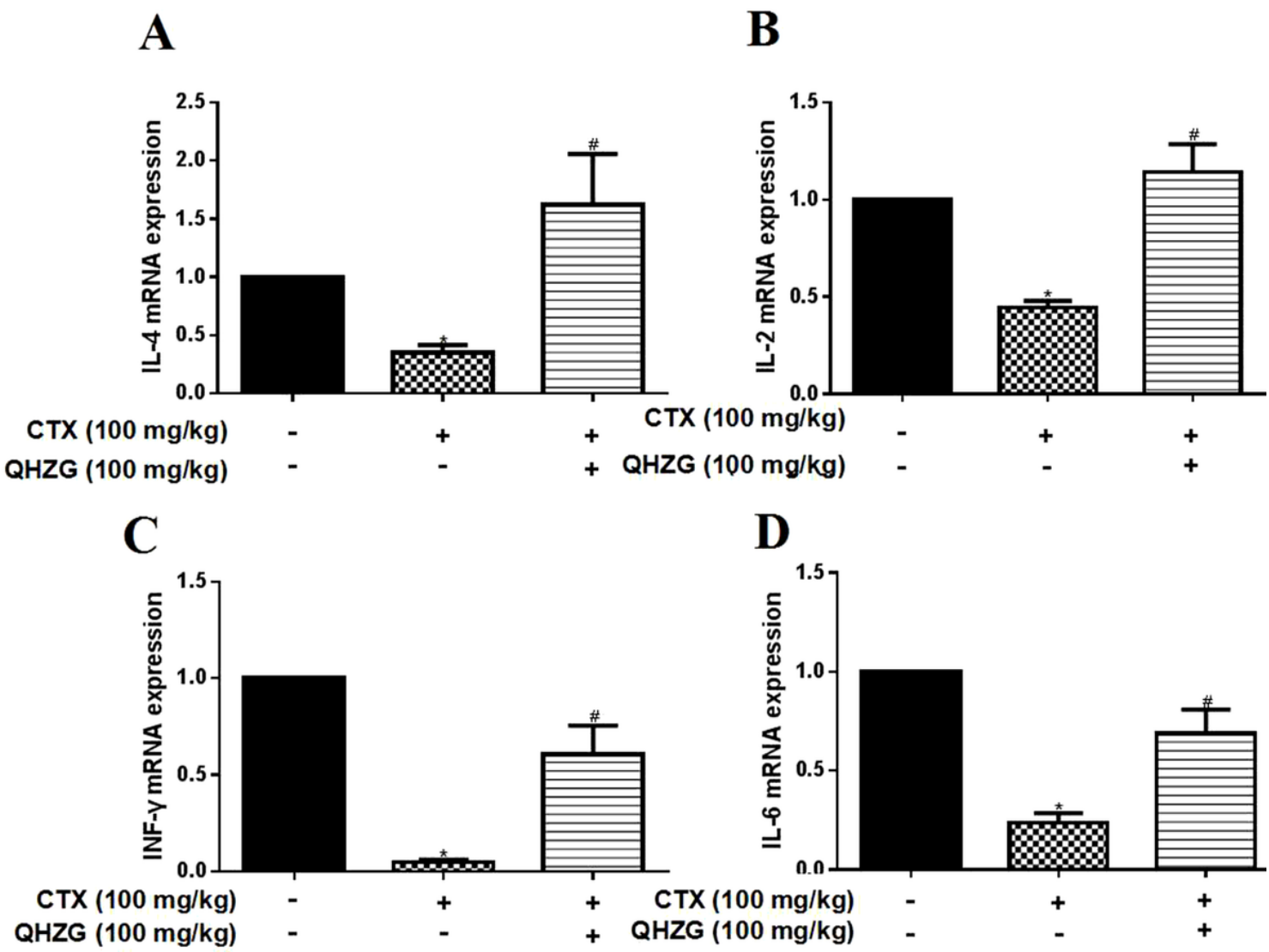

Figure 5

Effects of QHZG on the mRNA of cytokine. QRT-PCR analysis of relative (A) IL-4 (B) IL-2; (C) INF-γ (D) IL-6 with or without QHZG treatment in mice. Data were presented as the mean ± SD of five independent experiments. *$p < 0.05$, **$p < 0.01$ compared with indicated groups.

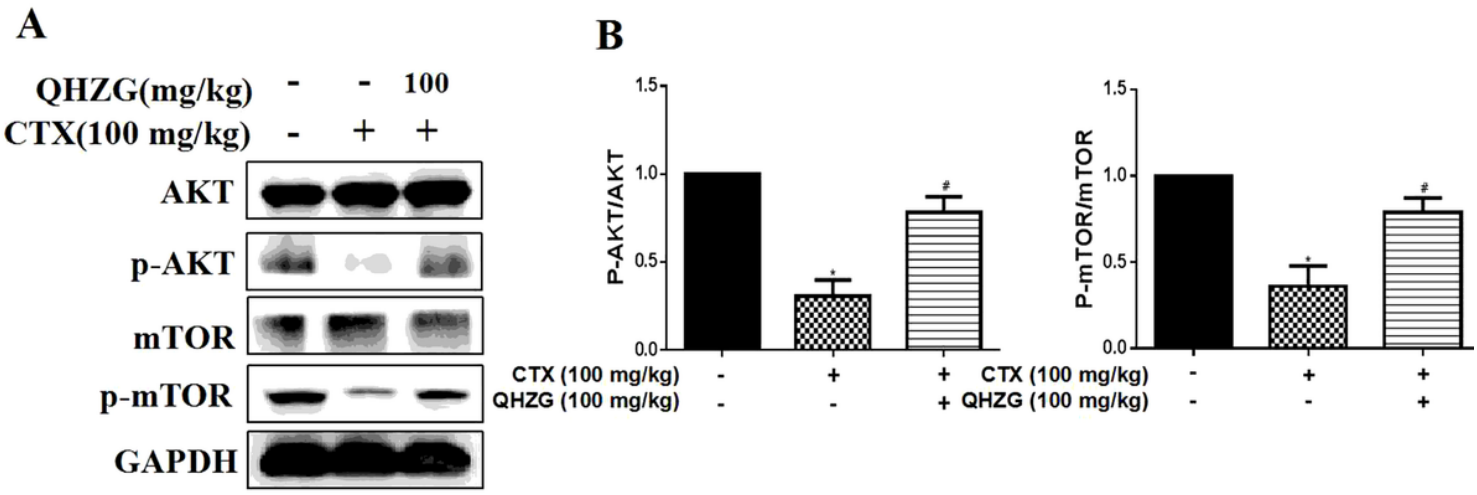

Figure 6

QHZG improved mice from CTX–triggered immunosuppression injury via PI3k/Akt/mTOR signaling pathway. (A) Immunoblot analysis of P-Akt and P-mTOR with or without QHZG treatment in mice. GAPDH was shown as a loading control. *P<0.05, **P<0.01, ***P<0.001 Data in (B) were presented as the mean ± SD of five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, compared with indicated groups.

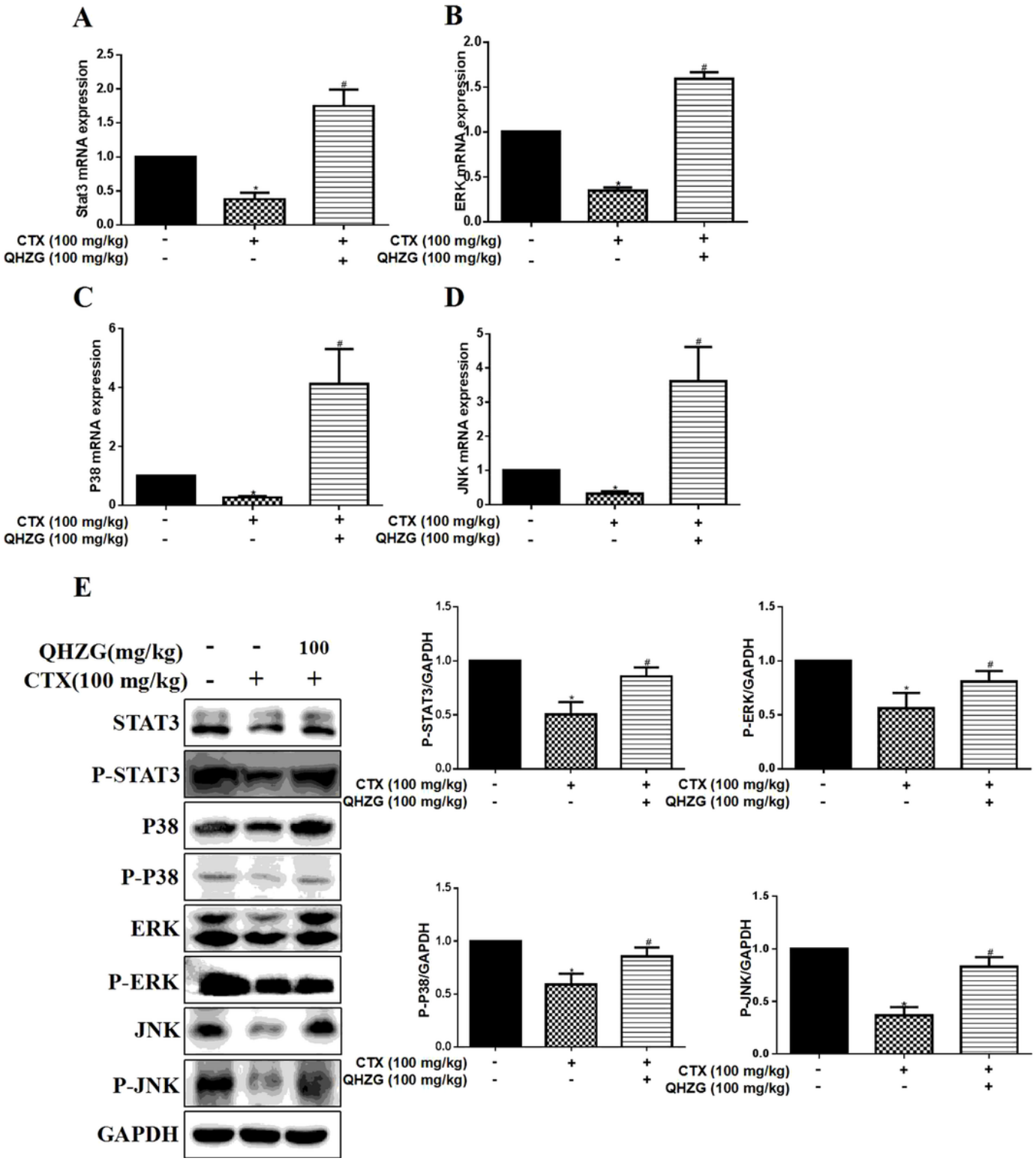

Figure 7

QHZ inhibited STAT3/MAPK signaling pathway in CTX-treated mice. BALB/c male mice were pretreated with GHZG, the spleens tissues of mice were harvested. (A-D) QPCR analysis of relative STAT3, ERK, P38 and JNK in mouse. (E) The proteins of P-STAT3, P-P38, P-ERK and P-JNK in mouse splenocytes were analyzed by western blot analysis. GAPDH was shown as a loading control. *P<0.05, **P<0.01,

***P<0.001 Data were presented as the mean±SD of five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, compared with indicated groups.

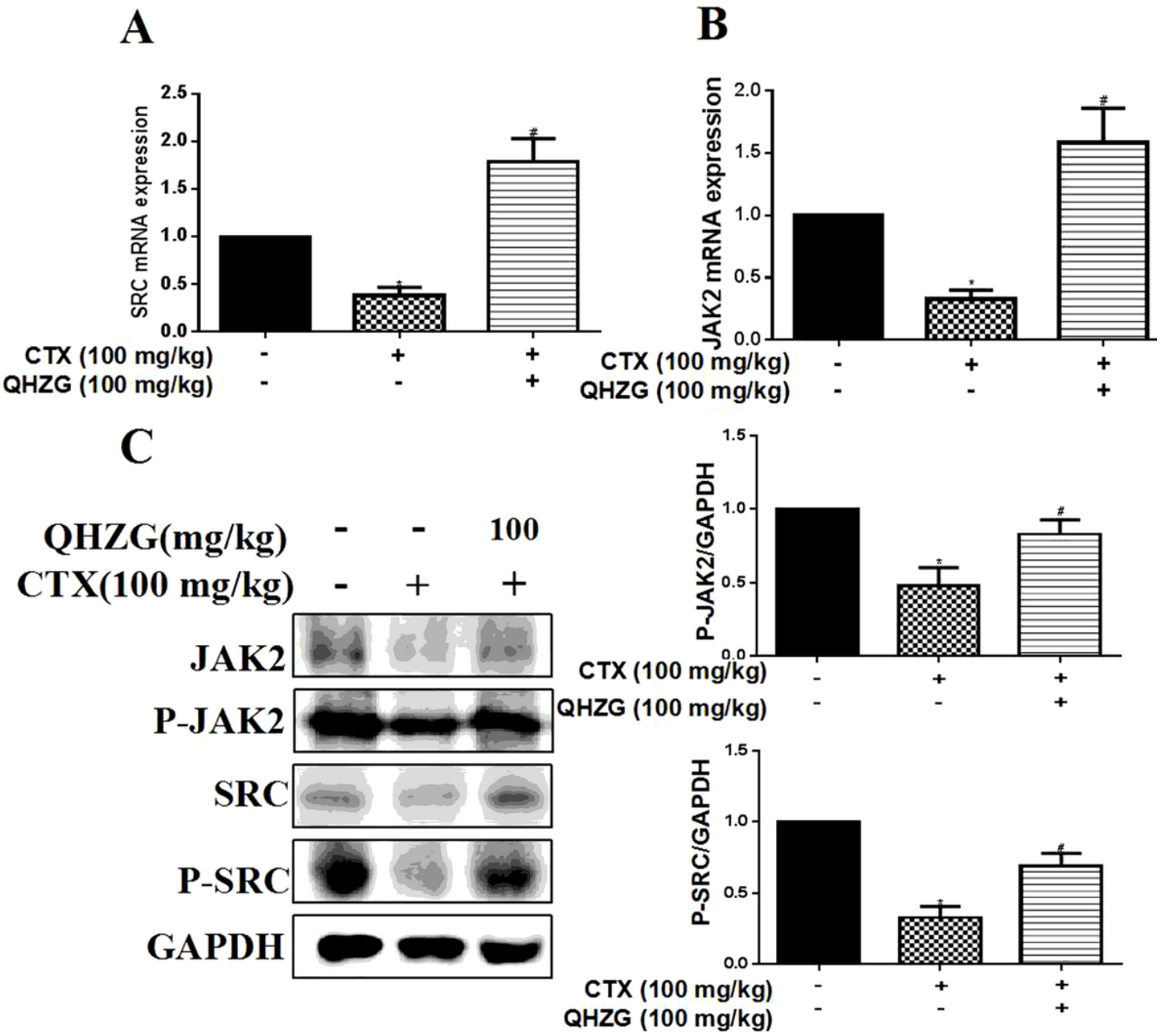

Figure 8

QHZG prevented mice from CTX-induced immunosuppression injury through JAK2/SRC signaling pathway. BALB/c male mice were pretreated with GHZG, the spleens tissues of mice were harvested. (A-C) qRT-PCR analysis of relative JAK2 and SRC in mouse. (D) The proteins of P-JAK2, P-SRC in mouse splenocytes were analyzed by western blot analysis. GAPDH was shown as a loading control. *P<0.05, **P<0.01, ***P<0.001 Data in were presented as the mean ± SD of five independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, compared with indicated groups.

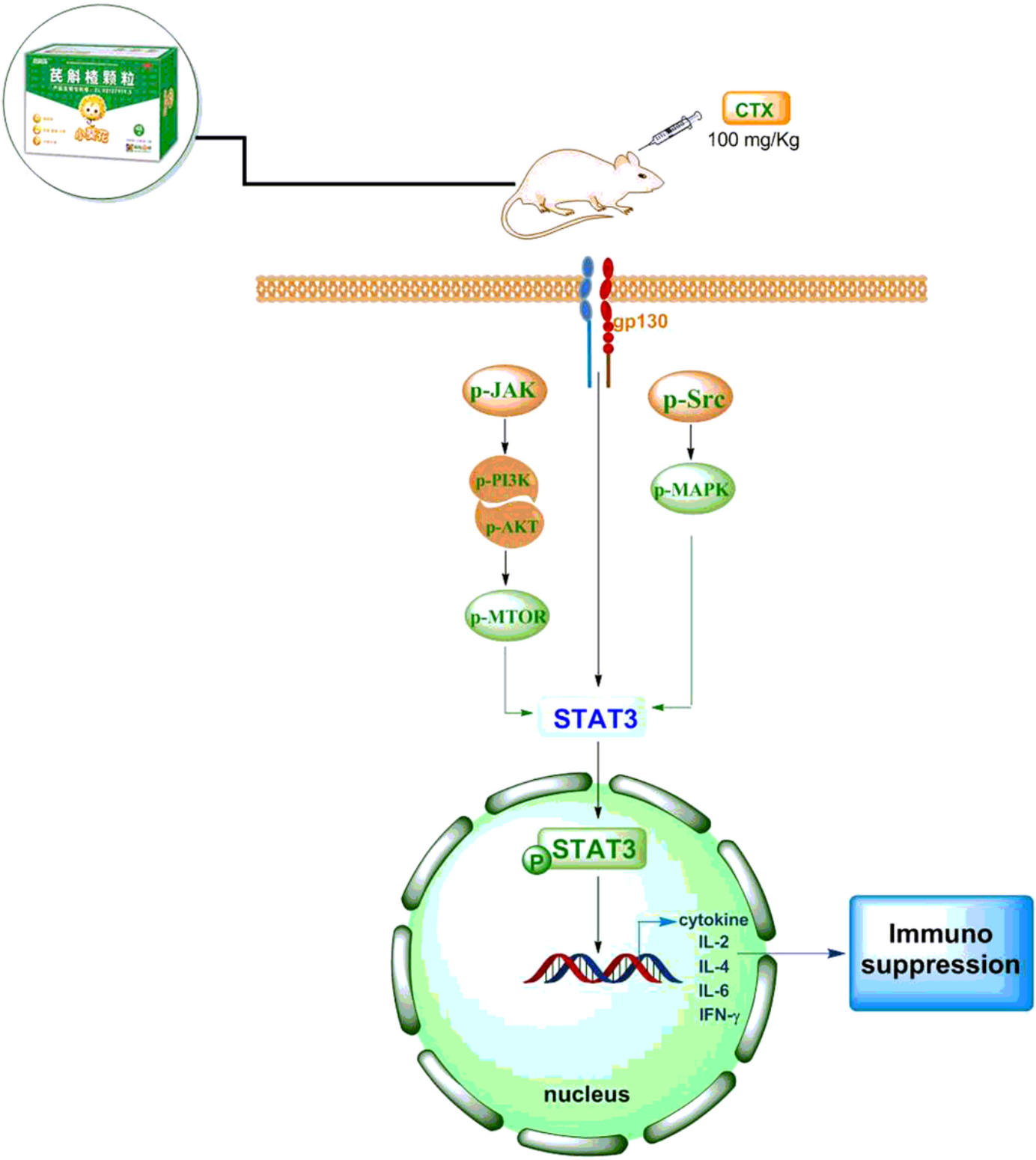

Figure 9

Proposed working model for QHZG in CTX-induced immunosuppression injury via JAK2/SRC/MAPK/STAT3 and PI3k/Akt/mTOR signaling pathway. QHZG significantly alleviated CTX-induced immunosuppression injury and showed protective effects against immunosuppression in mice by JAK2/SRC/MAPK/STAT3 and PI3k/Akt/mTOR signaling pathway.

Supplementary Files

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