Icariin Suppresses Activated Macrophages-Mediated Inflammatory Response Inhibition of mTOR/S6K1 and NF-κB Signaling

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Abstract

Background: This study aimed to explore icariin on macrophage polarization and the underlying molecular mechanism.

Methods: Macrophages were polarized to M1/M2 macrophages following stimulation with LPS, IFN- γ and IL-4. Meanwhile, they were intervened by icariin. ELISA was performed to detect TNF- α and IL-10 secretion to observe the effect of icariin on macrophages polarization at the protein level. RT-PCR was performed to detect mRNA expression of TNF- α , IL-6, iNOS, Arg1 and CD206 to observe the effect of icariin on macrophages polarization at the mRNA level. Next, flow cytometry were used to observe the effect of icariin on the proportions of polarized macrophages (M1, M2). Western blot was performed to examine the phosphorylation of mTOR, S6K1, NF- κ B p65, IKK β and I κ B α to observe the effect of icariin on mTOR/S6K1 and NF- κ B signaling pathway of M1 macrophages.

Results: Icariin can inhibit the polarization of M1 macrophages and promote M2 polarization of macrophages, and inhibit the expression of tumor necrosis factor α (TNF- α), interleukin 6 (IL-6) and inducible nitric oxide synthase (iNOS) to reduce the inflammatory response, and promote the expression of interleukin 10 (IL-10), mannose receptor C type 1 (CD206) and arginase 1 (Arg1) to anti-inflammatory and promote tissue repair. Icariin can inhibit the polarization of M1 macrophages and promote M2 polarization of macrophages. Additionally, western blot and immunofluorescence staining (IF) results found that it can inhibit expression of p-mTOR, p-S6K1, p-NF- κ B p65, p-IKK β and p-I κ B α on M1 macrophages.

Conclusions: Our results indicate that icariin inhibited M1 macrophage activation and enhanced M2 macrophage activation, and affect the mTOR/S6K1 and NF- κ B signaling pathway of M1 macrophages.

1. Introduction

The inflammatory response plays an important role in the occurrence and development of many diseases. The conventional view is that macrophages are important immune cells, is also an important component of the innate immune response, and involving in the initiation of an inflammatory response. In recent years, the study have been conducted to according to the immune microenvironment, macrophage phenotypes can be reversed, exhibit distinct functional heterogeneity and plasticity, be referred as macrophage polarization[1]. The distinct states of polarization exert different effects during inflammatory response[2], and secreting a variety of pro-inflammatory mediators, including TNF- α , IL-6 and IL-10, and producing iNOS, Arg1 and CD206 contributes to inflammatory response[3-6].

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase, it belongs to the phosphatidylinositol-3-kinase-related protein kinase (PIKK) family. mTOR is usually assembled into several multiprotein complexes, such as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), involving in various biological phenomena including cell proliferation, survival, autophagy, metabolism and immunity[7, 8]. Accumulating studies have shown mTOR

is involved in the regulation of multiple network signaling pathways, and working together with each other, integrating diverse life signals, involving in metabolism of mammalian cells[9]. It has earlier been suggested that mTOR signaling pathways play a pivotal regulatory role in macrophages polarization[10].

Nuclear factor- κ B (NF- κ B), a family of transcription factors, including NF- κ B1p50, NF- κ B2 p52 and RELA (known as p65)[11]. Nuclear factor- κ B (NF- κ B)-mediated inflammatory pathway is a classic signaling cascade. First, the I κ B kinase (IKK) complex mediates I κ B protein phosphorylation, and it is composed of three subunits (IKK α , IKK β , IKK γ), where IKK α and IKK β are catalytic subunits, and IKK γ is the regulatory subunit[12, 13]. Subsequently, phosphorylation of I κ B α prompts it for ubiquitination leading eventually to the degradation of I κ B α and the release and nuclear translocation of NF κ B, including NF- κ B 1p50-RELA, NF- κ B 1p50-c-REL, etc[11, 14]. NF- κ B has been shown to have been implicated in multiple inflammation-associated diseases, and can affect the polarization to M1 [15-17]. In recent years, studies have revealed that the NF- κ B signaling pathway can interact with mTOR to regulate autophagy and inflammatory response[18]. Simultaneously, Lin et al. point out that mTOR/NF- κ B pathway activation stimulates M1 macrophages in vitro experiments[19].

Icariin (ICA) is the main active compound extracted from a traditional Chinese herb Epimedium, which belongs to the Berberidaceae family. It belongs to 8-Prenylflavonol glycosides in structure, and is one of the most intensively ingredients in medicinal mechanism research at present[20]. A considerable amount of research has indicated that ICA has various pharmacological effects, including cardiovascular and cerebrovascular effects, anti-oxidation, anti-neoplastic, anti-inflammatory, regulating immune and endocrine[21-24]. In recent years, increasing amounts of research have concentrated on immunoregulatory function of ICA, it can modulate the polarization of macrophages to improve the immune microenvironment of tumors, and inhibit the development of tumors[25]. Further, it can alleviate inflammatory response, to play a protective role in tissue and organs[26]. However, the specific mechanism is still not very clear. In this study, we have explored the specific pharmacological mechanism of icariin's immunomodulatory function. Our results show that Icariin inhibit the polarization of M1 macrophages and promote M2 polarization of macrophages, and inhibiting mTOR/S6K1 and NF- κ B pathway in M1 macrophages.

2. Materials And Methods

2.1. Chemicals and reagents

Icariin (10mmol/L) was purchased from Selleck chemicals (USA). LPS was from Escherichia coli was purchased from PeproTech (USA), the powder was dissolved in DMSO (Gibco, USA) to the final concentration of 10 mg/mL. IFN- γ and IL-4 were purchased from PeproTech (USA), the powder was dissolved in DMSO (Gibco, USA) to the final concentration of 20 μ g/ml. Cell culture reagents, including Dulbeccos modified Eagles medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (USA). The CCK-8 kit was purchased from Sigma (USA). mTOR, phospho-mTOR, S6K1 and phospho-S6K1 antibody were purchased from Abcam (USA). NF- κ B Pathway Sampler kit (#9936) was purchased from

Cell Signaling Technology (USA). β -actin,HRP-labeled goat anti-rabbit IgG was purchased from Servicebio (Wuhan,China).TNF- α ELISA kit and IL-10 ELISA kit were purchased from Shenzhen Xinbosheng Biotechnology Co.,Ltd. (China).Anti-CD11b,anti-F4/80,anti-CD86,anti-CD206,anti-Inos for FACS analysis were purchased from Invitrogen (USA).

2.2. Cell culture and treatment

The BALB/c mice (20-22g) used in this experiment were provided by Hunan Slake Jingda Experimental Animal Co.,Ltd.Mice were euthanized by cervical dislocation,and immobilized in supine position.The skin of the abdomen was cut open to expose the abdominal muscle after disinfection.Then the peritoneal cavity was washed with RPMI-1640 medium and massaged for 1 min.The lavage fluid was collected and centrifuged at 1000 rpm for 5min.Cells were resuspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin,and seeded into 6-well plates at a density of 1×10^6 cells/ml, 2ml/well.Finally, they were cultured in a 37 °C, 5% CO₂ incubator.After culturing for 6 h,they were used for experimental studies.To determine the effect of icaritin,LPS,IFN- γ and IL-4 on cell viability,the cells were then grouped and treated with LPS,IFN- γ ,IL-4 and different concentrations of icariin.After 24 hours,CCK-8 assay was performed.

Raw264.7 cells were obtained from Institute of Emergency Medicine in Hunan Provincial People's Hospital.They were cultured in complete medium (DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin).After 1 week, the experimental session took place.

2.3. Measurement of TNF- α and IL-10 levels in supernatant

The super-natants were collected from cell cultures in each group.Then,the cell debris was removed by centrifugation at 1000 rpm for 5 min at 4°C ,and the supernatant was divided and stored at -20°C for subsequent analysis.Finally,the levels of IL-10 and TNF- α in cell supernatants were quantified with ELISA kits.All kits and assays were performed according to the kit manufacturers' instructions.

2.4. RNA extraction and real-time polymerase chain reaction (real-time RT-PCR)

After treatment,the cell supernatant in each group were discarded,the cells were collected.TRIzol,chloroform and isopropanol were applied to isolate total RNA from cells.And it was quantified by measuring the absorbance ratios at 260/280 nm.Then,using TB Green two-step qRT-PCR kit (Takara Bio,USA) to perform reverse transcription.PCR amplification using the following murine specific primers:TNF- α ,F:5'-GGTGCCTATGTCTCAGCCTCTT-3' (forward) and R:5'-GCCATAGAACTGATGAGAGGGAG-3' (reverse);IL-6,F:5'-TACCACTTCACAAGTCGGAGGC-3' (forward) and R:5'-CTGCAAGTGCATCATCGTTGTTC-3' (reverse);iNOS,F:5'-CCCTTCCGAAG TTTC TGGCAGCAGC-3' (forward)

and R:5'-GGCTGTCAGAG CCTCGTGGCTTTG-3' (reverse);CD206,F:5'-TTCAGCTATTGGACGCGAGG-3' (forward) and R:5'-GAATCTGACACCCAGCGGAA-3' (reverse);Arg1,F:5'-CTGA GAGATTCAAGGCAAGAGG-3' (forward) and R:5'-GAACGCGCTATCTTACC CCAG-3' (reverse); β -actin,F:5'-TTGCAGCTCC TTCGTTGCC-3' (forward) and R:5'-GACCCATTCCCACCATCACA-3' (forward).Real-time PCR was conducted using a TB green two-step method (Applied Biosystems,Bio-rad Inc.USA) in 96-well plates.

2.5. Flow cytometry

Cells in each group were stained for surface markers for 30 min at 4°C,protected from light.2mL of fixative/permeabilization solution was added into the cell suspension,stood for 45 mins at 4°C, protected from light.Finally,Cells in each group were stained with intracellular antibodies for 30 minutes at 4°C,protected from light.Flow cytometry was used for detection,and analysis was performed with FlowJo 10.0.7 software.Cells were labeled with monoclonal antibodies to cell surface markers and intracellular markers,labeling scheme was as follows:M1:CD11b-AF700(0.25 μ g),iNOS-PE(0.06 μ g) and CD86-Super Bright 600/BV605(0.125 μ g);M2:CD11b-AF700(0.25 μ g),F4/80-PE-Cy7(0.5 μ g) and CD206-APC(0.25 μ g).

2.6. Western blot analysis

The cells in various groups were treated with corresponding treatments,and washed with ice-cold PBS for 3 times and treated with 0.4ml (per well) of lysis buffer (RIPA).Cells cultured in 6-well plates were harvested using cell scrapers and were lysed in a lysis buffer at 4°C for 30 minutes.This was followed by centrifugation at 12,000 rpm for 15 min at 4 °C,to obtain the supernatants.Then,the protein content was determined by measuring absorbance at 280nm,and the protein quality was assessed measuring the 260/280nm absorbance ratios.The protein was divided and stored at -20°C for subsequent analysis.Samples were then boiled for 5 min at 100°C to denature the proteins and separated in 8% or 10% SDS-PAGE gel.The fractionated proteins were then transferred onto a nitrocellulose membrane.The membranes were washed with TBS containing 0.2% Tween 20 (TBS-T) and were blocked with 5% nonfat dry milk in TBS-T for 1 hours at room temperature,and incubated overnight at 4 °C with the following primary antibody in TBS-T: β -actin (1:3000),phospho-mTOR (1:1000),mTOR (1:1000),phospho-S6K1 (1:1000),S6K1 (1:1000),phospho-NF- κ B p65 (1:1000),NF- κ B p65 (1:1000),phospho-IKK β (1:1000),IKK β (1:1000),phospho-IkB α (1:1000),IkB α (1:1000).Incubation was carried out overnight at 4°C under shaking.The membrane were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h (1:4000).The protein bands were visualized with a BIO-RAD System.They intensities were quantified using the gel analytical software.Increase or decrease multiples in protein levels were determined by comparison with the control group.

2.7.Immunofluorescence microscopy Staining

The cells were collected at 24 h post-treatment and fixed with 4% paraformaldehyde for 1 h. The samples were incubated with blocking buffer containing 2% goat serum and 0.1% Triton X-100 for 1 h at room temperature, and incubated with anti-p-NF- κ B antibody at 4 °C overnight. Subsequently, the samples were washed in phosphate buffer. They were incubated with goat anti-mouse DyLight 594, goat anti-rabbit DyLight 488 for 2 h. Finally, fluorescence was visualized with a multiphoton laser scanning microscope (Zeiss LSM 510 META).

2.8. Statistical analysis

All data were obtained from at least three independent experiments and all results are expressed as the mean $\pm$ standard error of the mean. Statistical analysis was performed using Graphpad Prism 7. Then differences between two groups were compared using an unpaired t-test. Additionally, One-way ANOVA was used for compared among multiple groups. $P < 0.05$ meant that difference was statistically significant.

3. Results

3.1. Effects of LPS, IFN- γ , IL-4 and icariin on macrophage viability

After the cells adhered to the wall, RAW 264.7 macrophages were divided into two main groups in 96-well plates. They were treated with LPS (100 ng/ml), IFN- γ (20 ng/ml) or icariin (5–80 μ M) for 24 h; the other were treated with IL-4 (20 ng/ml) or icariin (5–80 μ M) for 24 h. CCK-8 assay for assessing cell proliferation. With increasing icariin dosage, the cell proliferation rate declined significantly and rapidly, particularly at the concentrations of 40 μ M and 80 μ M (Fig. 1A,B). This illustrates that it can cause toxicity to RAW 264.7 macrophages ($>20 \mu$ M), with increasing icariin dosage. Meanwhile, to better exert the protective effect of icaritin, we choose 10 μ M as the optimal icariin concentration for this study.

3.2. Effects of icariin on the polarization of macrophages

3.2.1. Effects of icariin on the secretion of TNF- α by M1 macrophages and the secretion of IL-10 by M2 macrophages

Macrophages were treated with IFN- γ + LPS to induce polarization to M1. Macrophages were treated with IL-4 to induce polarization to M2. They were treated with LPS and IFN- γ , TNF- α levels increasing 100-fold in the cell supernatant when compared to cells in blank group ($p < 0.01$), subsequently treated with icariin, the protein expression levels of TNF- α were significantly decreased in the cell supernatant when compared to

cells in IFN- γ + LPS group ($p < 0.05$) (Fig. 2A). In addition, another were treated with IL-4, IL-10 levels increasing 1.2-fold in the cell supernatant as compared to blank group ($p < 0.01$), subsequently treated with icariin, the protein expression levels of IL-10 were further increased in the cell supernatant as compared to IL-4 group ($p < 0.05$) (Fig. 2B).

3.2.2. Effects of icariin on the expression of TNF- α , iNOS and IL-6 mRNA in M1 macrophages

With LPS/IFN- γ stimulation, macrophages polarized to M1 macrophages and released large quantities of proinflammatory cytokines, including TNF- α , IL-6 and iNOS. Primary mouse peritoneal macrophages and RAW264.7 cells were treated with IFN- γ in combination with LPS for 24 h, The TNF- α , IL-6 and iNOS mRNA expression have significantly increased ($p < 0.01$), subsequently treated with icariin, which have inhibited IFN- γ and LPS-induced inflammatory mediator (TNF- α , IL-6 and iNOS) mRNA expression ($p < 0.05$ or $p < 0.01$) (Fig. 3A,B,C,D,E,F). However, the IL-6 mRNA expression has significantly reduced, the difference between LPS+IFN- γ and LPS+IFN- γ +icariin groups were not statistically significant ($p = 0.6386$) (Fig. 3C,F).

3.2.3. Effects of icariin on the expression of CD206 and Arg1 mRNA in M2 macrophages

Meanwhile, with IL-4 stimulation, macrophages polarized to M2 macrophages and induced the expressions of M2 macrophage markers (CD206 and Arg1) [3, 27, 28]. Primary mouse peritoneal macrophages and RAW264.7 cells were treated with IFN- γ in combination with LPS for 24 h, They were treated with IL-4 for 24 h, the CD206 and Arg1 mRNA expression have significantly increased ($p < 0.01$ or $p < 0.05$), subsequently treated with icariin, which have further promoted the CD206 and Arg1 mRNA expression ($p < 0.01$ or $p < 0.05$) (Fig. 4A,B,C,D). However, the Arg1 mRNA expression has increased, the difference between IL-4 and IL-4+icariin groups were not statistically significant in RAW264.7 macrophages ($p = 0.1096$) (Fig. 4D).

3.2.4. Effects of icariin on M1 and M2 macrophage subpopulations in mice

3.2.4.1. Icariin inhibits M1 macrophage activation

We further investigated the effect of icariin in macrophage polarization by flow cytometric analysis. This experiment examined distinctive proportional changes in each macrophage subpopulation. iNOS and CD86 are markers for M1. And RAW264.7 cells were treated with IFN- γ in combination with LPS for 24

h,the results were found that the macrophages (iNOS⁺CD86⁺) constituted (30±1.143)% in LPS+IFN-γ group.Compared with the blank control group,iNOS⁺CD86⁺macrophages were clearly increased (p<0.01).The macrophages (iNOS⁺CD86⁺) constituted (25.567±1.470)% in LPS+IFN-γ+icariin group.Compared with LPS+IFN-γ group,iNOS⁺CD86⁺macrophages were decreased (p<0.05) (Fig. 5B).Primary mouse peritoneal macrophages were treated with IFN-γ in combination with LPS for 24 h,the macrophages (iNOS⁺CD86⁺) constituted (42.933±4.714)% in LPS+IFN-γ group,compared with the blank control group,iNOS⁺CD86⁺macrophages were significantly increased (p<0.01).The macrophages (iNOS⁺CD86⁺) constituted (33.000±1.208)% in LPS+IFN-γ+icariin group.compared with LPS+IFN-γ group,iNOS⁺CD86⁺macrophages were decreased (p<0.05) (Fig. 5A).These results suggested that icariin inhibite the differentiation of primary mouse peritoneal macrophages and RAW264.7 cells into the M1 subtype.

3.2.4.2. Icariin induces M2 macrophage activation

On the other hand,CD206 and F4/80 are markers for M2.RAW264.7 cells were treated with IL-4 for 24 h.Percentage of M2 (CD206⁺F4/80⁺) macrophages were (8.344±1.617)% in IL-4 group.compared with the blank control group,CD206⁺F4/80⁺macrophages were increased (p<0.01).Percentage of CD206⁺F4/80⁺macrophages were (11.304±1.485)% in IL-4+icariin group.Compared with IL-4 group,CD206⁺F4/80⁺macrophages were further increased (p<0.05) (Fig. 6B).Primary mouse peritoneal macrophages were treated with IL-4 for 24 h,percentage of CD206⁺F4/80⁺macrophages were (20.833±0.910)% in IL-4 group.Compared with the blank control group,CD206⁺F4/80⁺macrophages were significantly increased (p<0.01).Percentage of CD206⁺F4/80⁺macrophages were (33.533±4.692)% in IL-4+icariin group.compared with IL-4 group,CD206⁺F4/80⁺macrophages were also further increased (p<0.05) (Fig. 6A).These results suggested that icariin induces the differentiation of RAW264.7 cells and primary mouse peritoneal macrophages into the M2 subtype.

3.3. Effects of icariin on mTOR/S6K1 signaling pathway of M1 macrophages

mTOR/S6K1 signaling pathway plays an important role in the polarization of M1 macrophages[29-31].We further investigated whether icariin could inhibit mTOR/S6K1 signaling pathway to the effect of M1 polarization,inhibiting the expression of inflammatory factors resulting in an anti-inflammatory effect.Macrophages were treated with IFN-γ + LPS to induce polarization to M1.Compared with the blank control group,the phosphorylation level of mTOR and S6K1 protein increased obviously (p<0.05 or p<0.01).Cells were treated with icariin for 24 h,this phosphorylation was inhibited (p<0.05) (Fig. 7A,B,C).

3.4. The effect of icariin on the NF- κ B signaling pathway of M1 macrophages

We also explored the relationship between icariin, macrophage polarization and the NF- κ B signaling pathway. NF- κ B is a family of inducible transcription factors that regulate innate and adaptive immune functions, including NF- κ B p65, IKK β / α , I κ B α , etc [11]. Icariin can inhibit the secretion of TNF- α , IL-6 and iNOS to exert its anti-inflammatory effect [26]. Western Blot results showed macrophages were treated with IFN- γ + LPS to induce polarization to M1, compared with the blank control group, the expression of phosphorylated-NF- κ B p65, phosphorylated-IKK β , phosphorylated-I κ B α have significantly increased in IFN- γ + LPS groups ($p < 0.01$ or $P < 0.05$), subsequently treated with icariin, which have inhibited p-NF- κ B p65, p-IKK β and p-I κ B α expression ($p < 0.05$) (Fig. 8A,B,C,D). Meanwhile, the immunofluorescence staining also showed similar results. We found the translocation of phosphorylated-p65 from the cytoplasm to the nucleus was evident in IFN- γ + LPS group as compared to the blank control group. Macrophages were treated with icariin, the translocation consequently reduced, and were primarily in the cytosolic fraction (Fig. 8E).

4. Discussion

The results of the present study suggest that icariin can have a dramatic effect on macrophage activation. After treatment with icariin, the ability of LPS in combination with IFN- γ to induce activation of M1 macrophages were significantly weakened. In contrast, the ability of IL-4 to induce activation of M2 macrophages were enhanced. Thus exerting anti-inflammatory effects, we found that this may be related to the inhibition of mTOR/S6K1 and NF- κ B signaling pathway.

Our previous research showed that icariin had anti-inflammatory effects, the specific mechanism is not yet clear. A large number of studies have demonstrated that as an important component of innate immunity, the different functional of macrophages play important roles in the development of diseases. At the initial stage of the diseases, various pathogenic factors induces polarization of macrophages towards the M1 proinflammatory phenotype, which secreting proinflammatory cytokines and chemokines to exert both pro-inflammatory actions. Subsequently inducing polarization of macrophages towards the M2 anti-inflammatory phenotype, secreting anti-inflammatory factors, suppressing the inflammatory response, and participating in the tissue repair process [32, 33]. Previous research finds icariin has immunomodulatory effects [23, 34, 35]. And it can modify the tumor immune microenvironment by regulating macrophages polarization to exert an antitumor effect [25]. Hence, we speculate that it can affect the progression of inflammatory disease states by influencing the function of macrophages.

In vitro, M1 macrophages can be induced by IFN- γ and LPS [3, 28], and produce enormous quantities inflammatory factors of TNF- α , IL-6, and expression of characteristic biomarker iNOS. On the other hand, IL-4 can promote the differentiation of macrophages into M2 macrophages. When combined with IL-13, it can promote the differentiation of macrophages into M2a, inhibiting the production of pro-inflammatory mediator to the control of the inflammatory response, promoting angiogenesis and tissue repair [36, 37]. It

is characterized by high expression of CD206,Arg1[37].These are consistent with our observations. Macrophages were treated with LPS+IFN- γ ,pro-inflammatory cytokines (IL-6, TNF- α , and iNOS) were significantly increased. After icariin intervention, their expression was inhibited. Simultaneously, macrophages were treated with IL-4, expression of the M2-related markers Arg1 and CD206 were clearly increased. After icariin intervention, their expression was further increased. Our study illustrates that they suppress inflammation by inhibiting M1 macrophage polarization and promoting M2 macrophage polarization. The same phenomenon was found in the results for flow cytometric analysis.

mTOR can participate in a variety of biological processes, play central roles in several cellular functions (growth, metabolism, homeostasis and autophagy induction)[38].The activation of mTORC1 is associated with multiple complicated signaling pathways. Its downstream signal including S6K1,RPS6,etc[39].Furthermore, several studies demonstrated mTOR can modulate intestinal inflammatory responses through its upstream TLR4-MyD88-MAPK signaling and downstream NF- κ B pathway[18].NF- κ B, a central mediator of the inflammatory process, also participates in innate and adaptive immune responses, stress responses, B-cell development, and lymphoid organogenesis[40].In the classical NF- κ B pathway, the proinflammatory cytokines ,LPS, growth-factor receptors and antigen receptors activate IKK complex (IKK β +IKK α and NEMO),which phosphorylates I κ B to prompt it for ubiquitination and degrades proteasomes leading eventually to NF- κ B/Rel complex release. The complex is further modified (phosphorylation, glycosylation and acetylation),and transported to the nucleus. It alone or in combination with other transcriptional regulators co-induce target gene expression[11].It is generally accepted that the NF- κ B signalling pathway, which is a classic pathway that promotes the M1 polarization of macrophages, is closely related to the inflammatory response and phagocytosis[41].As evident from the results of Western Blot, macrophages were treated with LPS+IFN- γ , the expression of p-mTOR,p-S6K1,p-p65,p-IKK β ,p-I κ B α have significantly increased. Subsequently treated with icariin, which have inhibited their expression. The same results were achieved by immunofluorescence staining. Our study illustrates that icariin inhibited M1 macrophage polarization through the mTOR/S6K1 and NF- κ B signaling pathway inhibition.

5. Conclusion

All in all, icariin can control the inflammation response by inhibiting M1 macrophages and promoting M2 macrophages. And these are related to the inhibition of mTOR/S6K1 and NF- κ B signaling pathway.

Declarations

Acknowledgements

Not applicable.

Ethical Approval and Consent to participate

This study was approved by the Ethics Committee of animal Experiment Center of Xiangya Medical College, Central South University.

Consent for publication

Not applicable.

Availability of supporting data

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Founding Sources

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Authors' Contribution

All persons who meet the authorship criteria are listed as authors, and all authors certify that they participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing or revision of the manuscript.

Conflict of interest

Not applicable.

References

1. Cassetta L, Cassol E, Poli G: **Macrophage polarization in health and disease.** *ScientificWorldJournal*2011, **11**:2391-2402.
2. Labonte AC, Tosello-Tramont AC, Hahn YS: **The role of macrophage polarization in infectious and inflammatory diseases.** *Mol Cells*2014, **37**(4):275-285.

3. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili S-A, Mardani F, Seifi B, Mohammadi A, Afshari JT, Sahebkar A: **Macrophage plasticity, polarization, and function in health and disease.** *Journal of Cellular Physiology*2018, **233**(9):6425-6440.
4. Porta C, Riboldi E, Ippolito A, Sica A: **Molecular and epigenetic basis of macrophage polarized activation.** *Semin Immunol*2015, **27**(4):237-248.
5. Murray PeterJ, Allen JudithE, Biswas SubhraK, Fisher EdwardA, Gilroy DerekW, Goerdts S, Gordon S, Hamilton JohnA, Ivashkiv LionelB, Lawrence T *et al*: **Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines.** *Immunity*2014, **41**(1):14-20.
6. Mosser DM, Edwards JP: **Exploring the full spectrum of macrophage activation.** *Nature Reviews Immunology*2008, **8**(12):958-969.
7. Saxton RA, Sabatini DM: **mTOR Signaling in Growth, Metabolism, and Disease.** *Cell*2017, **168**(6):960-976.
8. Harwood F, Klein Geltink R, O'Hara B, Cardone M, Janke L, Finkelstein D, Entin I, Paul L, Houghton P, Grosveld GJSa: **ETV7 is an essential component of a rapamycin-insensitive mTOR complex in cancer.** 2018, **4**(9):eaar3938.
9. Hua H, Kong Q, Zhang H, Wang J, Luo T, Jiang Y: **Targeting mTOR for cancer therapy.** *J Hematol Oncol*2019, **12**(1):71.
10. Byles V, Covarrubias AJ, Ben-Sahra I, Lamming DW, Sabatini DM, Manning BD, Horng T: **The TSC-mTOR pathway regulates macrophage polarization.** *Nat Commun*2013, **4**:2834.
11. Hayden MS, Ghosh S: **Shared principles in NF-kappaB signaling.** *Cell*2008, **132**(3):344-362.
12. Häcker H, Karin MJSsSstke: **Regulation and function of IKK and IKK-related kinases.** 2006, **2006**(357):re13.
13. Devin A, Lin Y, Yamaoka S, Li Z, Karin M, Liu Z: **The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF.** *Mol Cell Biol*2001, **21**(12):3986-3994.
14. Sun SC, Ley SC: **New insights into NF-kappaB regulation and function.** *Trends Immunol*2008, **29**(10):469-478.
15. Yuan Q, Gao F, Yao Y, Cai P, Zhang X, Yuan J, Hou K, Gao L, Ren X, Gao X: **Gold Clusters Prevent Inflammation-Induced Bone Erosion through Inhibiting the Activation of NF-kappaB Pathway.** *Theranostics*2019, **9**(7):1825-1836.
16. Shen B, Zhao C, Chen C, Li Z, Li Y, Tian Y, Feng H: **Picroside II Protects Rat Lung and A549 Cell Against LPS-Induced Inflammation by the NF-κB Pathway.** *Inflammation*2017, **40**(3):752-761.
17. Rakariyatham K, Wu X, Tang Z, Han Y, Wang Q, Xiao H: **Synergism between luteolin and sulforaphane in anti-inflammation.** *Food & Function*2018, **9**(10):5115-5123.
18. Zhou M, Xu W, Wang J, Yan J, Shi Y, Zhang C, Ge W, Wu J, Du P, Chen Y: **Boosting mTOR-dependent autophagy via upstream TLR4-MyD88-MAPK signalling and downstream NF-kappaB pathway quenches intestinal inflammation and oxidative stress injury.** *EBioMedicine*2018, **35**:345-360.

19. Lin LR, Gao ZX, Lin Y, Zhu XZ, Liu W, Liu D, Gao K, Tong ML, Zhang HL, Liu LL *et al*: **Akt, mTOR and NF-kappaB pathway activation in Treponema pallidum stimulates M1 macrophages.** *Int Immunopharmacol*2018, **59**:181-186.
20. Hernández J, Barreto-Torres G, Kuznetsov A, Khuchua Z, Javadov SJJoc, medicine m: **Crosstalk between AMPK activation and angiotensin II-induced hypertrophy in cardiomyocytes: the role of mitochondria.** 2014, **18**(4):709-720.
21. Wu B, Feng J-y, Yu L-m, Wang Y-c, Chen Y-q, Wei Y, Han J-s, Feng X, Zhang Y, Di S-y *et al*: **Icariin protects cardiomyocytes against ischaemia/reperfusion injury by attenuating sirtuin 1-dependent mitochondrial oxidative damage.** *British Journal of Pharmacology*2018, **175**(21):4137-4153.
22. Fang J, Zhang Y: **Icariin, an Anti-atherosclerotic Drug from Chinese Medicinal Herb Horny Goat Weed.** *Frontiers in Pharmacology*2017, **8**.
23. Hao H, Zhang Q, Zhu H, Wen Y, Qiu D, Xiong J, Fu X, Wu Y, Meng K, Li J: **Icaritin promotes tumor T-cell infiltration and induces antitumor immunity in mice.** *European Journal of Immunology*2019, **49**(12):2235-2244.
24. Chen HA, Chen C-M, Guan S-S, Chiang C-K, Wu C-T, Liu S-H: **The antifibrotic and anti-inflammatory effects of icariin on the kidney in a unilateral ureteral obstruction mouse model.** *Phytomedicine*2019, **59**.
25. Zheng X, Li D, Li J, Wang B, Zhang L, Yuan X, Li C, Cui L, Zhang Q, Yang L *et al*: **Optimization of the process for purifying icariin from Herba Epimedii by macroporous resin and the regulatory role of icariin in the tumor immune microenvironment.** *Biomedicine & Pharmacotherapy*2019, **118**.
26. Xu C-Q, Liu B-J, Wu J-F, Xu Y-C, Duan X-H, Cao Y-X, Dong J-C: **Icariin attenuates LPS-induced acute inflammatory responses: Involvement of PI3K/Akt and NF-κB signaling pathway.** *European Journal of Pharmacology*2010, **642**(1-3):146-153.
27. Bashir S, Sharma Y, Elahi A, Khan F: **Macrophage polarization: the link between inflammation and related diseases.** *Inflamm Res*2016, **65**(1):1-11.
28. Sica A, Erreni M, Allavena P, Porta C: **Macrophage polarization in pathology.** *Cellular and Molecular Life Sciences*2015, **72**(21):4111-4126.
29. Chen S, Chen L, Ye L, Jiang Y, Li Q, Zhang H, Zhang R, Li H, Yu D, Zhang R *et al*: **PP2A-mTOR-p70S6K/4E-BP1 axis regulates M1 polarization of pulmonary macrophages and promotes ambient particulate matter induced mouse lung injury.** *Journal of hazardous materials*2022, **424**:127624.
30. Gajanayaka N, Dong S, Ali H, Iqbal S, Mookerjee A, Lawton D, Caballero R, Cassol E, Cameron D, Angel J *et al*: **TLR-4 Agonist Induces IFN-γ Production Selectively in Proinflammatory Human M1 Macrophages through the PI3K-mTOR- and JNK-MAPK-Activated p70S6K Pathway.** *Journal of immunology (Baltimore, Md : 1950)*2021, **207**(9):2310-2324.
31. Cosin-Roger J, Simmen S, Melhem H, Atrott K, Frey-Wagner I, Hausmann M, de Valliere C, Spalinger MR, Spielmann P, Wenger RH *et al*: **Hypoxia ameliorates intestinal inflammation through NLRP3/mTOR downregulation and autophagy activation.** *Nat Commun*2017, **8**(1):98.

32. Lech M, Grobmayr R, Ryu M, Lorenz G, Hartter I, Mulay SR, Susanti HE, Kobayashi KS, Flavell RA, Anders HJ: **Macrophage phenotype controls long-term AKI outcomes—kidney regeneration versus atrophy.** *J Am Soc Nephrol*2014, **25**(2):292-304.
33. Anders HJ, Ryu M: **Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis.** *Kidney Int*2011, **80**(9):915-925.
34. Li C, Li Q, Mei Q, Lu T: **Pharmacological effects and pharmacokinetic properties of icariin, the major bioactive component in Herba Epimedii.** *Life Sci*2015, **126**:57-68.
35. Arief ZM, Munshi AH, Shawl AS: **Evaluation of medicinal value of Epimedium elatum on the basis of pharmacologically active constituents, Icariin and Icariside-II.** *Pakistan journal of pharmaceutical ences*2015, **28**(5):1665-1669.
36. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M: **The chemokine system in diverse forms of macrophage activation and polarization.** *Trends in Immunology*2004, **25**(12):677-686.
37. Arora S, Dev K, Agarwal B, Das P, Syed MA: **Macrophages: Their role, activation and polarization in pulmonary diseases.** *Immunobiology*2018, **223**(4-5):383-396.
38. Ma XM, Blenis J: **Molecular mechanisms of mTOR-mediated translational control.** *Nature Reviews Molecular Cell Biology*2009, **10**(5):307-318.
39. Newton PT, Vuppapapati KK, Boudierlique T, Chagin AS: **Pharmacological inhibition of lysosomes activates the MTORC1 signaling pathway in chondrocytes in an autophagy-independent manner.** *Autophagy*2015, **11**(9):1594-1607.
40. DiDonato J, Mercurio F, Karin M: **NF-κB and the link between inflammation and cancer.** *Immunological reviews*2012, **246**(1):379-400.
41. Shi Y, Luo P, Wang W, Horst K, Blasius F, Relja B, Xu D, Hildebrand F, Greven J: **M1 But Not M0 Extracellular Vesicles Induce Polarization of RAW264.7 Macrophages Via the TLR4-NFκappaB Pathway In Vitro.** *Inflammation*2020, **43**(5):1611-1619.

Figures

Figure 1

The effects of LPS,IFN-γ,IL-4 and icariin on RAW 264.7 macrophages viability.(A) RAW 264.7 macrophages were incubated with LPS,IFN-γ or various concentrations of icariin for 24 h.(B) RAW 264.7 macrophages were incubated with IL-4 or various concentrations of icariin for 24 h.CCK-8 assay to detect the cells viability.Data are expressed as mean $\pm$ SE for

Figure 2

Icariin inhibited M1 macrophage related cytokines release (TNF-α),and promoted M2 macrophage related cytokines release (IL-10).(A) Macrophages were incubated with LPS,IFN-γ or icariin for 24 h.(B) Macrophages were incubated with IL-4 or icariin for 24 h.Subsequently,Cell supernatants were collected.Serum inflammatory factors TNF-α and IL-10 were detected by ELISA.Data are expressed as

mean ± SE from three independent experiments.**P<0.01 vs. Blank group;#P<0.05 vs. LPS+IFN-γ or IL-4 group.

Figure 3

Icaritin inhibited the expression of TNF-α,iNOS and IL-6 in M1 macrophages.(A-C) Primary mouse peritoneal macrophages were incubated with LPS,IFN-γ or icaritin for 24 h.(D-F) RAW 264.7 macrophages were incubated with LPS,IFN-γ or icaritin for 24 h.Cells were collected for total mRNA extraction.The mRNA expression of pro-inflammatory cytokines (TNF-α,IL-6 and iNOS) was detected by RT-PCR.Data are expressed as mean ± SE from three independent experiments.**P<0.01 vs. Blank group;##P<0.01 vs. LPS+IFN-γ group;#P<0.05 vs. LPS+IFN-γ group.

Figure 4

Icariin promoted the expression of CD206 and Arg1 in M2 macrophages.(A-B) Primary mouse peritoneal macrophages were incubated with IL-4 or icariin for 24 h.(C-D) RAW 264.7 macrophages were incubated with IL-4 or icariin for 24 h.Cells were collected for total mRNA extraction.The mRNA expression of CD206 and Arg1 was detected by RT-PCR.Data are expressed as mean $\pm$ SE from three independent experiments.**P<0.01 vs. Blank group;*P<0.05 vs. Blank group;##P<0.01 vs. IL-4 group;#P<0.05 vs. IL-4 group.

A

B

Figure 5

Icariin inhibits M1 macrophage activation.(A) Primary mouse peritoneal macrophages were incubated with LPS+IFN-γ or icariin for 24 h.(B) RAW 264.7 macrophages were incubated with LPS+IFN-γ or icariin for 24 h.The cells were collected,and iNOS and CD86 are markers for M1.It was estimated that the changes of their proportions by flow cytometric analysis.Data are expressed as mean ± SE from three independent experiments.**P<0.01 vs. Ctrl group;#P<0.05 vs. LPS+IFN-γ group.

A **B** **Figure 6**

Icariin induces M2 macrophage activation.(A) Primary mouse peritoneal macrophages were incubated with IL-4 or icariin for 24 h.(B) RAW 264.7 macrophages were incubated with IL-4 or icariin for 24 h.The cells were collected,and CD206 and F4/80 are markers for M2.It was estimated that the changes of their proportions by flow cytometric analysis.Data are expressed as mean $\pm$ SE from three independent experiments.** $P < 0.01$ vs. Ctrl group;# $P < 0.05$ vs. IL-4 group.

Figure 7

Icariin inhibits the mTOR/S6K1 signaling pathway of M1 macrophages. Primary mouse peritoneal macrophages were incubated with LPS+IFN- γ or icariin for 24 h. The cells were harvested, and the total protein was prepared. (A) Western blot analysis was performed to detect the protein expression levels of mTOR, phosphorylated-mTOR, S6K1 and phosphorylated-S6K1. (B-C) Quantitative analysis of phosphorylated-mTOR and phosphorylated-S6K1 protein bands by grayscale analysis. Data are expressed as mean $\pm$ SE from three independent experiments. ** $P < 0.01$ vs. Blank group; # $P < 0.05$ vs. LPS+IFN- γ group.

Figure 8

Icariin inhibits the NF-κB signaling pathway of M1 macrophages. Primary mouse peritoneal macrophages were incubated with LPS+IFN-γ or icariin for 24 h. The cells were harvested, and the total protein was prepared. (A) Western blot analysis was performed to detect the protein expression levels of NF-κB p65, phosphorylated-NF-κB p65, IKKβ, phosphorylated-IKKβ, IκBα and phosphorylated-IκBα. (B-D) Quantitative analysis of phosphorylated-NF-κB p65, phosphorylated-IKKβ and phosphorylated-IκBα protein bands by grayscale analysis. (E) The cells were fixed with 4% paraformaldehyde. Immunofluorescence staining was performed to examine the nuclear translocation and expression level of phosphorylated NF-κB p65. Data are expressed as mean ± SE from three independent experiments. **P<0.01 vs. Blank group; *P<0.05 vs. Blank group; #P<0.05 vs. LPS+IFN-γ group.

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