Moxibustion alleviates inflammation via SIRT5 post-translational modification and macrophage polarization

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Research Article

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Abstract

Background

Macrophage polarization plays an essential role in the anti-inflammation process. Moxibustion, a traditional Chinese medicine therapy, has been reported to have an anti-inflammatory effect via enhancing α-ketoglutarate (α-KG) and succinate levels. Succinate/α-KG ratio is a hallmark of M1 and M2 macrophage shift. Glutamate dehydrogenase 1 (GLUD1) is a vital enzyme for α-KG production and can be deacetylated by Sirtuin5 (SIRT5). Currently, the role of moxibustion in SIRT5-GLUD1-α-KG-related macrophage alteration in inflammatory diseases has not been discussed yet.

Methods

In this study, complete Freund’s adjuvant (CFA)-induced adjuvant arthritis models were established. On day 4 post-CFA, moxibustion and acupoint MC3482 injection were administered. Foot volume was measured before and after the model was established, and after the moxibustion and acupoint injection interventions. ELISA assays were then performed to quantify inflammatory factors, including IL-1β, TNF-α, IL-4, TGF-β, succinate, and α-ketoglutarate (α-KG). Flow cytometry (FCM) and immunofluorescence were used to test M1- and M2-like macrophage expressions in the right arthrodial cartilages of mice. Furthermore, western blotting and immunoprecipitation (IP) were used to detect SIRT5, GLUD1, and GLUD1 succinylation expressions.

Results

Moxibustion and SIRT5 desuccinylation inhibitor MC3482 decreased inflammation by increasing M2 macrophage and reducing M1 macrophage levels in CFA model. The potential mechanism may relate to the effects of moxibustion and SIRT5 inhibition, which could invert succinate and α-KG levels in the CFA group, which displayed low succinate, high α-KG and increased GLUD1 succinylation modification after treatment.

Conclusion

This study supports that moxibustion's anti-inflammation effects are related to the consequences of macrophage conversion after SIRT5 post-translational modification.

Introduction

Inflammation is the body's defensive response to infection or injury. While excessive or persistent inflammation can damage tissues and harm health. In particular, excessive production of inflammatory cytokines activated by immune cells like macrophages, is a significant sign of inflammation[1, 2].
Although traditional Chinese medicine (TCM) does not hold the concept of "inflammation", it recognizes the symptoms caused by inflammatory responses, such as redness, swelling, heat, pain, and tissue damage. Moxibustion is a traditional Chinese medicine technique that applies heat to acupuncture points. Recent researches suggest moxibustion is an important anti-inflammatory and analgesic method, and is widely used in clinic [3]. The anti-inflammatory effectiveness mechanism of moxibustion mainly results from the combined action of the nervous, endocrine, and immune systems. Researchers found that moxibustion increases α-ketoglutarate (α-KG), succinate, citrate, and other tricarboxylic acid (TCA) cycle intermediates to correct metabolic disorders and promote inflammation repair [4]. Moxibustion may target macrophages, endothelial cells, T cells, and other cells to treat rheumatoid arthritis by regulating the immune cell network [5].

As part of the innate immune system, macrophage activation contributes to inflammatory processes involved in pathogen recognition, clearance, wound healing, and antigen presentation to T cells [6, 7]. In response to various external signals, macrophages can polarize into two main subtypes: classically activated macrophages (M1) and alternatively activated macrophages (M2) [8]. The imbalance between pro- and anti-inflammatory cytokines is a key mechanism of macrophages polarization. M1 polarization is activated by lipopolysaccharide (LPS), pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ, leading to the progression of inflammation [9]. Whereas, anti-inflammatory IL-4, IL-13 and TGF-β trigger M2 polarization, that is involved in tumor progression, wound repair, tissue remodeling, and immunity to parasitic helminths [10, 11].

Previous studies have shown that M1 macrophages have lower levels of α-ketoglutarate (α-KG) and higher succinate due to bypassing the TCA cycle [12]. Succinate accumulates in M1 macrophages and acts as an inflammation signal, while α-KG restores M2 polarization [13, 14]. Thus, the succinate/α-KG ratio modulates M1 and M2 macrophage activation, which is critical for inflammation [15, 16]. Glutamate dehydrogenase 1 (GLUD1) converts glutamate to α-KG [17]. In Glud1 knockout mice, macrophages were more M1 but less M2 [18].

Recent studies show that Sirtuin 5 (SIRT5) plays an essential role in macrophage polarization [19, 20]. SIRT5, a member of the SIRT family, possesses robust deacylase activity against lysine residues, including succinylation, malonylation, and glutarylation [21]. Many SIRT5 target proteins are involved in key metabolic pathways. Previous research found that SIRT5 deficiency increases succinylation of pyruvate kinase M2 (PKM2) protein, which increases HIF-1α levels and attenuates mitochondrial respiration and reactive oxygen species (ROS) accumulation [22, 23]. The latest research found that glutamate dehydrogenase 1 (GLUD1) can be deacetylated by SIRT5, allowing it to participate in Gln metabolism [24, 25]. GLUD1 is a key enzyme that generates α-KG from Glu in Gln metabolism [26]. GLUD1 activity mainly depends on changes in shape and chemical modifications [27]. Of these, SIRT5 is suggested to be key to regulating GLUD1 succinylation and activity. Studies show SIRT5 regulates GLUD1, an enzyme important for glutamine metabolism [28]. Without SIRT5, GLUD1 succinylation increases, reducing its activity. In brown fat, less active GLUD1 disrupts metabolism [24]. SIRT5 also activates GLUD1 in colorectal cancer, promoting tumor growth by enhancing glutamine breakdown [29].
These studies suggested that SIRT5 interaction with GLUD1 impacts glutamine metabolism and related processes. Therefore, it is reasonable to hypothesize that GLUD1 succinylation could critically influence anti-inflammatory regulation via macrophages polarization. Considering the significant anti-inflammation role of moxibustion. This study was designed to investigate the role of GLUD1 succinylation in regulating macrophage polarization via moxibustion.

Therefore, it stands to reason that the succinylation of GLUD1 is instrumental in regulating the anti-inflammatory effects achieved through the polarization of macrophages. Given the substantial anti-inflammatory effects of moxibustion, the present study sought to explore how the succinylation of GLUD1 influences the polarization of macrophages. Specifically, we hypothesized that the succinylation of GLUD1 plays a critical role in driving macrophages towards an anti-inflammatory M2 phenotype, associated with decreased production of pro-inflammatory cytokines and increased release of anti-inflammatory cytokines. By promoting M2 macrophage polarization, the succinylation of GLUD1 may inhibit excessive or prolonged inflammation. Conversely, reducing the succinylation of GLUD1 via SIRT5 could shift macrophages towards a pro-inflammatory M1 phenotype and sustain or intensify inflammatory responses. Overall, we aimed to determine how the succinylation status of GLUD1 affects macrophage polarization and subsequent regulation of inflammation. Elucidating this mechanism may reveal new therapeutic targets for controlling inflammation through the modulation of macrophage polarization.

Materials and methods

Animals

Adult male C57BL/6J mice (22 ± 2g) were purchased from Shanghai Model Organisms Co., Ltd. and housed under standard conditions (12-h light/dark cycle, 24°C, 40–50% humidity) with free access to food and water for 1 week before experiments. Mice were then randomly divided into 5 groups (n = 10 per group): control, CFA (Complete Freund's adjuvant), CFA + Moxi (moxibustion), CFA + MC3482, and CFA + MC3482 + Moxi. After interventions, mice were euthanized via inhalation of ethylether and cervical dislocation. All animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [30] and the ethical guidelines of the International Association for the Study of Pain [31]. All experimental procedures were approved by the Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine (reference number: AECCDUTCM-2018-11).

Adjuvant arthritis model

Adjuvant arthritis model was induced by injecting 20 µL complete Freund’s adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) into the right hind paw [32]. Local swelling and behavioral disability appeared within 24 h. Mice in control group were injected with 20 µL normal saline.

Moxibustion intervention
On day 4 post-CFA, moxibustion was applied at ST36 (Zusanli acupoint, 2 mm lateral to anterior tubercle of tibia, 4 mm distal to knee) for 15 days (5 days/week). Fur at ST36 was shaved and moxa sticks (Nanyang Hanyi Moxibustion Technology Development Co., Ltd.) were held 1.5 cm from the skin. Moxibustion was only applied to CFA + Moxi and CFA + MC3482 + Moxi groups.

**Acupoint injection**

On day 4 post-CFA, MC3482 (25 µM, 20 µL; MedChemExpress) was injected at ST36 in CFA + MC3482 and CFA + MC3482 + Moxi groups for 15 days (5 days/week). Saline was injected in control, CFA, and CFA + Moxi groups.

**Behavioral tests**

Foot volume was measured using a volume meter (ZH-LUO-G7, Zhenghua Biologic). The baseline of foot volumes were measured before the modeling, and after CFA induced adjuvant arthritis model, the foot volume measurements were applied on day 4, day 11, day 8, and day 25.

**ELISA assay**

Mice were anesthetized with an overdose of choral hydrate and intracardially perfused with saline. Right arthrodial cartilages were collected from mice. In compliance with the manufacturer's protocol, the amounts of IL-1β, TNF-α, IL-4, and TGF-β were quantified by ELISA kits according to the manufacturer's instructions.

**Flow cytometry (FCM)**

Right arthrodial cartilages were incubated in 0.2% collagenase, filtered, and stained for F4/80, CD86 and CD206. F4/80+CD86+ and F4/80+CD206+ macrophages were considered M1- and M2-like, respectively [33, 34]. Flow cytometry was performed using BD FACSCanto II (4.2.2) (BD Bioscience), and data were analyzed using FlowJo software (version 9.2). Antibodies used in this experiment including anti-F4/80 antibody (Proteintech), anti-CD86 (Proteintech), and anti-CD206 (Proteintech).

**Immunofluorescence**

The right arthrodial cartilages collected were decalcified in 13% EDTA (pH 7.3) for 3 days. After that, they were placed in 30% sucrose overnight and embedded in OCT at − 20°C the following day. Frozen sections were cut (30 µm) and placed on glass microslides coated with APS. The sections were post fixed in 4% paraformaldehyde for 3 minutes and incubated in blocking solution containing 3% BSA, 0.1% Triton X-100, and 0.02% NaN3 in PBS for 2 hours at room temperature. After blocking, sections were incubated with the appropriate primary antibodies in blocking solution at 4°C overnight. The primary antibodies used were: anti-SIRT5(1:100) anti-CD206 (1:100) and anti-86 (1:100) from ProteinTech. The secondary antibodies were goat anti-rabbit (1:500) and anti-mouse (1:500) antibody (ProteinTech Group, Chicago, USA). Slides were mounted with cover slips and visualized using a fluorescence microscope (CKX41 with an Olympus U-RFLT50 Power Supply Unit; Olympus, Tokyo, Japan).

**Western blotting**
The dermal tissues were immediately excised to extract proteins. Proteins were extracted by adding lysis buffer with 50 mM Tris–HCl (pH 7.4), 250 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 0.02% NaNs, and protease inhibitors (AMRESCO, Solon, OH, USA) to the samples. The samples were then homogenized using a Bullet Blender homogenizer (Next Advance, NY, USA). Extracted proteins (30 µg per sample, measured by BCA assay) underwent 8% SDS-Tris glycine gel electrophoresis and were transferred to a PVDF membrane. The membrane was blocked with 5% milk in TBS-T buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20), incubated with the primary antibody in TBS-T with 1% BSA, and incubated for 1 hour at room temperature. Then, a peroxidase-conjugated secondary antibody (1:5000) was used. Image intensities of specific bands were quantified using ImageJ software (Bethesda, MD, USA). Antibodies used were: anti-ACTIN (ProteinTech Group, Chicago, USA), anti-SIRT5 (ProteinTech Group, Chicago, IL, USA), anti-succinylation (PTM Biolab, Zhejiang, China) and anti-GLUD1 (ProteinTech Group, Chicago, USA). Bands were visualized using an enhanced chemiluminescence substrate kit (Biorad, Fuji Photo Film Co. Ltd, Tokyo, Japan).

**Immunoprecipitation (IP)**

Prepare 5 microliters of washed and mixed A + G beads (washed 3 times with lysis buffer, spun 1 minute at 3000 rpm, bead storage buffer replaced with lysis buffer) in each tube. Add ~ 5 microliters antibody against target protein to each tube; use corresponding IgG as control for each experiment. Prepare another set of tubes with 2X SDS sample buffer directly for input sampling. Take some supernatant after spinning for direct sampling; add remaining supernatant to IP tubes. Rotate overnight at 4 degrees Celsius on vertical mixer. Wash rotated beads with lysis buffer, discard supernatant. Sample with 1X SDS buffer. Detect results by Western blot. Prepare protein samples and new tubes for immunoprecipitation (IP). Add antibody-bead mixture to each tube. Prepare input sample tubes with 2X SDS buffer. Take supernatant sample after spinning, add rest to IP tubes. Rotate tubes overnight at 4°C. Wash beads, remove supernatant. Sample with 1X SDS buffer. Detect by Western blot.

**Statistical analysis**

Behavioral tests measurements were analyzed using two-way repeated measures ANOVA followed by Bonferroni’s post hoc test. One-way ANOVA followed by Fisher’s post hoc test for multiple comparisons or unpaired t test for comparisons between two groups were used for biochemical data. In all cases, \( p < 0.05 \) was considered significant.

**Results**

**Moxibustion and MC3482 intervention relieved inflammation of CFA-induced adjuvant arthritis models**

In paw capacity assessment, Fig. 1A shows that initially, all the mice had about the same capacity. Statistically, there were no major differences between the groups (\( p > 0.05 \)). The model group, moxibustion group, MC3482 group, and moxibustion + MC3482 group showed significant improvements.
in their paw capacities after CFA injection. On day 11 of the experiment, these 4 groups showed no significant changes in their paw abilities compared to day 4 \((p > 0.05)\). While, on day 25, compared to the model group, the moxibustion, MC3482, and moxibustion + MC3482 groups showed significantly decreased paw abilities \((p < 0.05)\).

Control control group, CFA adjuvant arthritis models, CFA + Moxi adjuvant arthritis models with moxibustion group, CFA + MC3482 adjuvant arthritis models with MC3482 ST36 injection group, CFA + Moxi + MC3482 adjuvant arthritis models with moxibustion and MC3482 ST36 injection group. Data are expressed as mean ± SEM \((n = 10)\). \# \(p < 0.05\), vs Control group, \(* p < 0.05\), vs CFA group.

To determine the anti-inflammatory effect of the intervention of Moxi and MC3482, the levels of pro-inflammatory cytokines IL-1\(\beta\) and TNF-\(\alpha\), which activate M1 macrophages, and anti-inflammatory cytokines IL-4 and TGF-\(\beta\), which activate M2 macrophages, were tested via ELISA (Fig. 2). Compared to the control group, the levels of IL-1\(\beta\) increased and TNF-\(\alpha\) rose in the CFA group \((p < 0.05)\), while the expression of IL-4 and TGF-\(\beta\) slightly decreased without statistical differences \((p > 0.05)\) (Fig. 2A-C). In the CFA + Moxi, CFA + MC3482, and CFA + MC3482 + Moxi groups, the expression of IL-1\(\beta\) and TNF-\(\alpha\) decreased, and the expression of IL-4 increased compared to the CFA group \((p < 0.05)\) (Fig. 2A-C). Moreover, TNF-\(\alpha\) remained relatively high in these three groups compared to the control group \((p > 0.05)\) (Fig. 2B). Regarding TGF-\(\beta\) expression, no significant differences were found between the control, CFA, CFA + Moxi, CFA + MC3482, and CFA + MC3482 + Moxi groups \((p > 0.05)\) (Fig. 2D).

**M1 and M2 macrophage expression changes with Moxibustion and MC3482 intervention**

The results of the flow cytometer (FCM) analysis showed that while the expression of CD86+ (a marker specific to M1 macrophages) in the CFA group increased slightly, there were no significant differences in M1 macrophage expression among the Control, CFA, CFA + Moxi, CFA + MC3482 and CFA + Moxi + MC3482 groups (Fig. 3A, C). In comparison, the expression of CD206+ (a marker specific to M2 macrophages) in the CFA + Moxi, CFA + MC3482 and CFA + Moxi + MC3482 groups increased compared to the CFA group \((p < 0.05)\). There was no difference in expression between the CFA and CFA + Moxi + MC3482 groups (Fig. 3B, C). The M2 to M1 macrophage expression ratio suggested that the CFA + Moxi, CFA + MC3482 and CFA + Moxi + MC3482 groups significantly increased compared to the CFA group. Although there was no statistically significant difference in the M2 to M1 ratio between the Control and CFA groups \((p > 0.05)\) (Fig. 3C).

The results from immunohistochemistry showed CD86 and CD206 expressions (Fig. 4A) in control, CFA, CFA + Moxi, CFA + MC3482, and CFA + MC3482 + Moxi groups. The statistical analysis revealed that compared with CFA group, CD86 expressions in the control, CFA + Moxi, CFA + MC3482, and CFA + MC3482 + Moxi groups decreased significantly (Fig. 4A, B). In comparison, CD206 expressions increased significantly in CFA + Moxi, CFA + MC3482, and CFA + MC3482 + Moxi groups (Fig. 4A, C).
Succinate and α-ketoglutarate expressions with Moxi and MC3482 intervention

The levels of succinate and α-ketoglutarate(α-KG) were analyzed, and it was found that compared to the CFA group, the succinate expressions decreased in the CFA + Moxi, CFA + MC3482, and CFA + MC3482 + Moxi groups \( (p < 0.05) \) (Fig. 5A). Conversely, the α-ketoglutarate expressions increased in these three groups \( (p < 0.05) \) (Fig. 5B). In the control group, although the α-ketoglutarate expressions significantly increased compared to the CFA group \( (p < 0.05) \) (Fig. 5B), succinate expression decreased without significant difference \( (p > 0.05) \) (Fig. 5A).

GLUD1 expression with Moxibustion and MC3482 intervention at ST36 in CFA-Induced adjuvant arthritis models Mice

Western blot analysis showed that GLUD1 expression in the dermal tissues of ST36 acupoints in the CFA group slightly decreased but did not differ significantly from other groups \( (p > 0.05) \) (Fig. 6A,B). Similar results were observed in the toes, where GLUD1 expression in the model group slightly decreased without significant difference compared to other groups \( (p > 0.05) \) (Fig. 6C,D).

IP results (Fig. 7A, B) showed that compared to the control group, GLUD1 succinylation modification expression in the dermal tissues of ST36 acupoints in the CFA group decreased \( (p < 0.05) \). After intervention, GLUD1 succinylation modification expression values increased significantly in the CFA + Moxi, CFA + MC3482, and CFA + Moxi + MC3482 groups compared to the model group \( (p < 0.05) \). Regarding GLUD1 succinylation modification expression in the toes, the CFA group showed an increase compared to the control group \( (p < 0.05) \) (Fig. 7C,D). With intervention, GLUD1 succinylation modification expression decreased significantly in the CFA + Moxi, CFA + MC3482, and CFA + Moxi + MC3482 groups compared to the CFA group \( (p < 0.05) \).

Discussion

Inflammation can arise from infections, injuries, exposure to harmful substances, immune system problems, and genetics. Ongoing inflammation harms healthy tissues and organs [35]. Inflammatory chemicals damage tissues, causing scarring and hardening that leads to organ damage and loss of function. Reducing inflammation is key to managing inflammatory diseases and improving outcomes. Treatment usually involves anti-inflammatory drugs like corticosteroids to suppress the immune system, immunosuppressants, and biological therapies targeting inflammatory pathways [36]. Long-term or high doses of these drugs can cause harmful side effects like stomach issues, high blood pressure, kidney damage, bone loss, and increased infection risk. When used alongside standard medical treatment, alternative therapies can be a safe way to boost wellness and promote health. Moxibustion is a TCM technique using burning dried mugwort on or over acupoints. It is thought to stimulate the flow of qi and blood, strengthen the body, and maintain health. Studies show moxibustion has anti-inflammatory, pain-
relieving, and warming effects. Moxibustion increases blood flow to inflamed areas, stimulates the immune system, and boosts the body's ability to fight infection and heal itself, eliminating inflammatory chemicals and byproducts [37–39]. This study confirms previous findings, the results show that moxibustion treatment can relieve inflammation in CFA-induced adjuvant arthritis models.

MC3482 is a specific SIRT5 desuccinylating inhibitor that inhibits SIRT5 extracellular expression levels[40]. SIRT5 is a mitochondrial enzyme that predominantly operates through an NAD+-dependent deacylated mechanism, catalyzing the removal of succinyl, glutaryl, acetyl, and malonyl groups from lysine residues [41, 42]. It is a crucial regulator of cellular homeostasis and modulates the activity of proteins involved in various metabolic pathways, including the TCA cycle, the electron transport chain, glycolysis, fatty acid oxidation, generation of ketone bodies, nitrogenous waste management, and detoxification of ROS. Reports indicated that SIRT5 have a protective function by decreasing keratinocyte proliferation and the production of inflammatory proteins.[43–46]. In this study, the results show that SIRT5 inhibitor MC3482 intervention, and a combination of moxibustion and MC3482 at ST36 site can relieve inflammation in CFA-induced adjuvant arthritis models. The results indicate that moxibustion treatment, MC3482 intervention, and combining moxibustion and MC3482 can help relieve inflammation and improve mobility in CFA-induced adjuvant arthritis mouse models. The combination of moxibustion and MC3482 appears particularly effective, suggesting a synergistic effect.

With the anti-inflammatory effects of moxibustion and inhibition of SIRT5 desuccinylolation at ST36 acupoints, inflammatory factors like interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and transforming growth factor-β (TGF-β) were tested. IL-1β and TNF-α are pro-inflammatory cytokines produced by macrophages [47]. These cytokines stimulate the production of M1 macrophages and other inflammatory chemicals like prostaglandins and leukotrienes [48]. Excessive or chronic production of IL-1β and TNF-α contributes to inflammatory diseases like rheumatoid arthritis, inflammatory bowel disease, and atherosclerosis [49–51]. IL-4 is an anti-inflammatory cytokine produced by macrophages, T cells, mast cells and basophils. It inhibits the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. By suppressing the inflammatory response, IL-4 helps promote tissue healing and repair [52]. In inflammatory diseases, increasing IL-4 can help shift the balance from a pro-inflammatory to an anti-inflammatory state, facilitating disease remission and recovery [53, 54]. TGF-β has both pro-inflammatory and anti-inflammatory effects. Excessive TGF-β activity early in the inflammatory process leads to tissue damage, scarring and fibrosis [55]. Limited TGF-β later on impairs resolution of inflammation and healing. Maintaining the appropriate balance of TGF-β is important for controlling inflammation and enabling recovery [56]. In chronic inflammatory states, the production of IL-4 and TGF-β can increase the production of M2 macrophages to reduce inflammation and promote healing [57]. The study results show that moxibustion treatment, MC3482 intervention, and a combination of moxibustion and MC3482 can relieve inflammation by regulating inflammatory cytokines expression. The levels of pro-inflammatory cytokines IL-1β and TNF-α increased in the CFA-induced adjuvant arthritis models, while the expression of anti-inflammatory cytokines IL-4 decreased. Moxibustion, MC3482 intervention, and a combination of moxibustion and MC3482 treatments decreased IL-1β and TNF-α expression, and increased IL-4 expression compared with the CFA-induced
adjuvant arthritis models. Regarding TGF-β expression, moxibustion and a combination of moxibustion and MC3482 treatments increased TGF-β level compared with the CFA group.

Macrophages are innate immune cells that serve as gatekeepers to recognize and respond to infection and tissue injury. Hyperactive macrophages are predominantly detected after injury, with increased production of pro-inflammatory factors, such as IL-1β, TNF-α, and IL-6. In addition, macrophages exhibit a highly plastic nature in injured tissues [2]. Phenotypic polarization represents a typical form of macrophage plasticity, normally characterized by two extreme switches termed M1 and M2 macrophages. M1 polarization is induced by proinflammatory mediators that confer host defense against inflammation, while M2 polarization is activated by the increased production of anti-inflammatory cytokines, such as IL-4 and TGF-β, favouring the transition into resolution of inflammation and tissue repair [58]. The results of FCM analysis showed no significant differences in M1 macrophage expression among groups. Instead, CD206 + expression (M2 macrophages) increased in Moxibustion, MC3482 intervention, and a combination of moxibustion and MC3482 treatments. These results indicate that moxibustion treatment, MC3482 intervention, and a combination of moxibustion and MC3482 can relieve inflammation in CFA-induced adjuvant arthritis models by regulating macrophage polarization, especially by promoting M2 macrophages to resolve inflammation.

In addition to external stimuli that determine macrophage phenotype, intrinsic regulatory mechanisms also play a critical role in macrophage polarization and maintaining the balance between immune response and tissue integrity [59]. Metabolic rewiring is a key feature and inherent mechanism of macrophage polarization [60]. M1 macrophages mainly rely on glycolysis to meet their energy demands and produce metabolites such as citrate, lactate, succinate, and nitric oxide to provoke inflammatory and antimicrobial reactions [61]. On the other hand, M2 macrophages maintain intact oxidative phosphorylation (OXPHOS) to meet their energy demands and couple with other pathways, such as glutamine and polyamine metabolism, to promote inflammation resolution and tissue remodeling [62]. Glutamine is a major source of both carbon and nitrogen in metabolism and biosynthesis [63]. Typically, glutamine fuels the tricarboxylic acid (TCA) cycle by providing its main metabolite α-KG through a process called glutaminolysis, which serves as a critical source to maintain intact OXPHOS [64, 65]. Glutamine metabolism mainly regulates macrophage phenotype by regulating α-ketoglutarate (α-KG) content. High α-KG/succinate ratio promotes M2 macrophage expression, while low α-KG/succinate ratio promotes M1 macrophage production [66]. α-KG is derived from the oxidative decarboxylation of isocitrate in the TCA cycle and is produced from glutamine and glutamate metabolism or external sources, which play an important role in the polarization of macrophages by providing an energy source for damaged tissues [67, 68]. Researchers have found that glutamine metabolism is an important pathway to regulate M1 and M2 macrophage polarization, due to the differences in bioenergetic demands between M1 and M2 macrophages [69]. However, the current research results are not sufficient to fully explain the regulatory mechanism of α-KG on M2 macrophages polarization, and there is a need to further investigate its potential effects on macrophages. In this study, we analyzed the expressions of succinate and α-KG with moxibustion and SIRT5 desuccinylation inhibition at ST36. The results showed that succinate expression increased, although not significantly different from the control group, in CFA-
induced adjuvant arthritis, while α-KG expression significantly decreased. With moxibustion treatment, MC3482 intervention, and a combination of moxibustion and MC3482, the expressions of succinate were reduced, and α-KG was enhanced. This suggests that moxibustion and SIRT5 desuccinylation inhibition at ST36 can regulate M1 and M2 macrophages through the glutamine pathway.

SIRT5 has robust lysine desuccinylase activity and is the primary regulator of the mitochondrial succinylome [28]. Mechanistically, SIRT5 was shown to activate GLUD1, increasing the α-KG production [70]. GLUD1 is an isoform of Glutamate dehydrogenase (GDH) that catalyzes the reversible deamination of l-glutamate to α-KG and ammonia using NAD + and NADP + as cofactors [70]. In mammals, GDH is a key enzyme for metabolism and is considered essential for processes such as amino acid and carbohydrate metabolism, energy production, ammonia management, neurotransmitter recycling and insulin secretion [64]. GLUD1 is normally thought to predominantly localize in the mitochondrial matrix, its main metabolic function is to fuel aerobic metabolism through the Krebs cycle for energy production, interconnecting in parallel amino acid and carbohydrate metabolism [26]. In addition, it contributes to other important tissue and non-tissue-specific processes, including autophagy, neurotransmitter recycling, ammonia management and insulin secretion [71, 72]. In this study, western blot analysis showed that GLUD1 expression in the dermal tissues and the toes did not change. On the other hand, GLUD1 succinylation modification expression values increased significantly in the dermal tissues of ST36 site with moxibustion and MC3482 injection at ST36 acupoint. While, GLUD1 succinylation expression decreased in the toes with moxibustion and MC3482 intervention. These results implied that moxibustion treatment, MC3482 intervention, and a combination of moxibustion and MC3482 might alter GLUD1 succinylation to regulate succinate and α-KG in GDH process. Thereby, TCA cycle and macrophage polarization process might be regulated. While, in this study, GLUD1 succinylation is not directly be blocked or overexpressed to analyze the feet volume of mice, and macrophage polarizations expressions. And this study implied the relation between SIRT5 and GLUD1 succinylation in the mechanism of anti-inflammation in moxibustion, but the specific correlation between SIRT5 and GLUD1 succinylation need further exploration.

Conclusions

Overall, this study revealed that moxibustion, MC3482 injected at ST36, and the combination of these two interventions at ST36 reduced inflammation in CFA-induced adjuvant arthritis, and GLUD1 succinylation might play a crucial role in macrophage M2 polarization in the anti-inflammatory mechanism of moxibustion and MC3482 intervention at ST36. Clinical trials are also needed to determine whether these treatments are effective and safe for human patients.

Abbreviations

SIRT5  Sirtuin5
α-KG   α-ketoglutarate
**GLUD1** glutamate dehydrogenase 1

**CFA** complete Freund's adjuvant

**FCM** Flow cytometry

**IP** immunoprecipitation

**TCM** traditional Chinese medicine

**TCA** tricarboxylic acid

**ROS** reactive oxygen species

**IL-1β** interleukin-1β

**TNF-α** tumor necrosis factor-α

**IL-4** interleukin-4

**TGF-β** transforming growth factor-β

**OXPHOS** oxidative phosphorylation

**Declarations**

**Ethics approval and consent to participate**

All experimental procedures were approved by the Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine (reference number: AECCDUTCM-2018-11).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

Chuan-yi Zuo conceived and designed the experiments, performed them, contributed reagents and materials, analyzed data, and wrote the paper. Cheng-shun Zhang conceived and designed the experiments, performed them, analyzed data, and wrote the paper. Han-xiao Zhang, Xiao-qin Dai, and Si-rui Lin performed the experiments, and analyzed and interpreted the data. Chun-yan Gou and Feng-wei Tian conceived and designed the experiments. Hong Lei and Zhu-xing Wang performed the experiments and contributed reagents and materials.

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Availability of data and material

The research data used to support the findings are available from the corresponding author upon request.

References


Figures
Figure 1

Paw capacity of mice with Moxi and MC3482 intervention on adjuvant arthritis models.

Control control group, CFA adjuvant arthritis models, CFA+Moxi adjuvant arthritis models with moxibustion group, CFA+MC3482 adjuvant arthritis models with MC3482 ST36 injection group, CFA+Moxi+MC3482 adjuvant arthritis models with moxibustion and MC3482 ST36 injection group. Data are expressed as mean ± SEM (n = 10). # p 0.05, vs Control group, *p 0.05, vs CFA group.
**Figure 2**

Inflammatory cytokines expression changes with moxibustion and MC3482 intervention.

A. IL-1β expressions, B. TNF-α expressions, C. IL-4 expressions, D. TGF-β expressions. Data are expressed as mean ± SEM (n = 6). # p 0.05, vs Control group, *p 0.05, vs CFA group.
Figure 3

Moxibustion and MC3482 intervention with M1 and M2 macrophage expression by FCM.

A. The proportion of macrophages (F4/80) cells was detected by flow cytometry. B. The proportion of M1 (CD86+) macrophages and M2 (CD206+) macrophage cells was detected by flow cytometry. C. Percentages of M1 macrophage cells, M2 macrophage cells and the ratio of M2 to M1 macrophage cells are shown in the bar. Error bars show mean ± SEM (n = 6). *p 0.05, vs CFA group, #p 0.05, vs CFA+MC3482+Moxi group.
Figure 4

CD86 and CD206 expressions with Moxibustion and MC3482 intervention by Immunofluorescence.

A. Immunofluorescence staining expressions of CD86 (green), CD206 (red), and DAPI (blue). Scale bars 40 μm. B. Average density of CD86 in the Control, CFA, CFA + Moxi, CFA + MC3482, CFA + MC3482 + Moxi groups. C. Average density of CD206 in these 5 groups. *p 0.05, vs CFA group.
Figure 5

Succinate and α-ketoglutarate expressions change with moxibustion and MC3482 intervention.

A. succinate expressions, B. α-ketoglutarate expressions. Data are expressed as mean ± SEM (n = 6). # p 0.05, vs Control group, * p 0.05, vs CFA group.
Figure 6

GLUD1 expressions with moxibustion and MC3482 intervention.

A,B. GLUD1 expressions and statistical analysis in the dermal tissues of ST36 acupoints. C,D. GLUD1 expressions and statistical analysis in the toes. Data are expressed as mean ± SEM (n = 4).
Figure 7

GLUD1 succinylation expressions with moxibustion and MC3482 intervention.

A,B. GLUD1 succinylation expressions and statistical analysis in the dermal tissues of ST36 acupoints. C,D. GLUD1 succinylation expressions and statistical analysis in the toes. Data are expressed as mean ± SEM (n = 4). # p 0.05, vs Control group, *p 0.05, vs CFA group.