Itaconate reduces proliferation and migration of fibroblast-like synoviocytes and ameliorates arthritis models

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CONFLICT OF INTEREST

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Abstract

Fibroblast-like synoviocytes (FLS) play critical roles in rheumatoid arthritis (RA). Itaconate (ITA), an endogenous metabolite derived from the tricarboxylic acid (TCA) cycle, has attracted attention because of its anti-inflammatory, antiviral, and antimicrobial effects. This study evaluated the effect of ITA on FLS and its potential to treat RA. ITA significantly decreased FLS proliferation and migration in vitro, as well as mitochondrial oxidative phosphorylation and glycolysis measured by an extracellular flux analyzer. ITA accumulates metabolites including succinate and citrate in the TCA cycle. In rats with type II collagen-induced arthritis (CIA), intra-articular injection of ITA reduced arthritis and bone erosion. *Irg1*-deficient mice lacking the ability to produce ITA had more severe arthritis than control mice in the collagen antibody induced arthritis. ITA ameliorated CIA by inhibiting FLS proliferation and migration. Thus, ITA may be a novel therapeutic agent for RA.

Keywords

Rheumatoid arthritis; Fibroblast-like synoviocytes; Itaconate; Intracellular metabolism

Abbreviations

CAIA, Collagen antibody-induced arthritis; CIA, Collagen-induced arthritis; FLS, Fibroblast-like synoviocytes; ITA, Itaconate; OXIPHOS, Oxidative phosphorylation; TCA; Tricarboxylic

acid; RA, Rheumatoid arthritis

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the joints [1]. Despite advances in RA treatment and the availability of effective targeted synthetic and biological disease-modifying antirheumatic drugs over the last three decades, many patients still have difficulty to achieve remission [2]. The increased susceptibility of treated patients to opportunistic infections and the high costs of existing therapeutics are additional challenges. Therefore, new treatment options for RA are required.

Fibroblast-like synoviocytes (FLS) play a critical role in RA pathophysiology [3]. Activated RA-FLS escape the growth limitation of contact inhibition, show enhanced migration, and acquire invasive abilities, similar to tumor cells [4]. However, currently, there are no approved therapies for RA that directly target these cells. Over the past ten years, evidence has demonstrated the critical roles of metabolic pathways in regulating immune and stromal cells, including FLS, in RA [4]. Several studies have shown that RA-FLS undergo a metabolic shift that favors glycolysis and mitochondrial dysfunction [4]. These metabolic changes in RA-FLS can provide novel targets for treatment.

Itaconate (ITA) is an endogenous metabolite derived from the mitochondrial tricarboxylic acid (TCA) cycle. Recently, ITA has attracted attention because of its anti-inflammatory, antiviral, and antimicrobial properties [5]. ITA inhibits the production of proinflammatory cytokines, including interleukin (IL)-1β and IL-6, through various

mechanisms in macrophages [5]. ITA exerts antimicrobial effects by directly inhibiting bacterial isocitrate lyase; further, it potently inhibits viral replication and excessive inflammatory responses to pathogenic viruses. Furthermore, ITA inhibits key glycolytic enzymes and impairs glycolysis in macrophages [5]. We have previously shown that ITA inhibits glycolysis and mitochondrial respiration in T cells [6]. Further, ITA alters methionine metabolism and modulates epigenetic changes in T cells, thereby ameliorating experimental autoimmune encephalomyelitis [6]. However, the effects of ITA on RA-FLS remain to be elucidated. In this study, we aimed to identify the effect of ITA on FLS and its potential to treat RA.

2. Materials and Methods

2.1 Preparation of synovial tissue and FLS

Synovial tissue samples were obtained from patients who underwent synovectomy or joint replacement surgery. All patients provided written informed consent prior to participation. Patient characteristics and clinical features are summarized in Supplementary Table 1. This study was approved by the Human Ethics Committee of Hokkaido University Hospital (reference number: 021-0070). All patients with RA fulfilled the American College of Rheumatology/European League Against Rheumatism 2010 classification criteria for RA [7]. Synovial tissues were harvested and collected in phosphate-buffered saline (PBS). FLS were

isolated by enzymatic digestion of synovial tissue, as described previously [8]. FLS from passages 4–8 were used in this study. The cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Sigma-Aldrich).

2.2 Proinflammatory cytokine stimulation of FLS and cell proliferation assay

Because of its major involvement in RA pathogenesis, the cytokine tumor necrosis factor α (TNFα) (Sigma Aldrich) was selected for treating FLS [9]. Based on previous studies [8], TNFα was added at a dose of 100 ng/ml. TNFα-treated FLS with or without ITA were evaluated using the tetrazolium/formazan and bromodeoxyuridine (BrdU) assays to assess the cell proliferation rate. Further information is provided in the Supplementary Methods.

2.3. Scratch assay

FLS were seeded at 2.0×10^4 cells/well in 24-well plates. After 24 h, 100 ng/ml TNFα (Sigma Aldrich) was added to the culture medium, incubated for 24 h at 37 ˚C, ollowed by addition of 6 mM ITA or PBS to the culture medium. After 24 h of incubation, the monolayers were scratched with a sterile 200 μl pipette tip to create a wound. After 18 h of incubation at 37 ˚C, the migration distance was photographed using a microscope (KEYENCE) at the indicated time points. The migration rate was calculated by measuring the distance between the wound

fronts after scratching as follows: rate of migration in % = [distance moved (migrating cell front)/total distance (wound margin)] × 100 [10].

2.4 Collagen-induced arthritis (CIA) in rats

Using the disease model developed by Earp et al. [8], CIA was induced in eight female LEW/CrlCrlj rats by immunizing each animal at the base of the tail with 200 μl of 1 mg/ml porcine type II collagen (Chondrex) dissolved in 0.05M acetic acid and emulsified 1:1 in Freund's incomplete adjuvant (Chondrex). After seven days, the animals received a booster injection of collagen antigen emulsified in Freund's incomplete adjuvant. All animal experiments were approved by the Animal Ethics Committee at Hokkaido University (reference number: 19-0039). On days 14 and 21, four rats received an intra-articular injection, into both the right and left ankle joints, of a 50 μl solution containing 6.6 mM ITA; the other four rats received PBS. Each day until day 23, rats were examined for visual signs of disease, defined as macroscopic evidence of increased paw thickness and ankle diameter. Arthritis was scored from 0 to 4 as follows: 0, normal; 1, mild, but definite redness and swelling limited to individual digits, regardless of the number of affected digits; 2, moderate redness and swelling of the ankle; 3, redness and swelling of the entire paw, including digits; 4, maximally inflamed limb with involvement of multiple joints [8]. The accuracy of the scoring system was verified using micro-computed tomography (micro-CT) coupled with histological analysis of the

representative arthritic limbs. Radiographic severity in the CIA rats was assessed in a blinded manner on day 28. Micro-CT of the ankles was performed for all animals. The treated rats were given a score of 0 to 3 for each ankle based on the extent of soft tissue swelling, joint space narrowing, bone destruction, and periosteal new bone formation (0, normal; 1+, soft tissue swelling only; 2+, soft tissue swelling and early erosion; 3+, severe erosion) [8].

2.5 Irg1-deficient mice

C57BL/6J (B6) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). *Irg1*-deficient mice (B6-Acod1em1(IMPC)J/J) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were bred in-house, maintained in temperature- and humidity-controlled facilities under pathogen-free conditions at Hokkaido University, and group-housed with free access to food and water under 12-h light/dark cycles. Male and female mice were used with appropriate age-matched controls at 6–10 weeks of age.

2.6 Collagen antibody-induced arthritis (CAIA) in Irg1-deficient mice

CAIA was induced in 13 C57BL/6J and 13 *Irg1*-deficient mice by intraperitoneal (i.p.) injection of a 1.5 mg anti-Collagen II arthritogenic monoclonal antibody cocktail (Chondrex) on day 0, followed by 50 μg lipopolysaccharide (LPS) (Chondrex) i.p. injection on day 3 [11]. Each day until day 16, mice were examined for visual signs of disease, defined as macroscopic

evidence of increased paw thickness and ankle diameter. Arthritis was scored from 0 (no erythema and swelling) to 4 (feet and digits or ankylosis of the limb) as in CIA rats [8]. Radiographic severity in mice with CAIA was assessed in a blinded manner on day 16. Micro-CT of the ankles was performed for all animals. The treated mice were assigned a score of 0 (normal joint) to 3 (severe cartilage and bone erosion) for each ankle as in CIA rats [8].

2.7 Extracellular flux analysis

A Seahorse XFp Extracellular Flux Analyzer (Agilent Technologies) was used to measure extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) [12]. FLS were cultured in an XFp microplate at a density of 6.0×10^3 cells. After 24 h, FLS were stimulated with 100 ng/ml TNFα for 24 h and treated with 6 mM ITA or PBS for 24 h. A Glycolytic Rate Assay (Agilent Technologies) was performed to measure the ECAR, followed by sequential treatments with rotenone/antimycin A (Rot/AA: 0.5 μM) and 2-deoxyglucose (50 μM). A Cell Mito Stress test (Agilent Technologies) was performed to measure the OCR, followed by sequential treatments with oligomycin (1.5 μM), FCCP (0.5 μM), and Rot/AA (0.5 μM). The obtained data were analyzed using Seahorse Wave v 2.6.0 (Agilent Technologies).

2.8 Profiling of intracellular metabolites

Intracellular metabolites were extracted from 1.0×10^6 cells per sample. Cells were collected

by centrifugation (800 × *g* and 4 ℃ for 2 min), washed with 10 ml 5% mannitol solution, and then treated with 800 µl methanol and vortexed for 30 s to inactivate the enzymes. Next, the cell extract was treated with 550 µl Milli-Q water containing internal standards (Human Metabolome Technologies) and allowed to rest for another 30 s. The extract was obtained and centrifuged at 2300 × *g* and 4 ℃ for 5 min. Then, 800 µl of the upper aqueous layer was centrifugally filtered through a Milli-pore 5-kDa cutoff filter at 9100 × *g* and 4 ℃ for 120 min to remove the proteins. The filtrate was centrifugally concentrated and resuspended in 50 µl Milli-Q water for capillary electrophoresis time-of-flight mass spectrometry (CE-TOF MS) analysis [12]. The samples were measured and analyzed by Human Metabolome Technologies.

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Statistical significance was determined by t tests (two tailed) for two groups or one-way ANOVA with Bonferroni's multiple comparisons tests for three or more groups. Data are expressed as the mean ± SD or SEM. Parametric analysis was used to analyze data with a normal distribution, and nonparametric analysis was used to analyze data with a non-normal distribution.

3. RESULTS

3.1 The effect of ITA on RA-FLS proliferation and migration in vitro

To confirm the effect of ITA on RA-FLS isolated from the synovial tissue of patients with RA, their proliferation potency was evaluated. As previously reported, TNFα-stimulated FLS proliferated faster than untreated FLS (Fig 1A). ITA significantly reduced the proliferation of TNFα-stimulated FLS evaluated using tetrazolium/formazan (Fig 1A) and BrdU assays (Fig 1B). ITA also reduced the migration of TNFα-stimulated FLS as evaluated using a scratch assay (Fig 1C and D, Supplementary Fig 1).

3.2 The effect of ITA on the metabolism of RA-FLS

We have previously reported that ITA inhibits glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) in T cells [6]. Acordingly, we evaluated its metabolic function using an extracellular flux analyzer to unveil the mechanisms underlying the inhibitory effect of ITA on FLS proliferation and migration. The glycolytic rate assay and mito stress test were performed using TNFα-stimulated FLS with or without ITA (Fig 1E and F). ITA significantly inhibited basal and compensatory glycolysis (Fig 1E). ITA also decreased the maximal OCR and spare respiratory capacity (Fig 1F). Overall, these results indicate that ITA inhibits glycolysis and mitochondrial OXPHOS in RA-FLS.

3.3 Metabolic reprogramming in ITA-treated FLS

To further explore the metabolic profile, we performed metabolomic analysis using CE-TOF

MS to evaluate the intracellular metabolites in ITA-treated FLS (Supplementary Fig 2). Previous studies have reported that ITA inhibits the enzymatic activity of succinate dehydrogenase (SDH) in macrophages [13]; consistently, we found accumulation of succinate due to SDH inhibition (Fig 2E). Citrate also accumulated significantly, and other intermediate products of the TCA cycle tended to be similar.

3.4 CAIA in Irg1-deficient mice

Next, we established the CAIA model in *Irg1*-deficient mice, lacking the ability to induce ITA from cis-aconitate, to evaluate the effects of endogenous ITA on arthritis in vivo. The clinical arthritis score was significantly lower in *Irg1*-deficient mice than in C57BL/6J mice (Fig 2A). Moreover, micro-CT analysis revealed that the erosion score was significantly higher in *Irg1*-deficient mice than in the wild-type mice (Fig 2B and C).

3.5 The effect of intra-articular ITA injection on CIA rats

To investigate the potential of ITA as a candidate treatment for RA, we used CIA rats. CIA was induced in rats, and an intra-articular injection of ITA or PBS into both ankles was administered on days 14 and 21. The intra-articular injection of ITA significantly improved the clinical arthritis scores of CIA rats (Fig 2D). Moreover, micro-CT revealed that the arthritic rats injected with ITA had significantly lower erosion scores than those of the rats injected

with PBS (Fig 2E and F).

4. DISCUSSION

In this study, we showed that ITA inhibited the proliferation and migration of FLS. Secondary, ITA inhibited glycolysis and mitochondrial OXPHOS in RA-FLS. We finally revealed that ITA ameliorated CIA in rats. To the best of our knowledge, this is the first report to demonstrate the effect of ITA on RA-FLS.

In RA, FLS acquire an aggressive pathogenic phenotype with the ability to migrate, adhere, and invade articular cartilage, leading to the induction of bone resorption [4]. This destructive phenotype of RA-FLS is associated with a shift in their cellular metabolic profile, with changes in glycolysis, the pentose phosphate pathway, oxidative phosphorylation, and amino acid metabolism compared to that in osteoarthritis-associated synovial fibroblasts [4]. In macrophages, the endogenous metabolite ITA inhibits the TCA cycle enzyme SDH and blocks reactive oxygen species production from complex I, leading to the inhibition of hypoxia-inducible factor-1α activity and IL-1β production [13]. ITA has also been reported to activate nuclear factor erythroid 2-related factor 2, leading to a boost in the antioxidant response and driving the activation of transcription factor 3 expression, consequently limitting the production of cytokines, such as IL-6 [13]. Increased ITA in the plasma of patients with RA correlates with improved DAS44 scores and decreased C-reactive protein levels [5].

Previously, we showed that ITA inhibited glycolysis and mitochondrial respiration in T cells, ameliorating experimental autoimmune encephalomyelitis [6]. However, the effects of ITA on FLS remain to be elucidated. In this study, we showed that ITA suppressed glycolysis and mitochondrial respiration in RA-FLS as well as in T cells. In RA-FLS, glucose deficiency inhibits proliferative and invasive function [4]. Blockade of glycolysis by inhibition of the enzyme hexokinase with 2-deoxyglucose reduced the secretion of RA biomarkers, MMP1, MMP3, and MCP-3 [14]. In addition, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), a glycolytic enzyme, is upregulated in RA-FLS, and its inhibiton reduces RA-FLS invasion, migration, and proinflammatory cytokine production [15]. In macrophages or T cells, ITA inhibits glycolysis by suppressing various enzymes, including aldolase A and glyceraldehyde-3-phosphate dehydrogenase. ITA also inhibits fructose-6-phophate 2-kinase, a compornent of PFKFB3, and decreases fructose-2,6-biphosphate which contributes to the inhibition of glycolysis in LPS-activated macrophages [16]. Therefore, ITA may suppress glycolysis in RA-FLS by a similar mechanism. Dimethyl fumarate inhibits SDH and decreases FLS proliferation [17]. Our data indicated that ITA suppressed FLS proliferation by inhibiting SDH. *Irg1*-deficient mice, in the absence of the ITA productivility, showed exacerbated CAIA. We also demonstrated that intra-articular injection of ITA ameliorated arthritis in CIA rats. Dimethyl malonate, the derivatives of ITA, ameliorated LPS-induced sepsis mouse model and an ulcerative colitis mouse model [5]. It has also been reported that *Irg1*-deficient mice

increased inflammatory cytokine and chemokine levels, resulting in severe dextran sulfate sodium-induced colitis in mice [5]. ITA exerts an antimicrobial effect via direct inhibition of bacterial isocitrate lyase. Recently, a breakdown product of ITA was also shown to limit the growth of *Mycobacterium tuberculosis* and to suppress SARS-CoV-2 replication. Therefore, ITA could be considered a new antirheumatic drug that targets FLS and along with exerting antimicrobial effects [5].

This study had several limitations. ITA could also affect other immune cells in vivo. Further research is thus needed to confirm the mechanism of ITA action in FLS. In addition, the mechanism by which ITA alters enzyme activity should be assessed in detail.

In summary, we demonstrated that ITA reduced the proliferation and migration of FLS in vitro, and diminished arthritis and joint destruction in a rat disease model. Thus, ITA is an attractive treatment option with a low risk of infection in patients with RA.

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Figure Legends and Tables

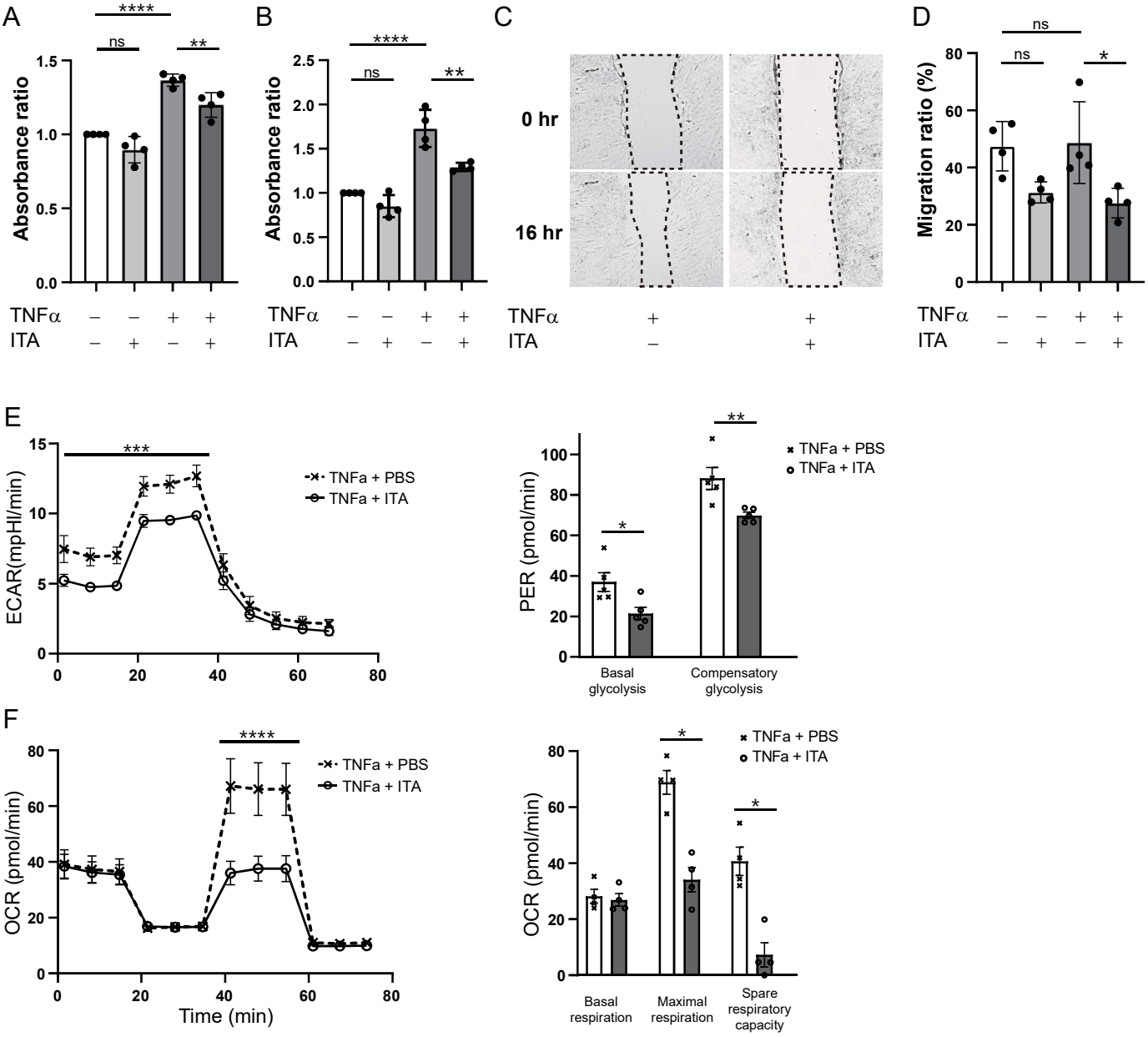
Fig 1. The effect of ITA on the proliferation, migration, and intracellular metabolism of FLS

FLS (n = 4) were stimulated with or without TNFα for 24 h and treated with ITA or PBS for 48 h. Cell proliferation rate of FLS measured using tetrazolium/formazan (A) or BrdU assays (B). Migration ratio(%) measured using a scratch assay (C and D). ECAR of TNFα-stimulated FLS (n = 5) with ITA or PBS measured using a glycolytic rate assay (E). Basal glycolysis and compensatory glycolysis calculated based on the ECAR (E). Summarized curves of OCR for TNFα-stimulated FLS (n = 4) with ITA or PBS measured using a mito stress test (F). Baseline respiration, maximal respiration, and respiratory spare capacity calculated based on OCR (F). Abbreviations: ITA, itaconate; FLS, fibroblast-like synoviocytes; TNFα, tumor necrosis factor alpha; ECAR, Extracellular acidification rate; PER, Proton efflux rate; OCR, oxygen consumption rate; TCA, tricarboxylic acid. In A, B, D, E, and F symbols represent individual samples; bars indicate the mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, using Student's t-test for single comparisons or one-way analysis of variance (ANOVA) test for multiple comparisons with 95% confidence intervals.

Fig 2. CAIA in *Irg1*-deficient mice and the effect of ITA intra-articular injection on CIA in rats.

CAIA was induced in C57BL/6J mice (n = 13) and *Irg1*-dificient mice (n = 13) by intraperitoneal injection (i.p.) of an anti-collagen II arthritogenic monoclonal antibody cocktail on day 0, followed by LPS i.p. injection on day 3. Arthritis scores were monitored for 16 days (A). Representative micro-CT images of arthritic mice (B). Bone erosion scores calculated from the micro-CT images (C). CIA was induced in female LEW/CrlCrlj rats (n = 8 rats), and intra-articular injection of ITA or PBS into both ankles (n = 8 legs per group) was performed on days 14 and 21. Arthritis scores were monitored for 23 days (D). Representative micro-CT images of arthritic rats treated with PBS or ITA (E). Bone erosion scores calculated from micro-CT images (8 legs per group) (F).

Abbreviations: CAIA, Collagen antibody-induced arthritis; ITA, itaconate; CIA, Collagen-induced arthritis; LPS, Lipopolysaccharide; micro-CT, microcomputed tomography; Ti, Tibia; Ta, Talus. In A, C, E, and G, symbols represent individual samples; bars represent the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001, by Student's t-test for single comparisons and two-way analysis of variance (ANOVA) test for multiple comparisons with 95% confidence interval.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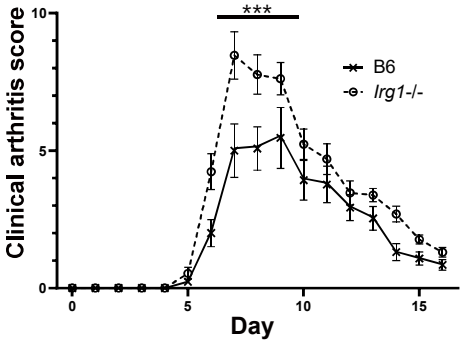
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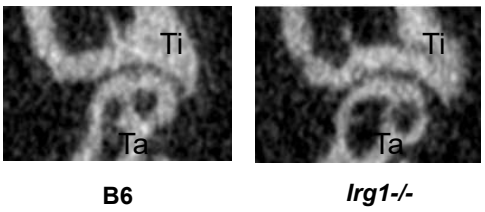
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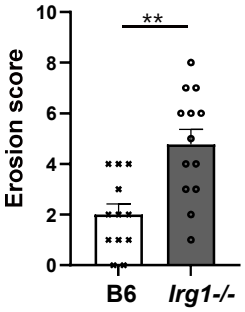
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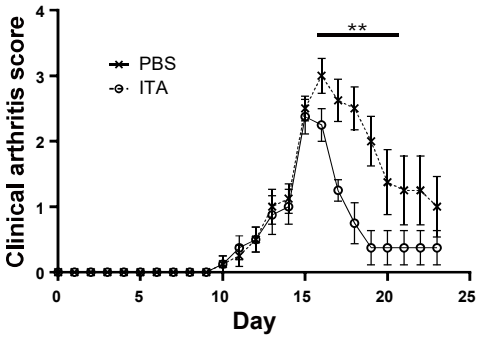
Erosion score
B6
Irg1-/-

D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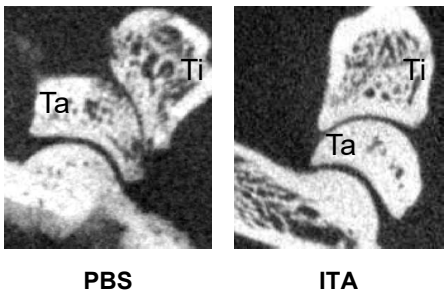
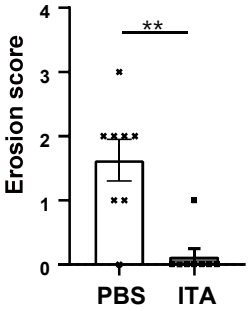
PBS
ITA
Clinical arthritis score
Day

E


Ti
Ta
Ti
Ta
PBS
ITA

F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Erosion score
PBS
ITA