

STAT1 itaconation prevents macrophage cytotoxic mtDNA -induced inflammation in Wear Particle-Induced Aseptic Loosening

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Research Article

Keywords: aseptic loosening, osteolysis, macrophage inflammatory, IRG1, 4-OI, itaconylation

Posted Date: September 12th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7476581/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Cell Communication and Signaling on April 16th, 2026. See the published version at <https://doi.org/10.1186/s12964-026-02874-4>.

Abstract

Prosthetic wear particle-driven macrophage inflammation severely limits the long-term efficacy of total joint replacements through aseptic loosening. However, the specific mechanisms by which wear particles induce macrophage inflammation remain incompletely elucidated. Itaconic acid produced by the Krebs cycle is markedly up-regulated in TiPs-stimulated macrophages, which may modulate mitochondrial metabolism via itaconation or competitive inhibition of specific proteins. Here, using the 4-octyl itaconate (4-OI, a derivative of itaconic acid), we demonstrate that itaconate functions as an endogenous metabolic regulator that suppresses succinate dehydrogenase (SDH) activity, thereby significantly inhibiting STING pathway activation. Moreover, 4-OI can alkylate STAT1, preventing its phosphorylation and relieving transcriptional repression of the mitochondrial transcription factor TFAM; which stabilizes mitochondrial homeostasis and attenuates macrophage inflammation. In a murine calvarial osteolysis model, 4-OI reversed bone destruction induced by TiPs and TFAM knockdown. Collectively, our findings establish itaconate as a critical endogenous metabolite that alleviates wear particle-mediated inflammation and osteolysis by reprogramming macrophage metabolism.

Introduction

Arthroplasty is the most effective method for treating end-stage bone and joint diseases, with over 5 million prostheses implanted globally each year[1]. However, studies report that aseptic loosening—now the leading cause of joint revision—significantly limits long-term success[2]. Mechanistically speaking, prosthesis wear particles can activate macrophages, trigger inflammatory responses, and release cytokines (e.g., TNF- α , IL-6)[3, 4], which further activate osteoclasts to promote bone resorption[5], ultimately leading to the development of this disease. Therefore, in-depth investigation of wear particle-induced macrophage inflammation and identification of the key factors involved will offer a novel therapeutic strategy for preventing the progression of aseptic loosening and prolong the lifespan of prostheses.

Mitochondria have long been recognized as cellular powerhouses, where metabolites are oxidized through the TCA cycle and subsequently generate ATP via the electron transport chain[6]. However, recent studies reveal that mitochondrial metabolic reprogramming upon stimulation regulates macrophage immunophenotypes, with mitochondrial products such as mtROS and mtDNA serving as key indicators of macrophage polarization and activation[7]. Specifically, leakage of mitochondrial DNA (mtDNA) activates the cytosolic cGAS-STING signaling pathway, perpetuating a self-amplifying inflammatory cascade[8]. Succinate dehydrogenase (SDH), as an indispensable key enzyme in the TCA cycle, can catalyze the conversion of succinic to fumaric to participate in the electron respiratory

chain transport process.[9]. Previous studies have shown that SDH modulates mtDNA release via ROS production, indirectly regulating STING/TBK1-mediated inflammatory factor expression[10]. Simultaneously, SDH controls macrophage M1 polarization by altering succinate levels in the TCA cycle[11]. These findings collectively position SDH as a pivotal regulator of mitochondrial immunometabolism.

Immune responses hinge on mitochondrial metabolism. Under excessive stimulation, the compensatory increase of metabolites such as itaconate is a typical manifestation [12, 13]. Immune-responsive gene 1 (IRG1) encodes cis-aconitate decarboxylase of the tricarboxylic acid cycle (TCA) in mitochondria and catalyzes the decarboxylation of cis-aconitate to itaconic acid (ITA)[14]. Emerging evidence indicates that the induction of IRG1 is reliant upon the activation of the STING signaling pathway elicited by diverse stimuli[15, 16]. Itaconate exerts multifaceted anti-inflammatory effects through covalent protein modification (termed 'itaconylation') and metabolic reprogramming[17]. Previous studies reveal structural similarity between ITA and succinate, and demonstrate ITA's high binding efficacy to the succinate catalytic site on the α -subunit of SDH via electrostatic interactions[18]. ITA inhibits the catalytic conversion of succinate to fumarate, thereby reducing ROS generation and suppressing hypoxia-inducible factor (HIF-1 α) along with its downstream pro-inflammatory cytokines such as IL-1 β [19]. Concurrently, Endogenous itaconate and 4-OI have been demonstrated to alkylate cysteine residues on a variety of proteins, include KEAP1[20], GAPDH\ [21], ALDOA[22], and NLRP3[23]. This mode of alkylation, termed itaconation constitutes an essential component of its anti-inflammatory properties—for example, itaconation of KEAP1 activates Nrf2 and thereby suppresses the STING-driven inflammatory pathway [20]. Furthermore, ITA regulates mitochondrial transcription factor A (TFAM) expression to mitigate mtDNA leakage[24]. Nevertheless, the specific role of IRG1/ITA in wear particle-mediated aseptic loosening of prosthetic joints remains unclear.

Here, we elucidate a previously unrecognized ITA-STAT1-TFAM axis that safeguards mitochondrial integrity during wear particle stimulation. Throughout this study, using 4-OI together with TiPs, we found that IRG1 up-regulation acts as a metabolic checkpoint that suppresses inflammatory osteolysis. Specifically, we observed that the IRG1 gene of macrophages was significantly upregulated after stimulation with TiPs, with altered mitochondrial metabolism. We demonstrated that ITA, encoded by IRG1, modulates macrophage metabolic reprogramming by inhibiting SDH activity, significantly reducing STING pathway activation and M1 polarization, thereby regulating TiP-induced inflammation. Furthermore, we discovered that ITA can also affect the transcriptional expression of TFAM by alkylating STAT1, stabilizing mtDNA leakage. This metabolic-immune crosstalk disrupts the STING-NF- κ B amplification loop, providing a unified explanation for itaconate's dual anti-inflammatory and mitochondrial protective effects. Additionally, animal experiments have shown that 4-OI targeted injection can alleviate the

osteolysis and inflammation of the mouse skull caused by TiPs and targeted knockdown of TFAM. In conclusion, our research results indicate that IRG1/ITA may be a potential target for preventing periprosthetic AL.

Materials and methods

1.Clinical Specimen. The synovial tissue samples were taken from the Department of Orthopedics, Sun Yat-sen Memorial Hospital, Sun Yat-sen University. The interfacial membranes around the hip joint prosthesis were collected from 6 patients undergoing revision surgery for aseptic loosening of the hip joint prosthesis, and the synovial tissues of the hip joint were collected from 6 patients with femoral head necrosis (FHN) undergoing primary total hip arthroplasty (THA). This study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University (SYSKY-2025-566-01), and all experiments were conducted with the consent of the patients.

2.Titanium particles. Sterile TiPs were prepared as previously described in our study. Titanium particle (TiP) solutions were purchased from Alfa Aesar (<20 μ m, W08A030) and further diluted with sterile water. Titanium particles with a size of 1.2 to 10 μ m were filtered using Isopore membranes (pore sizes of 10 μ m/1.2 μ m, TCTP04700/RTTP04700, Millipore). Subsequently, the particles were sterilized by high-pressure steam sterilization to eliminate endotoxins and other pathogenic microorganisms. Then the particles were weighed and diluted to 1 mg/ml with PBS (YJ0014, YONG JIN).

3.Cell and Culture. RAW264.7 cells (CL-0190, Procell) were routinely cultured in the special medium for RAW264.7 cells (TCM-G766, HyCyte). 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution had been pre-added. The culture conditions were 37 ° C and 5% CO₂. Before stimulation, inoculate the cells onto the plate for 24 hours until the fusion reaches 70-80%.

4.RNA Sequencing. After 6 hours of TiPs stimulation, macrophages were lysed with TRIzol™ reagent (15596018,Invitrogen). The collected total RNA was removed with rRNA to retain mRNA and ncRNA for constructing specific libraries and sequencing. After reverse transcription and PCR amplification detection of the processed RNA solution, sequencing was performed using Illumina HiSeq 4000. Remove the original readings that contain adapters or are of low quality (Q value $\leq$ 20). The Reads were mapped to the mouse genome database (GRCm38), and differentially expressed genes (DEGs) were defined as FDR<0.05 and log₂FC >1.

5.Lentiviruses and Stable Cell Lines' Establishment. Mouse TFAM overexpressing lentivirus (oe-TFAM) and low-expressing lentivirus (sh-TFAM/sh-STAT1) were designed and synthesized by GenePharma (Shanghai, China). The vector (Mock) and scramble sequence (sh-NC) were used as the control groups for overexpression and knockdown.

RAW264.7 24-well plates were spread at a rate of 1×10^5 cells per well. Then, a medium containing 8 $\mu\text{g/mL}$ Polybrene of lentivirus was added. After 24 hours, the medium was changed. After 48 hours, the positive rate of GFP was preliminarily observed by fluorescence microscopy ($> 70\%$ is preferred). Continuous screening with 2-5 $\mu\text{g/mL}$ puromycin for 7 days; During this period, the liquid should be changed every 2 to 3 days to retain the resistant clones. The expression of the target gene was verified by PCR and western blot (WB), and the most effective knockdown lentivirus was selected for further experiments.

6. Real-Time qPCR. Total RNA of cells was extracted using the EZ-press RNA purification Kit (B0004D, EZBioscience), and RNA concentration was determined using the NanoDrop instrument (ND2000, Thermo Fisher Scientific). According to the determined concentration, the RNA solution was mixed in proportion with the Color reverse transcription Kit (A0010CGQ, EZBioscience), and then reverse transcribed using a thermal cyclator (ETC811, EASTWIN) to obtain the corresponding cDNA solution. Subsequently, the cDNA solution was proportionally mixed with the Color qPCR Kit (A0012-R2, EZBioscience), specific primers and RNase-Free Water, and real-time qPCR was performed on the real-time PCR instrument (CFX Connect, Bio-Rad). The gene expression was normalized according to GAPDH, and the quantitative method was $2^{-\Delta\Delta C_t}$. The provided primer sequences were as follows:

TNF- α : 5'-CAGGCGGTGCCTATGTCTC-3' (forward)

5'-CGATCACCCCGAAGTTCAGTAG-3' (reverse);

IL-6: 5'-CTGCAAGAGACTTCCATCCAG-3' (forward)

5'-CTGCAAGAGACTTCCATCCAG-3' (reverse);

P65: 5'-GGCCTCATCCACATGAACTT-3' (forward)

5'-CACTGTCACCTGGAAGCAGA-3' (reverse);

IRF3: 5'-GATGGAGAGGTCCACAAGGA-3' (forward)

5'-GAGTGTAGCGTGGGGAGTGT-3' (reverse);

STING: 5'-TCGCACGAACTTGGACTACTG-3' (forward)

5'-CCAACTGAGGTATATGTCAGCAG-3' (reverse);

TBK1: 5'-CCAACTGAGGTATATGTCAGCAG-3' (forward)

5'-ATGGTAGAATGTCACTCCAACAC-3' (reverse);

STAT1 5'-GAAAAGCAAGCGTAATCTCC -3' (forward)

5'- GGTCTCTGCAACAATGGTGA-3' (reverse);

TFAM: 5'-TGATTCACCGCAGGAAAAGC -3' (forward)

5'- TTGTGCGACGTAGAAGATCC-3' (reverse);

IRG1: 5'-CGCGCATGTTTGGAGAAGTT -3' (forward)

5'-TGCTTTGTCAAGACCAATTCCC-3' (reverse);

GAPDH: 5'-TGACCTCAACTACATGGTCTACA -3' (forward)

5'-CTTCCCATTCTCGGCCTTG-3' (reverse);

7.Detection of mtDNA Levels. RAW264.7 cells were inoculated in 12-well plates to detect the presence of mtDNA in the cytoplasm. After the cell fusion exceeded 80%, the adherent cells were pipeted with PBS, and the cells resuspended in PBS were centrifuged at 1000 rpm for 5 minutes. Discard the supernatant, resuspend the cells in 100µL of PBS, and then extract DNA using the TIANamp Genomic DNA Kit (DP304-02, TIANGEN). The level of mtDNA was detected by RT-qPCR. The provided primer sequences were as follows:

D-LOOP: 5'- AATCTACCATCCTCCGTGAAACC -3' (forward)

5'- TCAGTTTAGCTACCCCCAAGTTTAA-3' (reverse);

ND1: 5'- CGGGCTACTACAACCCTTCG -3' (forward)

5'- GCGATGGTGAGAGCTAAGGT-3' (reverse);

ND4: 5'- CAGCCACATAGCCCTCGTAG-3' (forward)

5'- CCCGTGGGCGATTATGAGAA-3' (reverse);

8.Enzyme-Linked Immunosorbent Assay (ELISA). Following the stimulation of ASC-transfected RAW 264.7 cells with TiPs for 4 hours, the cell culture supernatant was collected. The concentrations of TNF-α and IL-1β were measured using mouse-specific enzyme-linked immunosorbent assay kits (E-EL-M3063/ E-EL-M0037 -48T, Elabscience). Subsequently, optical density was measured using a microplate reader (Synergy H1, Biotek) at the appropriate wavelength, and the resulting data were analyzed accordingly.

9.Western Blot. RAW264.7 was seeded in 6-well plates and cultured until the cell density was 80%. Specific doses of the protease inhibitor mixture (CW2200S, CW Biotech) and the phosphatase inhibitor mixture (CW2383S, CW Biotech) were prepared in Strong RIPA lysis

buffer (cw2333s,CW Biotech) for cell lysis. After collecting the lysate, centrifuge at 12,000 g for 30 minutes, collect the supernatant, and determine the protein concentration by the bisglobulin acid (BCA) method (p0011,Beyotime). After incubating with SDS loading buffer (CW0027S,CWBiotech) at 95°C for 5 minutes, the protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Transfer the isolated protein samples on the gel to a PVDF (IPVH00010, Merck Millipore) membrane with a pore size of 0.45 mm in an ice bath and incubate for 30 minutes with protein-free rapid blocking solution (G2052-500 ml, Servicebio). Subsequently, the primary antibody was incubated at room temperature for 2 hours or overnight at 4 °C. After three TBST membrane washes, the secondary antibody was incubated at room temperature for 1 hour. Protein bands were visualized using a chemiluminescent substrate (WBKLS 0100, Merck millipore), and immunoblot results were obtained using a digital imaging system (Syngen G: BOX Chmi XT4). The quantification of blot intensity was conducted using ImageJ software. The antibodies used are as follows: TNF- α (R1203-1,HUABIO), IL-6 (ab9324, Abcam), GAPDH(ET1601-4, HUABIO), STING(13647, CellSignaling), TBK1(ab40676, Abcam), IRF3(ET1612-14, HUABIO), P-STING(AF7416, AFFINITY), p-TBK1(5483, Cell Signaling), P-IRF3(AF2436, AFFINITY), IRG1(HA722819,HUABIO), TFAM(BS61387,Bioworld), HIF1- α (HA721997,HUABIO), Stat1(14994,Cell Signaling), β -tubulin (ET1602-4, HUABIO), HRP conjugated alpaca anti-rabbit IgG FC, Recombinant VHH antibody(HA1031, HUABIO), 488 conjugated goat anti-rabbit IgG antibody(HA1121, HUABIO), 594 conjugated goat anti-rabbit IgG antibody(HA1122, HUABIO), Anti-mouse IgG (H+L), F(ab')₂ Fragment (Alexa Fluor® 647 Conjugate) (4410S, Cell Signaling).

10. CHIP-qPCR. Chromatin immunoprecipitation was performed using a ChIP kit (P2080S, Beyotime). After collecting the cells, formaldehyde cross-linking was used to stabilize the interactions between proteins and DNA within the chromatin. Subsequently, ultrasonication was employed to lyse the cells, shearing the genomic DNA into fragments of 200-1000 bp in size. Specific antibodies against STAT1 (14994, Cell Signaling) were then used to bind the target protein. Washing steps were conducted to remove non-specifically bound proteins and other contaminants, and the cross-links were reversed to dissociate the DNA fragments. Finally, the DNA was purified and analyzed by qPCR to measure TFAM binding.

11. Flow cytometry. RAW264.7 cells were seeded in six-well plates until the cell density reached 80%. Cells were pipeted off with PBS and fixed with 4% paraformaldehyde common tissue fixative (YJ0002, YONG JIN) for 15 minutes. After centrifuging at 1000rpm to remove the supernatant, fix with 0.1% Triton X-100 for 15 minutes until the cell membrane is permeable. Subsequently, add the PE-Cy7-conjugated iNOS antibody (25-5920-82,Thermo Fisher Scientific) and avoid light Incubate at 20°C for 30 minutes. After centrifugation, remove the supernatant and resuspend the cells with PBS. Flow cytometry

was performed on RAW264.7 cells suspended in PBS using BD FACSVerser (BD FACSVerser, BD Biosciences), and data were processed using FlowJo (version: 10.6.2).

12. Immunofluorescence (IF) Staining. After seeding RAW264.7 cells into confocal dishes (BDD011035, Jet bifiltration Co), when the cell density reached 50%, the cells were stimulated with TiPs for 2 hours. After fixation in 4% paraformaldehyde (PFA) for 15 minutes, incubate in a shaker at room temperature with 0.1% Triton X-100 for 15 minutes. After washing with PBS, add goat serum for blocking (ZLI-9056, ZS) to block the cells for 30 minutes, and incubate overnight with the primary antibody at 4 ° C. After thorough washing with PBS, add the secondary antibody at room temperature for 1 hour. Finally, nuclear counterstaining was performed for 5 minutes using DAPI (C0065, Solarbio) staining. After removing DAPI, an anti-fluorescence quench (S2110-25, Solarbio) was added and observed under a confocal microscope (FV3000, Olympus).

The sectioned tissues were first heated in a constant temperature box at 37°C for 1 hour. Then it was transferred to a constant temperature box at 60°C and heated for another hour. The sections were washed twice in sequence with xylene and anhydrous ethanol for 10 minutes each time, and then with 90% ethanol, 80% ethanol, 70% ethanol, 60% ethanol and PBS for 3-5 minutes. Next, use an immunohistochemical pen (BC004, Biosharp) to circle the tissue on the section, and cover the tissue with pepsin (G0142, servicebio). Then place these sections back in a 37°C incubator for antigen remediation for 30 minutes. Wash the sections three times with PBS and then block them at room temperature with goat serum (SL038, Solarbio) for 1 hour. Subsequently, the primary antibody was prepared and incubated overnight at 4 ° C . The secondary antibody was prepared, washed with PBS and incubated at room temperature in a dark room for 1 hour. Finally, DAPI and anti-fluorescence quencher (S2110-25, Solarbio) were added, and the sections were observed under an inverted fluorescence microscope (Olympus IX73).

13.Click chemistry. RAW264.7 cells were seeded in 6-well plates. After the cell density grew to 80%, they were incubated with ITalk (HY-133870, MCE) (100μM) for 12 hours. Protease inhibitors cocktail (B14001, Selleck) and phosphatase inhibitors cocktail (CW2383S, CW Biotech) were added to IP lysis buffer (P0013J, Beyotime) to lyse the cells for 20 minutes. Then, the cells were disrupted in an ice bath with ultrasonic waves, and the supernatant was collected at 4 °C(16000 g, 10 minutes) to remove cell debris. The protein concentration was determined by the BCA method and proportioned to 2mg /mL. Subsequently, the cell lysate was reacted with 1mm CuSO₄ (451657-10G, Sigma-Aldrich), 100 mm TBTA ligand (HY-116677, MCE), 100 mm azide biotin (762024-10MG, Sigma-Aldrich) and 1mm TCEP (ST045-5g, Beyotime) on a room-temperature rotator for 1 hour. Centrifuge at 4000×g for 5 minutes to precipitate the protein. After discarding the supernatant, wash the protein twice with cold methanol and dissolve the protein precipitate in 1.2% SDS solution using ultrasound. Heat the sample at 95°C for 5 minutes,

retain 50µl of the sample solution as input, and dilute the remaining part with PBS (0.2%SDS) to a final volume of 1.5 mL. Then the final sample was incubated with 40 µl streptavidin Magnetic beads (HY-K0208, MCE) on a rotator for 6 h at 4°C. Add 1 ml of washing buffer (PBS, 0.05% tween-20) to the EP tube, close the tube cap, vortex the beads for 15 seconds, separate on a magnet and discard the supernatant. Repeat this wash four times. Add 50µL of 1×SDS-PAGE Loading Buffer to the magnetic beads, heat at 95 °C for 5 minutes for elution, then place the beads on a magnetic rack to separate them, and collect the supernatant for subsequent analysis.

14. In Vivo Experiments. The animals used in this research were sourced from the Animal Experiment Center of Sun Yat-sen University (Approval Number: SYSU-IACUC-2023-001033). Under the guiding principles of the Animal Ethics and Welfare Committee of Sun Yat-sen University, this experiment selected 10-week-old C57BL/6J male mice for animal experiments. Throughout the entire surgical procedure, every step was carried out strictly in accordance with the pre-set standards for animal surgery and welfare. The method for establishing a skull osteolysis model was described in our previous research. Specifically, we randomly divided 36 mice into 6 groups. The following are the grouping situations. SHAM group: 100 µL of PBS, TiPs group: 100 µL of PBS containing 5 mg of TiPs, 4-OI group: 100 µL of PBS containing 200 mM 4-OI, 4-OI & TiPs group: 100 µL of PBS containing 200 mM 4-OI and 5 mg of TiPs, TiPs & oe-TFAM group: 100 µL of PBS containing 5 mg of TiPs with 0.5×10⁶ oe-TFAM stably-knockdown RAW264.7, TiPs & sh-TFAM group: 100 µL of PBS containing 5 mg of TiPs with 0.5×10⁶ sh-TFAM stably-knockdown RAW264.7, TiPs & sh-TFAM & 4-OI group: 100 µL of PBS containing 5 mg of TiPs with 0.5×10⁶ sh-TFAM stably-knockdown RAW264.7 and 200 mM 4-OI. A sagittal incision about 15mm long was made along the midline of the skull, and gelatin sponges (0.5*0.5*0.3 cm³) were implanted for localized injections according to group assignments. No complications or mortalities occurred during the 14-day postoperative period. The mice were sacrificed under anesthesia, their skulls were isolated, all soft tissues were removed for subsequent analysis. Data analysis was conducted on the skulls of mice in each group, including bone mineral density (BMD), bone volume (BV), and total volume (TV). After micro-CT scanning, the skull was decalcified, dehydrated and embedded for histological analysis. Detect the levels of IL-6 and TNF-α to assess the inflammatory response.

15 Statistics. Experimental data were analyzed using GraphPad Prism (version 9.0) and displayed in the form of mean±SEM. All values were assessed for normality using the Kolmogorov-Smirnov test. Comparisons between two groups were performed using the T-test, while comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered statistically significant. All experiments were repeated at least three times to ensure reliability.

Results

1. Inflammatory responses were detected in AL synovium and in TiP-stimulated macrophages.

To verify the association between AL (aseptic loosening) and inflammation, we first collected synovial tissues from patients with AL and FHN (femoral head necrosis). We then detected TNF- α and IL-6 in the synovial tissues using immunohistochemical fluorescence (IHF). The results showed that both TNF- α and IL-6 in the AL group were significantly upregulated compared with the FHN group (Fig. 1A). Quantitative analysis of fluorescence intensity revealed that IL-6 and TNF- α were higher in the AL group (Figure S1C).

Given that TiPs can exacerbate synovial tissue inflammation, to verify the role of macrophages in wear particle-induced inflammation, we stimulated macrophages with TiPs to simulate the initiation of inflammation *in vitro*. [25]. The qPCR results showed that TiPs stimulation led to an increase in the mRNA expression of TNF- α and IL-6 with the increase of stimulation time (Fig. 1B). Meanwhile, the results of WB determination showed that the expressions of TNF- α and IL-6 increased after TiPs stimulation, especially between 4 and 6 hours (Fig. 1C). Similarly, IF staining showed that the fluorescence of TNF- α and IL-6 was stronger in the TiPs treatment group (Fig. 1D). We collected cell culture supernatants for ELISA analysis, which confirmed that the secretion levels of TNF- α and IL-1 β were higher in macrophages stimulated by TiPs (Fig. 1E). Additionally, flow cytometry analysis indicated an increased proportion of iNOS-positive cells in the TiPs-stimulated group (Fig. 1F), demonstrating a transition of macrophage phenotype to the M1 type. These findings suggest that the synovial inflammation surrounding AL prostheses is exacerbated, and that TiPs stimulation of macrophages similarly induces an inflammatory response.

2. TiPs-stimulation Macrophages initiate metabolic reprogramming to activate the STING pathway and simultaneously upregulate IRG1 expression

Next, we explored the potential mechanisms underlying TiPs activation of macrophages. We applied TiPs to stimulate macrophages and extracted protein lysates for WB determination. As expected, the phosphorylation levels of STING, TBK1 and p65 were upregulated with the extension of stimulation time (Fig. 2A), and qPCR results demonstrated that mRNA levels of STING, TBK1 and p65 target genes were up-regulated (Fig. 2B), indicating the activation of the STING-NF- κ B pathway.

Next, we performed RNA-seq using Illumina HiSeq 4000 to explore the gene expression profile of RAW264.7 macrophages stimulated by TiPs for 6 hours. We observed that under

the stimulation of TiPs, the mRNA expression of IRG1 was upregulated, while the expression of SDH-related genes decreased (Fig. 2C). Previous studies have confirmed that itaconate competitively inhibits SDH, leading to succinate accumulation[26], which blocks reverse electron transport (RET) and reduces the production of reactive oxygen species (ROS)[27]. This, in turn, decreases HIF1 α -mediated IL-1 β transcription[28] and the subsequent inflammatory cascade.

Subsequently, to investigate whether IRG1 is involved in the inflammatory response of macrophages, we conducted in vitro and in vivo experiments and found that IRG1 was highly expressed in the synovium of the AL group (Fig. 2D). WB and qPCR analyses indicated that IRG1 expression was upregulated in TiPs-stimulated macrophages (Fig. 2E,F). At the same time, we also found that the concentration of itaconate in the cytoplasm was significantly increased (Fig. 2G). We extracted the supernatant from TiPs-stimulated macrophage cultures for SDH activity assays, and the results showed decreased SDH activity and intracellular succinate accumulation following TiPs stimulation (Fig. 2H,I). These findings suggest that IRG1-mediated metabolic reprogramming of macrophages is closely associated with TiPs-induced macrophage inflammation.

3. TFAM inhibits the activation of the STING pathway in TiPs-stimulated macrophages by stabilizing mtDNA.

Mitochondrial DNA (mtDNA) cytoplasmic leakage, as a damage-associated molecular pattern (DAMP), markedly activates the STING pathway and triggers a series of inflammatory responses. We therefore investigated whether TiPs induce cellular stress by promoting mtDNA release. Initially, we extracted genomic DNA using TIANamp Genomic DNA Kit, and qPCR results showed increased levels of mtDNA fragments such as D-LOOP, ND1, and ND4 upon TiPs stimulation (Fig. 3A). Concurrently, IF staining revealed increased co-localization of dsDNA with mitochondria in the cytoplasm, indicating mtDNA leakage upon TiPs stimulation (Figure S1F).

MtDNA stability is mainly mediated by mitochondrial transcription factor A (TFAM), which is an essential mtDNA packaging protein for mtDNA replication and transcription.[29]. Under pathological conditions, disruption of TFAM can lead to mtDNA depletion and mitochondrial bioenergetics defects[30]. We then performed WB and qPCR analyses and found that TFAM expression was downregulated following TiPs stimulation (Fig. 3B,C). Subsequently, we generated macrophages with TFAM knockdown and TFAM overexpression. In TFAM knockdown macrophages, Western blot analysis revealed that the STING-mediated inflammatory signaling cascade would spontaneously activate without TiPs stimulation. Exposure to TiPs further amplified this activation (Fig. 3D). Quantitative

PCR also corroborated these findings (Fig. 3E). Flow cytometry analysis revealed an increased proportion of iNOS-positive cells (Figure S1G), and consistent qPCR data revealed a corresponding increase in mtDNA-encoded transcript abundance. (Fig. 3A). However, in TFAM-overexpressing macrophages stimulated with TiPs for 6 hours, WB and qPCR results indicated downregulation of STING pathway-related proteins (Fig. 4A,B). IF staining showed a reduction in nuclear translocation of P-STING and P-IRF3 (Fig. 3F, G), and this also decreased dsDNA co-localized with mitochondria (Fig. 3H). Flow cytometry analysis showed a decrease in the proportion of iNOS-positive cells (Fig. 3I).

Next, we performed in vivo experiments to further validate the role of TFAM. First, IHF staining of synovial tissues from patients with AL and FHN revealed reduced TFAM expression in the AL group (Fig. 4C,D). In our murine calvarial osteolysis model, TiPs stimulation significantly enhanced bone resorption, whereas lentiviral overexpression of TFAM alleviated this process and conferred higher BMD and BV/TV ratios. Conversely, TFAM knockdown exacerbated osteolysis (Fig. 4E-G). Histological analysis of calvarial sections showed increased fluorescence intensity of TNF- α (Figure S2A,B) and IL-1 β (Fig. 4H,I) in the sh-TFAM group, whereas TFAM overexpression attenuated these changes. H&E sections showed less inflammation in the TFAM-OE group (Fig. 4J,K). These findings suggest that TFAM can stabilize mtDNA, thereby inhibiting the activation of the STING pathway in TiPs-stimulated macrophages.

4. ITA regulates mitochondrial metabolism to Mitigate TiPs-stimulated Macrophage Inflammation and M1 Polarization

To investigate the specific role of itaconate (ITA), we conducted in vitro experiments by co-stimulating macrophages with 4-OI and TiPs. We prepared a working solution of 4-OI at a concentration of 200 μ M by dissolving it in DMSO[15]. As anticipated, after co-culturing with 4-OI for 6 hours, the activity of succinate dehydrogenase (SDH) in the cells was significantly reduced (Fig. 5A). We also measured the intracellular succinate concentration using a colorimetric assay (Fig. 5B). The results showed that 4-OI increased the intracellular succinate concentration, indicating its inhibitory effect on SDH activity.

Subsequently, we treated the macrophages with 4-OI co-culture for 2 hours followed by TiPs stimulation for 6 hours, while also setting up negative and positive control groups. The results of WB and qPCR showed that 4-OI down-regulated the expression of HIF1- α in TiP-stimulated macrophages. (Fig. 5C,D). Further investigation revealed that 4-OI inhibited the activation of STING pathway. WB and qPCR analyses showed reduced phosphorylation of STING, TBK1, and IRF3 (Fig. 5E,F), and IF staining showed the nuclear translocation of P-STING, as well as P-IRF3 was impaired by 4-OI (Fig. 5G). We then assessed the levels of iNOS, CD80, CD86 and ROS using flow cytometry and found that the

proportion of iNOS-positive cells was reduced with the addition of 4-OI (Fig. 5H), CD80⁺ CD86 and ROS-positive cells exhibited the same results (Fig. 6A), which indicate that 4-OI attenuates M1 polarization of macrophages. ELISA results indicated that 4-OI decreased the secretion of IL-1 β (Fig. 5I).

Likewise, we also conducted *in vivo* experiments to confirm the role of 4-OI. In the murine calvarial osteolysis model, administration of 4-OI markedly attenuated TiPs-induced bone resorption and resulted in elevated BMD and BV/TV ratios (Fig. 6B-D).

Immunofluorescence staining of calvarial sections revealed a significant reduction in TNF- α (Figure S4B,C) and IL-1 β (Fig. 6E,F) fluorescence intensity in the 4-OI group. Similarly, H&E staining also showed the same trend as the above results (Fig. 6G,H). These results suggest that ITA regulates metabolic reprogramming in macrophages, thereby modulating TiPs-induced macrophage inflammation.

5. ITA alkylates STAT1 to Promote TFAM Transcription which stabilizes mtDNA and regulates Macrophage Inflammation

Given that TFAM sustains mitochondrial homeostasis and that itaconate (ITA) modulates mitochondrial metabolism to suppress STING activation, we therefore investigated whether ITA suppresses STING pathway activation by modulating TFAM expression. After co-stimulating macrophages with 4-OI and TiPs for 6 hours, we extracted genomic DNA using TIANamp Genomic DNA Kit. Subsequent qPCR analysis revealed that mtDNA-encoded transcripts (D-loop, ND1, ND4) were reduced in the 4-OI group (Fig. 7C). Similarly, immunofluorescence (IF) staining showed reduced colocalization of mtDNA with mitochondria in the 4-OI group (Fig. 7D), indicating that 4-OI diminished mtDNA leakage. Further qPCR and Western blot (WB) results indicated that 4-OI upregulated the expression of TFAM (Fig. 7A,B).

We then conducted *in vivo* experiments to verify the regulatory effect of 4-OI on TFAM. Administration of 4-OI reversed the calvarial osteolysis induced by TFAM knockdown and restored elevated BMD and BV/TV ratios (Fig. 7E). IHF staining of calvarial sections showed that 4-OI markedly reduced the fluorescence intensity of IL-1 β and TNF- α (Fig. 7G), while H&E staining revealed that 4-OI alleviated the inflammatory response triggered by TiPs combined with TFAM knockdown (Fig. 7F). Collectively, these results demonstrate that 4-OI modulates TFAM to mitigate TiPs-induced inflammation both *in vivo* and *in vitro*.

By searching the Cistrome Data Browser database, we found that STAT1 can bind to the promoter sequence of TFAM (Fig. 7H), thereby regulating its transcription. Our ChIP-qPCR results showed enrichment of STAT1 at the TFAM promoter region (Fig. 7I).

Subsequently, we generated STAT1 knockdown macrophages. qPCR and WB analyses revealed that TFAM expression was upregulated following STAT1 knockdown (Fig. 7J,K), confirming that STAT1 negatively regulates TFAM transcription.

Next, we investigated whether 4-OI could modulate STAT1 to affect TFAM transcription. WB and qPCR results suggested that 4-OI downregulated the expression of p-STAT1 (Fig. 7L,M). Previous studies have shown that itaconate and its derivatives can undergo Michael addition reactions with target proteins such as KEAP1 and SYK, a process termed "itaconylation," thereby affecting the function of these proteins[17]. To further explore its ability to modify STAT1, we co-incubated macrophages with an itaconate-alkyne (ITalk) probe for 12 hours to capture itaconylated proteins in macrophages (Fig. 8G). Gratifyingly, WB results showed the presence of STAT1 in the proteins pulled down by streptavidin (Fig. 8B), indicating that STAT1 can be itaconylated. Next, we immunoprecipitated STAT1 from ITALK-co-incubated lysates and subjected the precipitated proteins to LC-MS/MS analysis, which identified itaconate modification of STAT1 (Fig. 8D-F). Subsequently, we applied a STAT1-selective transcriptional activator, 2-NP. WB results showed increased p-STAT1 expression in the 2-NP group, while co-incubation of 2-NP with 4-OI led to decreased p-STAT1 expression (Fig. 8A), indicating that 4-OI can reverse the effect of 2-NP. Subsequently, macrophages were co-incubated with ITALK for 12 h, followed by 4 h of TiPs stimulation. Western blot analysis revealed that itaconate modification attenuated STAT1 phosphorylation (Fig. 8C). Therefore, we hypothesize that 4-OI-mediated itaconation of STAT1 can inhibit its phosphorylation process. The above results indicate that 4-OI can itaconylate STAT1, thereby upregulating TFAM expression, stabilizing mtDNA, and reducing macrophage inflammatory responses.

Discussion

Total joint replacement is the most effective method for treating end-stage bone and joint diseases, yet the average lifespan of prostheses is currently only 15–20 years, with aseptic loosening being the primary cause of long-term failure, accounting for 5.2% and 28.1% of revisions in hip and knee replacements, respectively[32]. Postoperative sliding friction and micromotion between prostheses and bone generates wear particles[33]. Macrophages are the primary cells that recognize wear particles and initiate inflammatory responses, producing pro-inflammatory cytokines like IL-6 and TNF- α , which then activate osteoclasts and induce bone resorption[34]. Among various wear particles, TiPs have the strongest stimulatory effect[35], thus inhibiting TiPs-induced macrophage inflammation is crucial for delaying periprosthetic bone dissolution.

Wear particles stimulate macrophages to activate pattern recognition receptors (PRRs) such as TLRs and NLRs, thereby triggering downstream pathways like STING/TBK1 and NF- κ B to mediate the release of TNF- α and IL-1 β , as clarified in our previous research[36–

38]. Our recent focus has been on the mitochondrial metabolic processes of macrophages, which not only supply ATP to cells but also regulate macrophage inflammation[39]. In mitochondrial TCA, succinate dehydrogenase (SDH) catalyzes the conversion of succinate to fumarate and is a crucial molecule in modulating macrophage immune functions. Changes in SDH activity alter the succinate / α -ketoglutarate ratio in the TCA cycle, affecting ROS release and HIF1- α expression, thereby regulating macrophage mitochondrial metabolic reprogramming[11]. Itaconate is a significant TCA cycle derivative, playing extensive roles in bacterial/viral infections, metabolic reprogramming, and macrophage inflammation inhibition[40]. Studies show that ITA, encoded by IRG1 and structurally similar to succinate, binds to the succinate catalytic site on SDH's α -subunit via electrostatic interaction, significantly inhibiting its activity[18]. Concurrently, ITA can mediate a novel post-translational modification on lysine residues—itaconoylation—which in turn regulates the immune function of macrophages. Moreover, the level of this modification is significantly upregulated during the activation process of macrophages. Previous studies have shown that ITA can inhibit SYK through alkylation and suppress inflammation associated with gut microbiota dysbiosis induced by hvKP[12]. Additionally, ITA can activate the transcription factor TFEB via alkylation modification, inducing lysosomal biogenesis and thereby enhancing the antimicrobial innate immune capacity of macrophages[41]. Thus, we consider IRG1/ITA can modulate macrophage mitochondrial metabolism and repress downstream inflammatory pathways to alleviate TiPs-induced osteolysis by inhibiting SDH activity.

In this study, we compared the synovium of patients with femoral head necrosis and patients with aseptic loosening of artificial joints, and found that the expression of inflammatory factors was significantly increased in the aseptic loosening group, indicating that these patients had clinical inflammatory injury. Referring to enrichment of IRG1 in TiPs-induced macrophage RNA-seq analysis and previous studies linking IRG1 to various inflammatory diseases, we hypothesized that IRG1 plays a crucial role in the inflammation of macrophages induced by tips. In vitro, the high expression of IRG1 in AL synovium and TiPs-stimulated macrophages supports its involvement in the pathogenesis of AL. Subsequently, we found that 4-OI, on the one hand, could reduce the expression of HIF1- α by inhibiting the activity of SDH; On the other hand, inhibiting the STING pathway regulates the activation of inflammatory factors within macrophages and simultaneously inhibits the polarization of macrophages towards M1.

Following exploration along the STING pathway, we found that mtDNA, the most well-known inflammatory agonist within mitochondria, once it leaks into the cytoplasm, it is recognized as a mitochondrial damage-associated molecular pattern (DAMP), triggering the pathway and initiating a cascade of inflammatory effects[42]. Mitochondrial transcription factor A (TFAM) is a DNA-binding protein that stabilizes mtDNA and initiates its replication; studies have shown that TFAM deficiency significantly induces mtDNA

instability and promotes its escape into the cytoplasm, causing cytosolic stress[43, 44]. We found that TiPs induce mtDNA leakage in macrophages, leading us to hypothesize whether 4-OI could alleviate this process by promoting TFAM expression. Our results showed that the addition of 4-OI increased TFAM expression in macrophages, and subsequently, we observed reduced mtDNA leakage in the 4-OI group, confirming our hypothesis.

High levels of intracellular itaconate can undergo Michael addition to important cysteine residues on proteins via its electrophilic α,β -unsaturated carboxylic structure, a post-translational modification also known as "itaconylation"[17]. Previous studies have shown itaconylation at Cys147 of STING and at Cys66 of GPx4, modulating their activation[15]. We hypothesized whether itaconylation occurred during 4-OI regulation of mtDNA release. Using the Cistrome Data Browser, we found STAT1 binds the upstream promoter sequence of TFAM, inhibiting TFAM transcription. We subsequently confirmed this result by ChIP-qPCR. Following the methods of the aforementioned studies[45], we used ITalk to detect itaconate alkylation in living cells and, as expected, found STAT1 to be itaconylated. We also observed that both ITalk and 4-OI reduced STAT1 phosphorylation, leading us to propose that 4-OI inhibits STAT1 phosphorylation via itaconation. Subsequently, we engineered STAT1 knockout and observed increased TFAM expression and reduced cytoplasmic mtDNA leakage. Thus, we confirmed at the cellular level the regulatory effect of 4-OI on the inflammatory response of macrophages stimulated by wear particles. To further verify these findings, we established a mouse calvarial osteolysis model. Consistent with our previous research methods, injections of 4-OI, oe-TFAM lentivirus all reversed TiPs-induced cranial bone resorption and inflammation.

However, there are still some limitations in our research. Whether itaconate derivatives, such as 4-OI, can be completely hydrolyzed into itaconate by cellular esterases within cells and fully replace the function of endogenous itaconate remains to be elucidated[46]. Some studies have shown that the role of 4-OI is no different from endogenous itaconate[47, 48], but other studies have reported that 4-OI cannot be converted into cellular itaconate and shows different effects in the regulation of inflammasomes and Type I IFNs[49]. Therefore, using 4-OI as a representative of IRG1/ITA has certain limitations. In our iTALK click chemistry experiments, we found evidence of itaconylation of STAT1, but we did not further explore the specific sites of action. This needs to be further improved in future experiments.

In summary, our study indicates that IRG1/ITA modulates SDH activity in macrophage mitochondrial cycles, alleviating inflammation induced by TiPs; additionally, it promotes TFAM expression by alkylating STAT1 to stabilize mtDNA and reduce cytosolic stress, thereby mitigating TiPs-induced inflammatory responses. This also opens new avenues for the prevention and treatment of aseptic loosening in artificial joints.

Declarations

Funding:

This work was supported by the National Natural Science Foundation of China (82372415).

Author Contribution

Y.Y., T.L., and C.P. contributed equally to this work. Drafting of the article and design of study: Y.Y., T.L., C.P.; support of materials and techniques: Y.Y., T.L., S.L.; animal keeping, sample collection: Y.Y., T.L., H.S., H.L.; histological tests and data collection: Y.Y., H.L., K.W.; analysis, and interpretation of data: J.L., Z.C., S.T.; research supervision: Y.D, manuscript editing: Y.Y, C.L., Y.D.; All authors have read and approved the final submitted manuscript.

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Figures

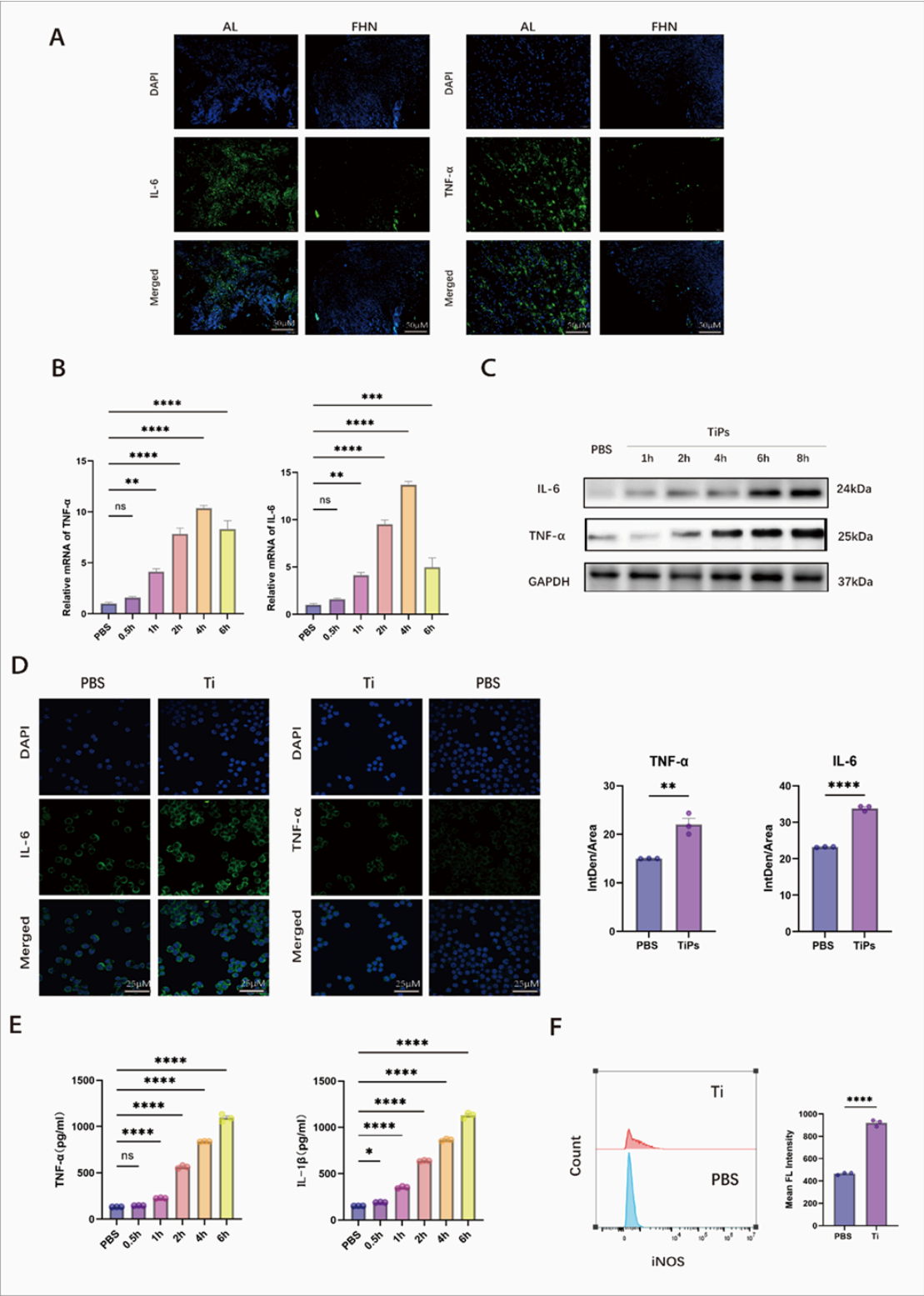


Figure 1

Inflammatory responses were detected in AL synovium and in TiP-stimulated macrophages. (A) Immunohistochemical fluorescence (IHF) staining images of TNF- α and IL-6 in synovial tissue sections from AL patients and FHN patients. Scale bar, 50 μ m. (B) qPCR analysis revealed that the mRNA expression levels of TNF- α and IL-6 in macrophages increased in a time-dependent manner following TiPs stimulation. (C) Western blot (WB) results indicated that the protein expression levels of TNF- α and IL-6 in macrophages increased in a time-dependent manner following TiPs stimulation. (D) Immunofluorescence (IF) staining results showed stronger fluorescence of TNF- α and IL-6 in the TiPs-stimulated group. Scale bar, 25 μ m. (E) ELISA analysis showed increased secretion of TNF- α and IL-1 β in TiPs-stimulated macrophages. (F) Flow cytometry analysis revealed that the proportion of iNOS-positive macrophages increased following TiPs stimulation. All data represent at least three independent experiments with similar results. Statistical data are presented as mean $\pm$ standard deviation and were analyzed using unpaired t-tests or one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant.

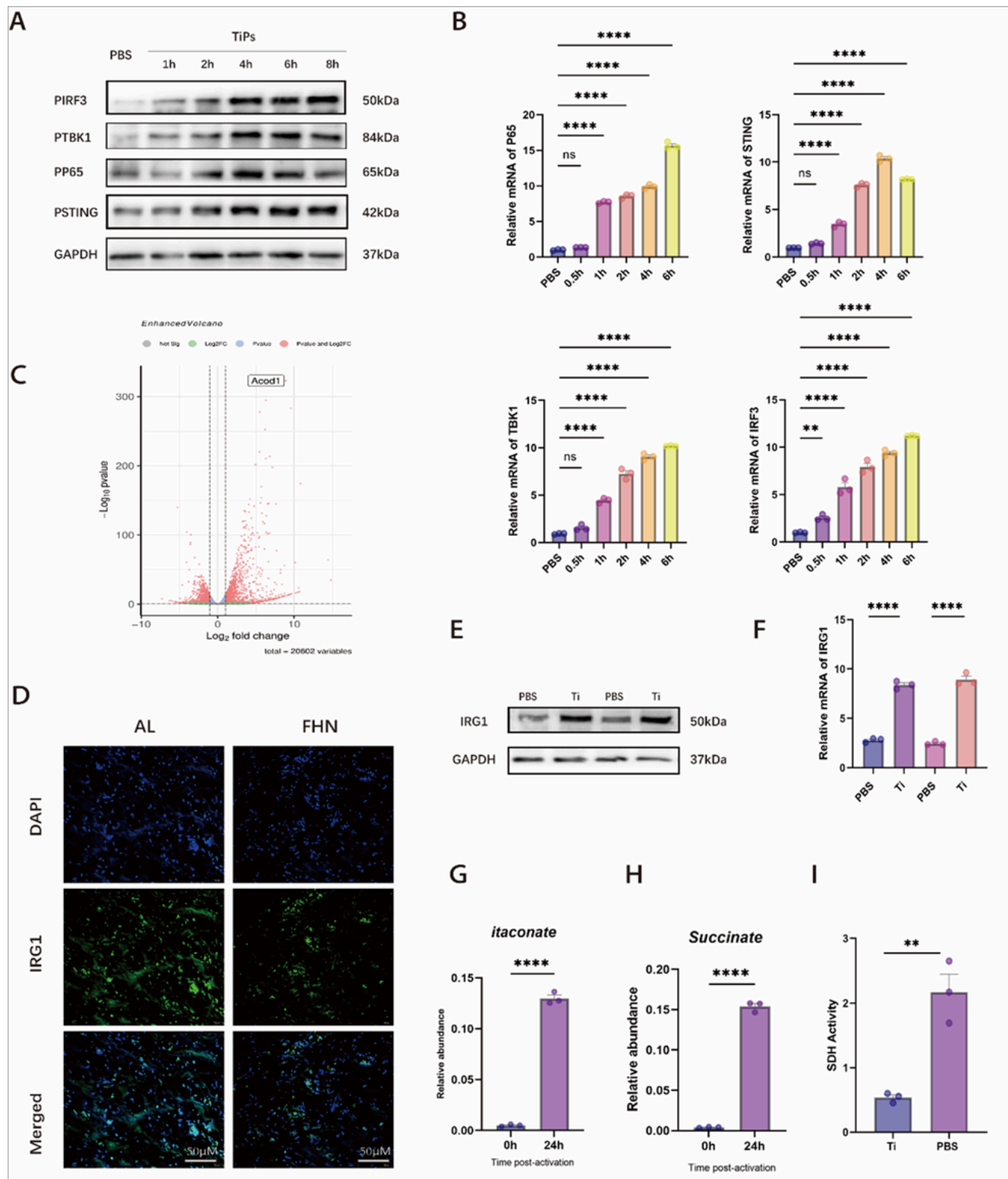


Figure 2

TiPs-stimulation Macrophages initiate metabolic reprogramming to activate the STING pathway and simultaneously upregulate IRG1 expression. (A) Western blot (WB) analysis revealed that the protein expression levels of PP65, P-IRF3, P-TBK1, and P-STING in macrophages increased in a time-dependent manner following TiPs stimulation. (B) qPCR analysis showed that the mRNA expression levels of PP65, P-IRF3, P-TBK1, and P-STING

increased in a time-dependent manner. (C) Volcano plot of differentially expressed genes. (D) Immunohistochemical fluorescence (IHF) staining images of IRG1 in synovial tissue sections from AL patients and FHN patients. Scale bar, 50 μ m. (E) WB analysis showed increased expression of IRG1 protein in macrophages following TiPs stimulation. (F) qPCR analysis showed increased mRNA expression levels of IRG1 in macrophages following TiPs stimulation. (G) Accumulation of itaconate in macrophages following TiPs stimulation. (H) Accumulation of succinate in macrophages following TiPs stimulation. (I) Decreased succinate dehydrogenase (SDH) activity in macrophages following TiPs stimulation. All data represent at least three independent experiments with similar results. Statistical data are presented as mean $\pm$ standard deviation and were analyzed using unpaired t-tests or one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant.

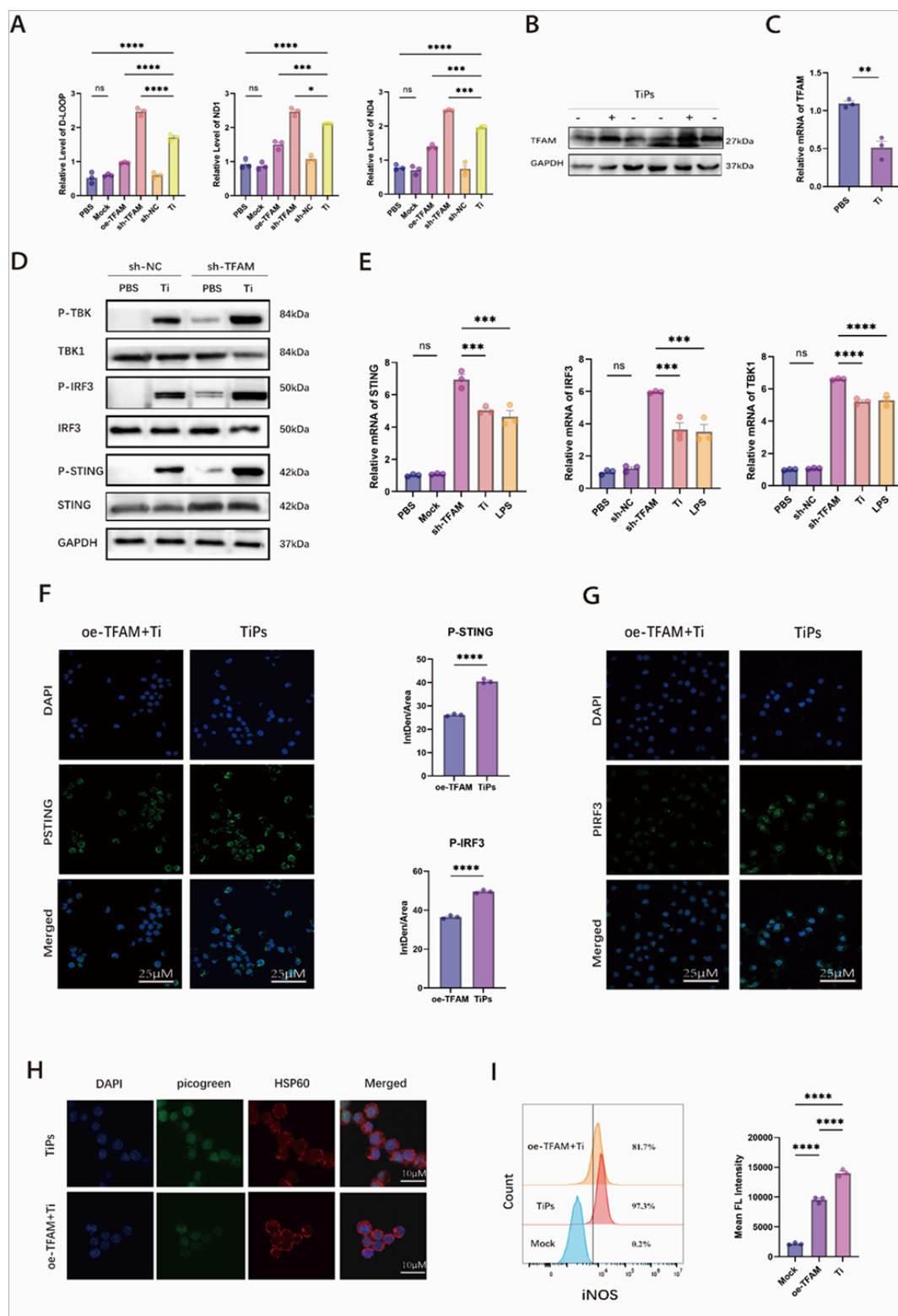


Figure 3

TFAM inhibits the activation of the STING pathway in TiPs-stimulated macrophages by stabilizing mtDNA. (A) mRNA expression levels of ND1, ND4, and D-LOOP in macrophages across different treatment groups. (B) Western blot (WB) analysis showed decreased expression of TFAM protein in macrophages following TiPs stimulation. (C) qPCR analysis revealed increased mRNA expression levels of TFAM in macrophages following TiPs

stimulation. (D) WB analysis showed elevated expression of STING pathway proteins in macrophages following TFAM knockdown. (E) qPCR analysis revealed increased mRNA expression levels of STING pathway components in macrophages following TFAM knockdown. (F–G) Immunofluorescence (IF) staining showed reduced staining intensity of P-STING and P-IRF3 in macrophages following TFAM overexpression. Scale bar, 25 μ m. (H) IF staining showed decreased mitochondrial DNA (mtDNA) content in cells following TFAM overexpression. Scale bar, 10 μ m. (I) Flow cytometry analysis showed a decreased proportion of iNOS-positive macrophages following TFAM overexpression. All data represent at least three independent experiments with similar results. Statistical data are presented as mean $\pm$ standard deviation and were analyzed using unpaired t-tests or one-way ANOVA. * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001; ns, not significant.

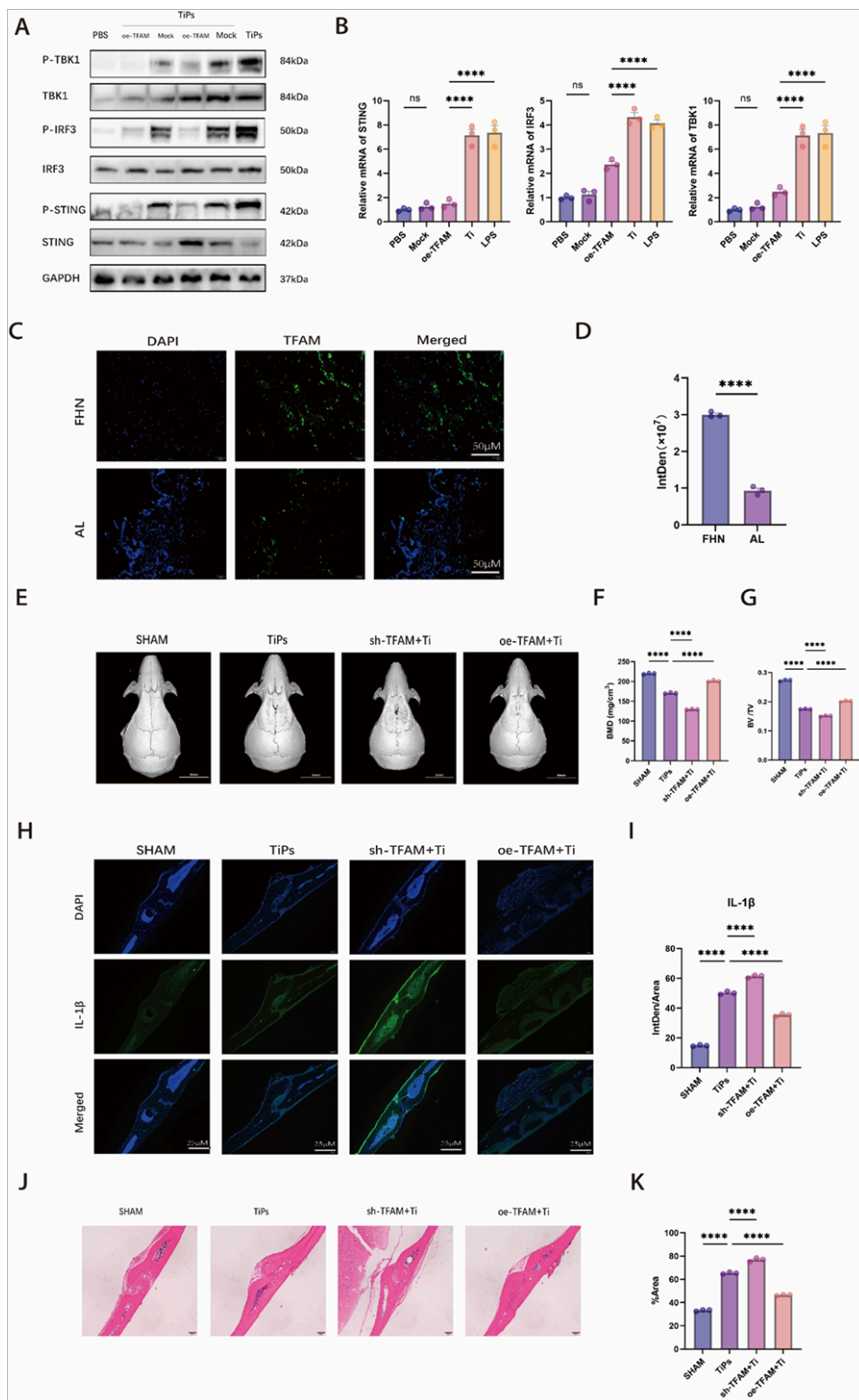


Figure 4

TFAM inhibits the activation of the STING pathway in TiPs-stimulated macrophages by stabilizing mtDNA. (A) WB analysis showed decreased expression of STING pathway proteins in macrophages following TFAM overexpression. (B) qPCR analysis revealed downregulated mRNA expression levels of STING pathway components in macrophages following TFAM overexpression. (C) Immunohistochemical fluorescence (IHF) staining

images of TFAM in synovial tissue sections from AL patients and FHN patients. Scale bar, 50 μ m. (D) The average intensity of TFAM IHF staining was calculated using ImageJ analysis. (E) 3D Reconstruction of the Skull in C57BL/6J Mice. (F,G) BMD and BV/TV Analysis Based on Micro-CT Scanning. The results are consistent with morphological bone dissolution. (H,I) Images of IL-1 β Staining in Mouse Skull Sections and the average intensity of IL-1 β was calculated using ImageJ analysis. (J,K) H&E staining results of mouse calvaria and their quantitative analysis. All data represent at least three independent experiments with similar results. Statistical data are presented as mean $\pm$ standard deviation and were analyzed using unpaired t-tests or one-way ANOVA. * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001; ns, not significant.

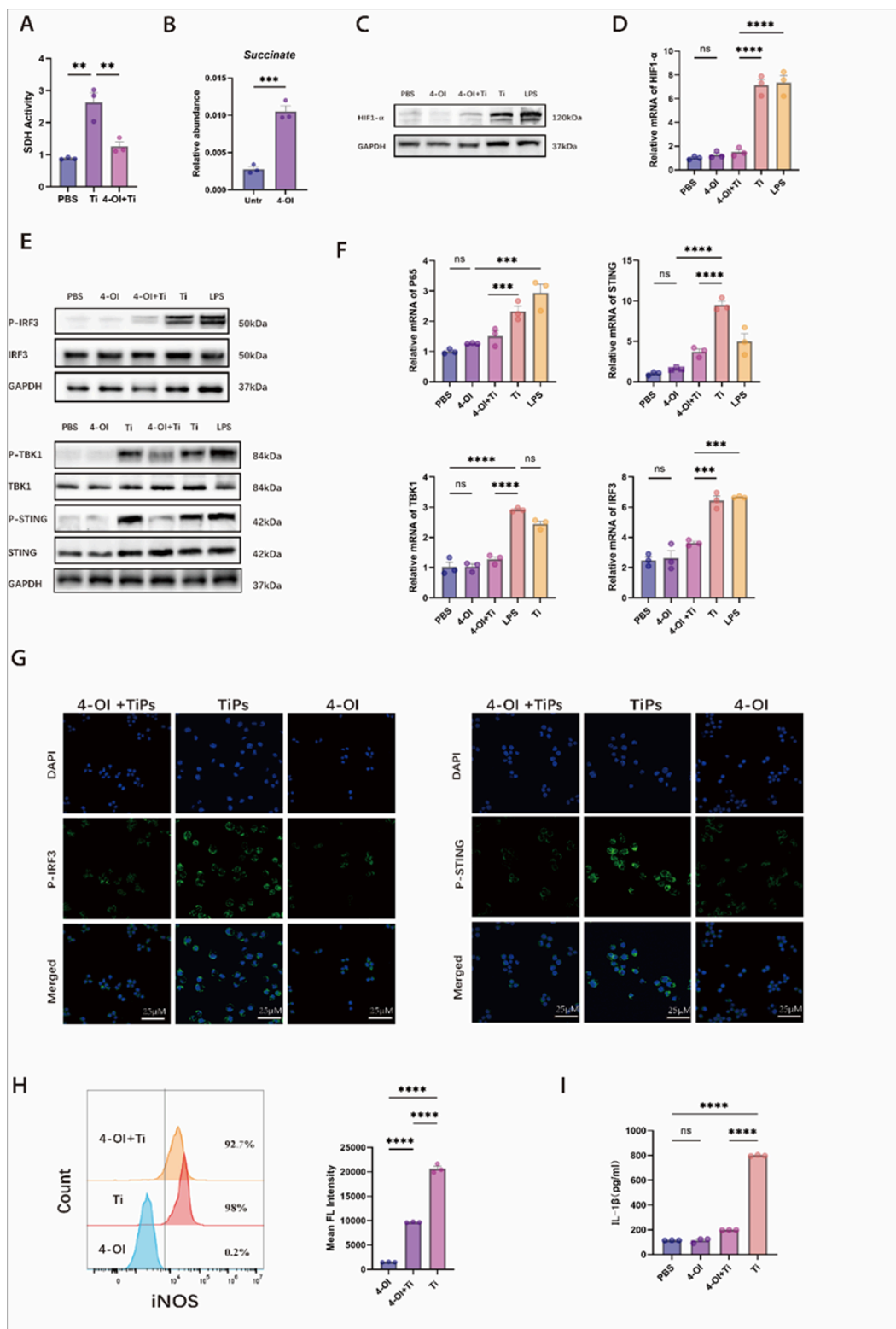


Figure 5

ITA regulates mitochondrial metabolism to Mitigate TiPs-stimulated Macrophage Inflammation and M1 Polarization. (A) Decreased succinate dehydrogenase (SDH) activity in macrophages following treatment with 4-OI. (B) Increased succinate levels in macrophages following treatment with 4-OI. (C,D) Western blot (WB) and qPCR analyses showed decreased levels of HIF-1 α in macrophages following treatment with 4-OI. (E) WB

analysis revealed decreased expression of STING pathway proteins in macrophages following treatment with 4-OI. (F) qPCR analysis showed decreased mRNA expression levels of the STING pathway in macrophages following treatment with 4-OI. (G) Immunofluorescence (IF) staining demonstrated reduced staining intensity of P-STING and P-IRF3 in macrophages following treatment with 4-OI. Scale bar, 25 μ m. (H) Flow cytometry analysis showed a decreased proportion of iNOS-positive macrophages following treatment with 4-OI. (I) ELISA analysis revealed reduced secretion of IL-1 β by macrophages following treatment with 4-OI. All data represent at least three independent experiments with similar results. Statistical data are presented as mean $\pm$ standard deviation and were analyzed using unpaired t-tests or one-way ANOVA. * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001; ns, not significant.

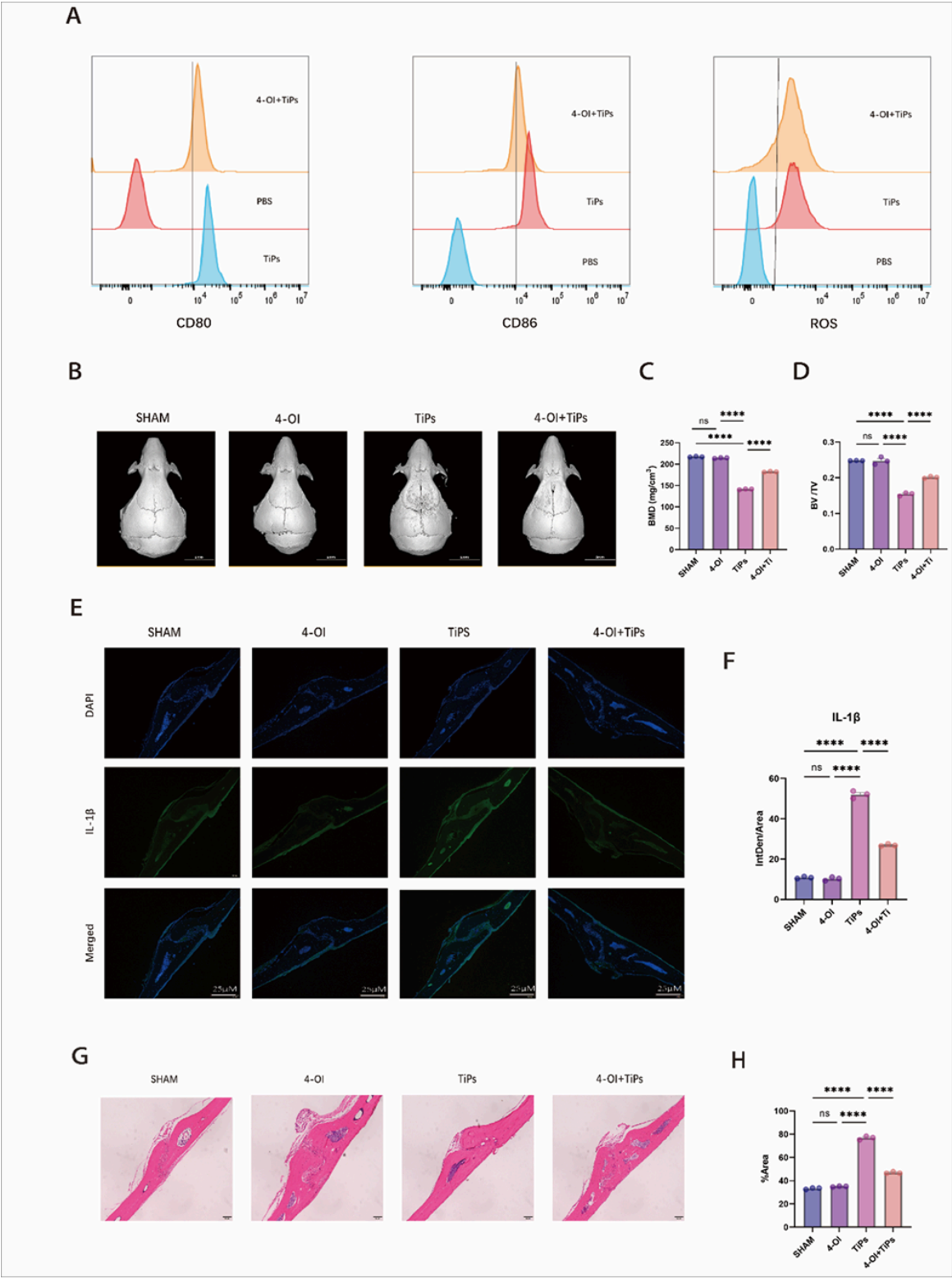


Figure 6

ITA regulates mitochondrial metabolism to Mitigate TiPs-stimulated Macrophage Inflammation and M1 Polarization. (A) Flow cytometry results revealed that 4-OI treatment reduced the proportions of CD80⁺, CD86⁺, and ROS⁺ macrophages; the data are presented with accompanying analysis. (B) 3D Reconstruction of the Skull in C57BL/6J Mice. (C,D) BMD and BV/TV Analysis Based on Micro-CT Scanning. The results are

consistent with morphological bone dissolution. (E,F) Images of IL-1 β Staining in Mouse Skull Sections and the average intensity of IL-1 β was calculated using ImageJ analysis. (G,H) H&E staining results of mouse calvaria and their quantitative analysis. All data represent at least three independent experiments with similar results. Statistical data are presented as mean $\pm$ standard deviation and were analyzed using unpaired t-tests or one-way ANOVA. * p <0.05, ** p <0.01, *** p <0.001, and **** p <0.0001; ns, not significant.

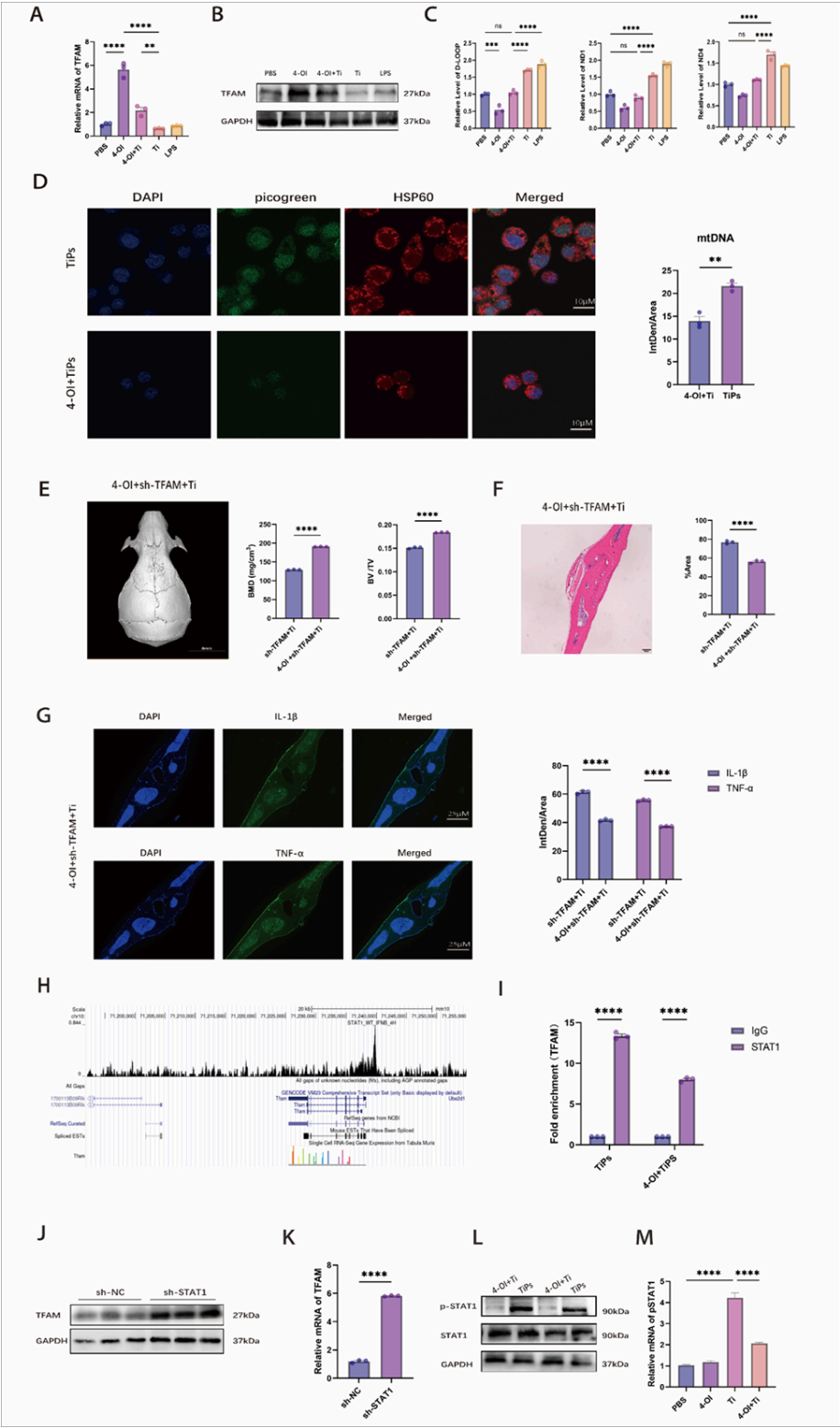


Figure 7

5. ITA Alkylates STAT1 to Promote TFAM Transcription which stabilizes mtDNA and regulates Macrophage Inflammation. (A,B) Western blot (WB) and qPCR analyses revealed increased expression levels of TFAM in macrophages following treatment with 4-OI. (C) mRNA expression levels of ND1, ND4, and D-LOOP in macrophages decreased following treatment with 4-OI. (D) Immunofluorescence (IF) staining showed reduced mitochondrial DNA (mtDNA) content in cells following treatment with 4-OI. Scale bar, 10 μ m. (E) 3D Reconstruction of the Skull in C57BL/6J Mice. BMD and BV/TV Analysis Based on Micro-CT Scanning. The results are consistent with morphological bone dissolution. (F) H&E staining results of mouse calvaria and their quantitative analysis. (G) Images of IL-1 β and TNF- α Staining in Mouse Skull Sections and the average intensity was calculated using ImageJ analysis. (H) A STAT1 binding peak within the TFAM promoter region, identified using the Cistrome Data Browser (Mouse GRCm38/mm10, chr10:71,192,029-71,286,528). (I) Chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) results showed that STAT1 was enriched in the TFAM promoter region, and 4-OI could reduce its binding. (J,K) WB and qPCR analyses showed increased expression levels of TFAM in macrophages following STAT1 knockout. (L,M) WB and qPCR analyses revealed decreased expression levels of P-STAT1 in macrophages following treatment with 4-OI.

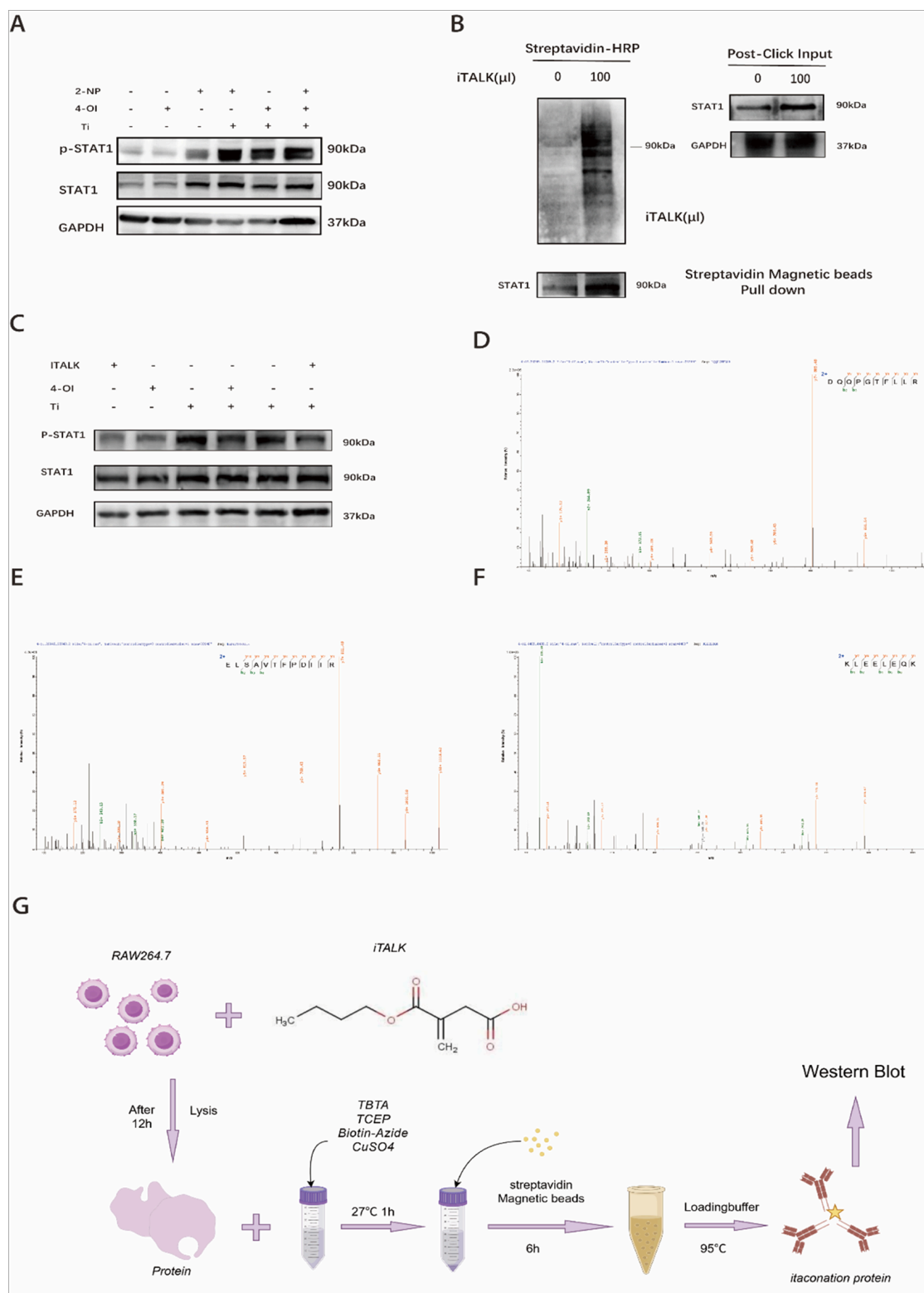


Figure 8

ITA Alkylates STAT1 to Promote TFAM Transcription which stabilizes mtDNA and regulates Macrophage Inflammation. (A) WB results showed that 4-OI reversed the effect of 2-NP. (B) WB analysis showed that iTALK binds to STAT1 protein. (C) Itaconate modification attenuated STAT1 phosphorylation. (D-F) LC-MS/MS analysis of 4-OI-alkylated peptides of STAT1. (G) Schematic diagram of the iTALK procedure. All data

represent at least three independent experiments with similar results. Statistical data are presented as mean $\pm$ standard deviation and were analyzed using unpaired t-tests or one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant.

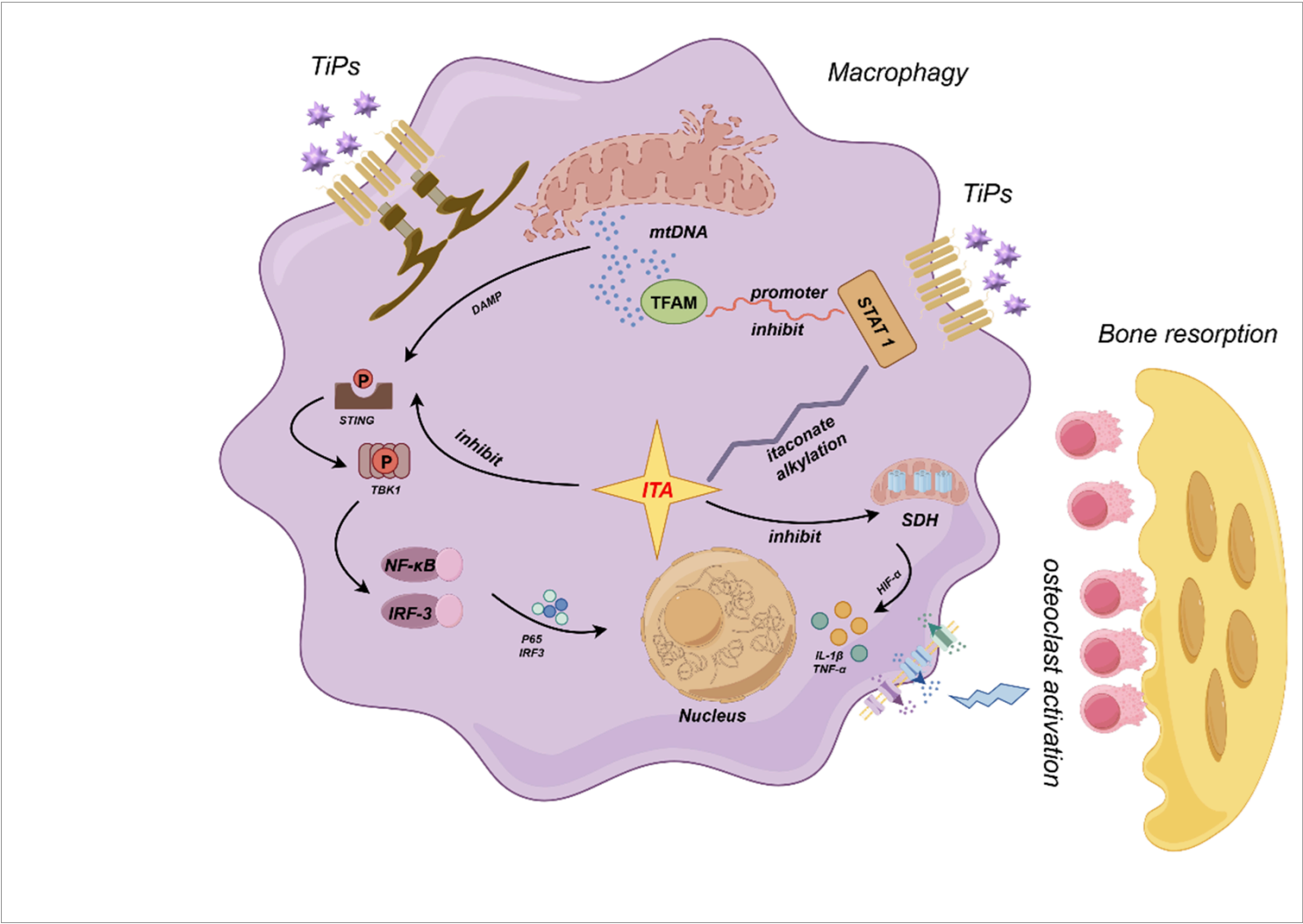


Figure 9

Schematic diagram: 4-OI itaconylates STAT1 to enhance TFAM transcription, thereby stabilizing mtDNA and attenuating macrophage inflammation.

Supplementary Files

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- [SupportingInformation.docx](#)
- [supplementaryfile.zip](#)