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ORIGINAL RESEARCH

4-Octyl Itaconate Modulates Dendritic Cells Function and Tumor Immunity via NRF2 Activation

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Objective: Dendritic cells (DCs) play a pivotal role in orchestrating anti-tumor immune responses. However, various factors can suppress DCs function and compromise anti-tumor immunity. Itaconate, a metabolite activated during inflammation and infection, has been identified to possess immunomodulatory properties, but its role on DCs remains largely unexplored. In this study, we aimed to investigate the role of itaconate in regulating the maturation and function of DCs and its underlying molecular mechanism.

Methods: Bone marrow-derived dendritic cells (BMDCs) were treated with 4-octyl itaconate (4OI). The expression levels of CD40, CD80, CD86, and MHC-II on BMDCs were analyzed by flow cytometry. The mRNA expression of cytokines was assessed using RT-qPCR. BMDCs with different treatment were adoptively transferred to B16-OVA tumor-bearing mice. The production of IFN-γ, IL-2, and TNF-α in $CD4^+$ T and $CD8^+$ T cells were analyzed by flow cytometry. The protein level of NRF2 in BMDCs was analyzed by Western blot.

Results: Treatment with 4OI represses DC maturation and function. Specifically, 4OI-treated DCs exhibited impaired phenotypic and functional maturation, characterized by decreased expression of co-stimulatory molecules CD40, CD80, and CD86, as well as lower levels of pro-inflammatory cytokines IL-12, IL-6, TNF-α and IL-1β. Furthermore, these DCs demonstrated a diminished capacity to stimulate T cell responses both in vitro and in vivo. Mechanistically, 4OI inhibits DCs maturation and function through enhancing and activating KEAP1/NRF2 pathway.

Conclusion: This study reveals that 4OI inhibits DC function through NRF2 activation, elucidating the immunomodulatory mechanisms of itaconate and emphasizing its pivotal role in developing targeted DC-based tumor immunotherapy strategies.

Keywords: 4-octyl itaconate, dendritic cells, immune response, tumor immunotherapy

Introduction

Dendritic cells (DCs) serve as specialized antigen-presenting cells that are key to integrating innate and adaptive immunity.[1,2] DCs play a critical role in initiating specific T cell-mediated anti-tumor immune responses by cross-presenting tumor-associated antigens to naïve T cells, thereby aiding in the suppression of tumor proliferation dissemination.[3] The maturation of DCs is triggered by appropriate stimuli, leading to the increased expression of costimulatory molecules CD80, CD86, and CD40, as well as the secretion of cytokines to promote inflammatory in the tumor microenvironment (TME).[4] The interaction of CD80 and CD86 on DCs with CD28 or cytotoxic T lymphocyte

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antigen 4 (CTLA4), respectively, regulates the activation or suppression of T cells.[5] Effector functions of T cells are dependent on cytokines from DCs, including IL-12 and type I interferons.[6] Thus, DCs are fundamental to the initiation of tumor immunity and play a critical role in amplifying anti-tumor effector functions throughout this process. Targeting DCs within the TME holds significant promise for enhancing anti-tumor immunity.

An immunosuppressive TME can hinder the functions of DCs by changing the phenotype and encouraging dysfunction and tolerance.[3,4] Various mechanisms, such as soluble mediators and cell-to-cell interactions, are responsible for these effects.[3,4] Many researches have reported that metabolites in the TME can hinder the cross-presentation function of DCs.[7–11] For example, in the TME, lipid peroxidation products initiate endoplasmic reticulum stress in DCs, resulting in lipid accumulation and the formation of cytosolic lipid droplets, which subsequently disrupt the transport of antigen peptide-MHC-I complexes.[7] In addition, increased lactate in the TME also inhibit the maturation and activation of DCs.[10,11]

Itaconate is an immunomodulatory metabolite produced catalytically by the metabolic enzyme aconitate decarboxylase (ACOD1), which is encoded by immune-responsive gene (IRG1).[12,13] Research has demonstrated that IRG1/itaconate functions to reduce inflammation in macrophages and is involved in the pathogenesis of various infectious and inflammatory conditions, including sepsis,[14,15] viral infections,[16,17] psoriasis,[18] ischemia/reperfusion injury,[19] and pulmonary fibrosis.[20] Recent findings indicate that IRG1/itaconate contributes tumor growth by boosting the immunosuppressive capabilities of tumor-associated macrophages and reducing the cytolytic function of $CD8^+$ T cells within TME.[21–23] Itaconate is proposed to be a crucial immunomodulatory element in the TME, contributing to tumor development. However, the effect of itaconate on the antitumor activity of DCs and its molecular mechanism remains largely unknown.

NRF2 is a transcription factor initially identified as a key player to maintain the reduction-oxidation balance. Over the past years, studies have elucidated its crucial role in the innate and adaptive immunity.[24,25] The activation of NRF2 leads to increased proliferation and enhanced effector functions of $CD8^+$ T cells, thereby reinforcing the antitumor response in preclinical animal experiments.[26] Furthermore, NRF2 signaling inhibits the maturation and function of DCs by inducing heme oxygenase-1 (HO-1) and downregulating the expression of inducible nitric oxide synthase (iNOS).[27] These findings underscore the essential role of NRF2 in immune regulation during inflammatory diseases. As the activator of NRF2, itaconate inhibits ferroptosis of macrophage via NRF2 in acute lung injury.[28] It has been reported that itaconate attenuates autoimmune hepatitis and airway inflammation via inhibiting DCs maturation and immune-priming function.[29,30] However, whether itaconate exerts its effects on DC maturation and function through NRF2 activation remains to be fully elucidated.

In this study, we report that a cell-permeable itaconate derivative, 4OI inhibits phenotypic and functional maturation of DCs. This inhibition is characterized by lower expression of co-stimulatory molecules like CD40, CD80, and CD86, along with diminished production of pro-inflammatory cytokines such as IL-12, IL-6, TNF-α, and IL-1β. In addition, 4OI-treated DCs show an impaired ability to stimulate T cell responses. Mechanistically, 4OI represses DCs activation and maturation through activating KEAP1/NRF2 pathway. Thus, we assert that 4OI curtails DCs functions and blocks vigorous T cell responses, which weakens host immune surveillance and permits tumors to escape immune detection. Our study reveals new understandings of itaconate's role in tumor immunity, indicating itaconate could be a target for cancer immunotherapy.

Materials and Methods

Mice

Eight-week-old female C57BL/6 mice were obtained from the Experimental Animal Center at Yangzhou University (SCXK(SU)2021–0013). OT-I and OT-II mice were obtained from The Jackson Laboratory. In a facility devoid of specific pathogens, mice were bred. All experiments received approval from Jiangsu University's Committee on the Use of Live Animals in Research and Teaching (UJS-IACUC-AP-20190307016). All animal experiments followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.5–23, revised 1996; NIH Bethesda, MD, USA).

BMDCs Culture

BMDCs were cultured by extracting bone marrow cells from 8-week-old C57BL/6 mice and cultivating at a density of 1×10^6 cells/mL in the RPMI-1640 medium containing 10% (v/v) FBS, penicillin-streptomycin (100 U/mL), recombinant

mouse GM-CSF (20 ng/mL, Peprotech) and recombinant mouse IL-4 (5 ng/mL, Peprotech). The same volume of medium was added on days 3 and 5, and non-adherent cells were collected on day 7.

Cell Apoptosis Assay

BMDCs were re-suspended in 500 μL of binding buffer with 5 μL of Annexin V FITC and 10 μL of PI, then incubated for 15 minutes at room temperature in the dark. Subsequently, Flow cytometry on the BD FACS Canto II was used to analyze apoptosis levels within an hour.

Tumor-Bearing Mouse Model Preparation

BMDCs were pretreated with 4OI (250μM) (SML2338, Sigma-Aldrich) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) (L2630, Sigma-Aldrich) for 3 hours, washed and pulsed with OVA (50μg/mL) for 5 hours, and then 4OI and OVA treated BMDCs were adoptively transferred into wildtype (WT) mice i.v. at day −7, then 5×10^5 B16-OVA cells were inoculated subcutaneously on day 0, and tumor growth was monitored every 2–3days. The size of the tumor was calculated on the specified days using the formula: length × width × width × 0.5.

Flow Cytometry and Intracellular Cytokine Staining

For the purpose of surface marker identification, anti-mouse CD11c (N418), CD40 (3/23), CD80 (16–10A1), CD86 (PO3), MHC-II (M5/114.15.2), CD4 (RM4-5), and CD8 (53–6.7) from Biolegend, anti-mouse CD45 (30-F11) from eBioscience were used. For intracellular staining, cells were incubated with PMA (Sigma-Aldrich, 50 ng/mL), ionomycin (Sigma-Aldrich, 1 μg/mL), and Brefeldin A (Biolegend, 1000×) for five hours. According to the intracellular staining procedure, cells were stained with antibodies for surface markers, fixed, permeabilized, and then stained with anti-IFN-γ mAb (XMG1.2, Biolegend), anti-IL-2 mAb (JES6-5H4, Biolegend), and anti-TNF-α mAb (MP6-XT22, Biolegend). The BD FACSCanto II was employed for flow cytometry, and the data was analyzed using FlowJo software.

T Cells Isolation

The Dynal mouse CD4 negative isolation kit (Biolegend) was used to enrich $CD4^+$ T cells from OT-II mice spleens via magnetic separation. $CD8^+$ T cells were extracted from the spleens of OT-I mice using a Dynal mouse CD8 negative isolation kit (Biolegend) through magnetic separation. The sorted cells were then labeled with CFSE at 37 °C for 10 minutes, washed, and counted prior to being cultured with BMDCs.

T Cells Proliferation and Activation Assay

BMDCs were pretreated with 4OI (250 μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) for 3 hours, and then 4OI and LPS treated BMDCs were cultured in 96-well plates. For OT-II stimulation, 5×10^4 BMDCs were incubated with endotoxin-free OVA protein (50μg/mL) or $OVA_{323\text{-}339}$ peptide (5μg/mL) for 5 hours, washed and incubated with 1×10^5 OT-II T cells. For OT-I stimulation, 5×10^4 BMDCs were incubated with endotoxin-free OVA protein (50μg/mL) or $OVA_{257\text{-}264}$ peptide (1ng/mL) for 5 hours, washed and incubated with 1×10^5 OT-I T cells. Flow cytometry was used to evaluate T cell proliferation after 72 hours for OT-II T cells and 48 hours for OT-I T cells. After 36 hours, T cell activation was evaluated using flow cytometry, followed by a 5-hour stimulation with PMA, ionomycin, and Brefeldin A.

RT-qPCR

TRIzol (Invitrogen) was used to extract total RNA, and cDNA was synthesized with HiScript III Reverse Transcriptase (Vazyme, R323-01) following the manufacturer's guidelines. Gene transcripts were measured using real-time quantitative PCR with ChamQ SYBR qPCR Master Mix (Vazyme, Q711-02). The primer details are provided in Supplemental Table 1.

RNA-Sequencing Analysis

BMDCs were pretreated with 4OI (250μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) for 3 hours. Total RNA was isolated from 4OI-treated BMDCs using TRIzol (Invitrogen) and sequenced with the HiSeq or Novaseq 2×150 platform by AZENTA Life Sciences (Suzhou). Differentially expressed genes were analyzed using

EdgeR, applying a cutoff of fold change greater than or equal to 2 and a p-value less than 0.05. We utilized the R package clusterProfiler and software for functional enrichment analysis of MSigDB gene sets (KEGG and GO) to pinpoint signaling pathways enriched in various samples.

Cell Transfection

NRF2-specific siRNA was purchased from GenePharma (Shanghai, China), with the following sequences: si-NC sense: 5′-AUGGCAUCAUAAGCUGCACAC-3′; si-NRF2: 5′-UGAAAGCACAGCAGAAUUTT-3′. Bone marrow-derived dendritic cells (BMDCs) were cultured until day 5 and transfected with siRNA using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following the manufacturer's instructions. Briefly, cells were seeded in 12-well plates at a density of 3×10^5 cells/well and transfected with 20 nmol/L siRNA. After 24 hours, the medium was replaced with fresh culture medium. Forty-eight hours post-transfection, cells were pretreated with 4OI for 2 hours and subsequently stimulated with LPS (100 ng/mL) for 3 hours. Finally, cells were harvested for qPCR analysis.

Western Blotting

Cells underwent two washes with ice-cold PBS before being broken down with RIPA buffer containing PMSF and a cocktail inhibitor. Cell lysates were separated by SDS-PAGE, then transferred to nitrocellulose membranes, and subsequently blotted. Antibodies including: anti-NRF2 (12,721, 1:1000, Cell Signaling Technology), and anti-GAPDH (sc-365062, 1:1000, Santa Cruz).

Statistical Analysis

The statistical significance was assessed using either the two-tailed unpaired Student's *t*-test or one-way/two-way ANOVA. All data analyses were conducted with GraphPad Prism 7.0 software, and p values less than 0.05 were deemed statistically significant.

Results

4OI Suppresses the Expression of Co-Stimulatory Molecules and Inflammatory Cytokines in DCs

4OI (Figure S1B), which is a permeable itaconate derivative, can be efficiently converted to itaconate (Figure S1A) intracellularly, thereby exerting immunomodulatory properties. To explore whether 4OI would affect the survival of DCs in vitro, the BMDCs were treated with different concentrations of 4OI, and the frequencies of apoptotic cells were analyzed. As shown in (Figure S1C), the percentages of apoptotic BMDCs did not change significantly with either low or high concentrations of 4OI treatment compared with that of the control, suggesting that 4OI did not influence the survival of BMDCs in vitro.

It is well accepted that DCs highly and continually express membrane co-stimulatory molecules, including CD40, CD80, and CD86, after activation in response to LPS stimulation, directly impact the function and stage of DCs in their process of antigen presentation and T-cell activation. We first investigated the effect of 4OI on DCs activation and maturation. As shown in (Figure 1A–D), 4OI-treated BMDCs had decreased expression of MHC class II and co-stimulatory molecules CD40, CD80, and CD86 in response to LPS stimulation. Moreover, BMDCs treated with 4OI exhibited a reduction in CD11c expression, accompanied by a diminished capacity for antigen phagocytosis (Figure S2A and B). Activated DCs release significant amounts of inflammatory cytokines such as IL-12, IL-6, and IL-1β, which facilitate T-cell differentiation driven by DCs. We also observed that 4OI-treated BMDCs expressed a lower mRNA level of IL-12a, TNF-α, IL-6 and IL-1β in response to LPS stimulation (Figure 2A–D). Together, these data imply that 4OI hinders the maturation phenotype and the expression of proinflammatory cytokines in DCs.

4OI Inhibits DCs Antigen Presentation Capabilities in vitro

We next analyzed whether 4OI regulates the ability of DCs to promote T-cell proliferation in vitro. 4OI-treated BMDCs were pulsed with whole ovalbumin (OVA) or OVA peptide and co-cultured with OVA-specific $CD4^+$ or $CD8^+$ T cells

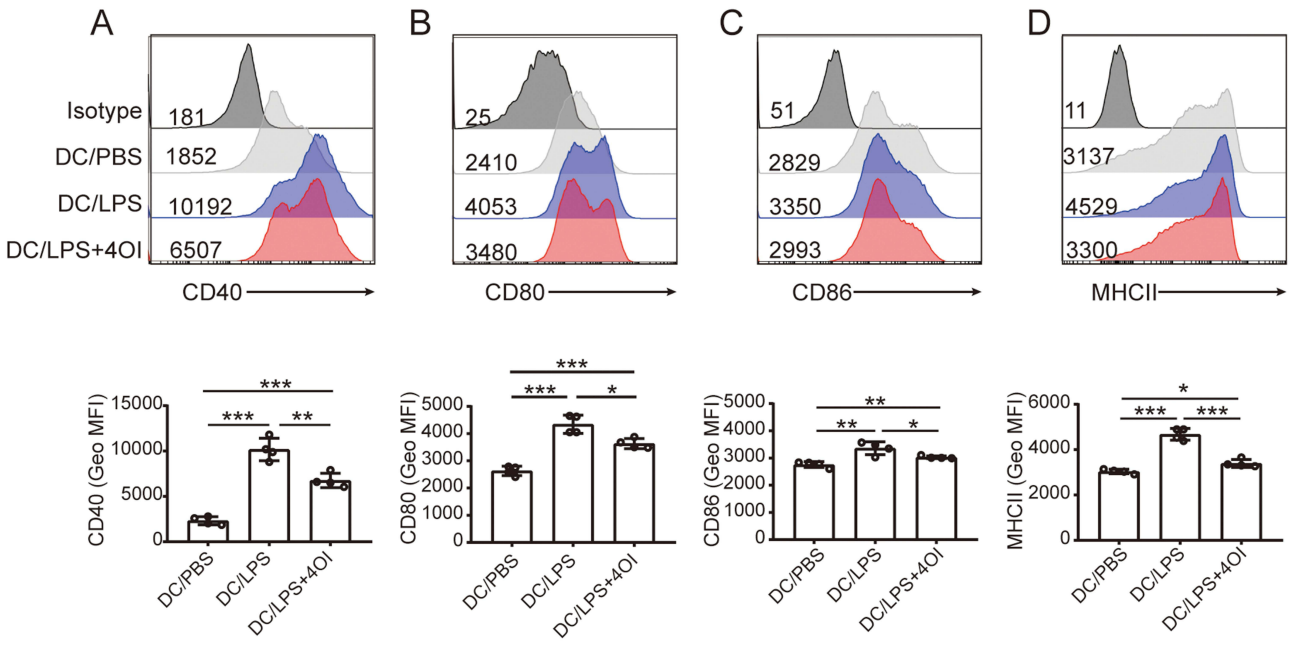

Figure 1 4OI represses the expression of co-stimulatory molecules in BMDCs. (**A**–**D**) BMDCs were pretreated with 4OI (250μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) for 24 hours. The expression of CD40, CD80, CD86, and MHC-II. on BMDCs in three groups was analyzed by flow cytometry. Data are shown as mean ± s.e.m from three independent experiments (n=4). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

from transgenic mice (OT II and OT I, respectively). As expected, 4OI reduces OVA and $OVA_{323-339}$ pulsed BMDC-induced $CD4^+$ T cell proliferation (Figure 3A). Similarly, 4OI also reduces OVA and $OVA_{257-264}$ pulsed BMDCs-induced $CD8^+$ T cell proliferation compared with that of the control (Figure 3B). These results demonstrate that 4OI represses DCs function in antigen presentation and promotion of T cell proliferation in vitro.

4OI Inhibits T-Cell-Priming Capacity of DCs in vitro

It is widely acknowledged that the cytokines secreted by DCs are vital for the differentiation and operation of effector T cells. To further investigate how 4OI influences the capacity of DCs to promote T-cell responses in vitro. 4OI-treated BMDCs were pulsed with OVA and incubated with OVA-specific $CD4^+$ or $CD8^+$ T cells from OT II and OT I mice respectively. Fewer IFN-γ, TNF-α, and IL-2-secreting cells were detected after $CD4^+$ T cells were co-cultured with 4OI-treated BMDCs compared to the untreated cells (Figure 4A). In similar experiments with $CD8^+$ T cells, IFN-γ, TNF-α, and IL-2 expression were also significantly decreased in OT I $CD8^+$ T cells co-cultured with 4OI-treated BMDCs (Figure 4B). Furthermore, 4OI demonstrates a dose-dependent inhibition of the T-cell-priming capacity of DCs (Figure S3A and B) These data suggested that the 4OI treatment inhibits DCs T-cell-priming function in vitro.

4OI Attenuates the Antitumor Function of BMDCs

By cross-presenting antigens from tumor cells to naïve T cells, DCs were key in developing specific T-cell responses that help control tumor growth and dissemination. To investigate whether 4OI treatment affected the antitumor function of BMDCs in vivo. As shown in Figure 5A, 4OI pretreated BMDCs were added with OVA protein and transferred into WT mice via intravenous injection on day-7 before B16-OVA cells inoculation. As expected, 4OI-treated BMDCs-immunized mice showed faster growth of B16-OVA tumors than that of the untreated control group (Figure 5B). Tumor volume and mass were evaluated on day 19 after tumor inoculation. It was shown that compared to the untreated control group, tumor volume and mass in mice immunized with 4OI-treated DCs were significantly increased (Figure 5C and D). Collectively, these findings imply that the anti-tumor function of 4OI-treated BMDCs was attenuated in vivo.

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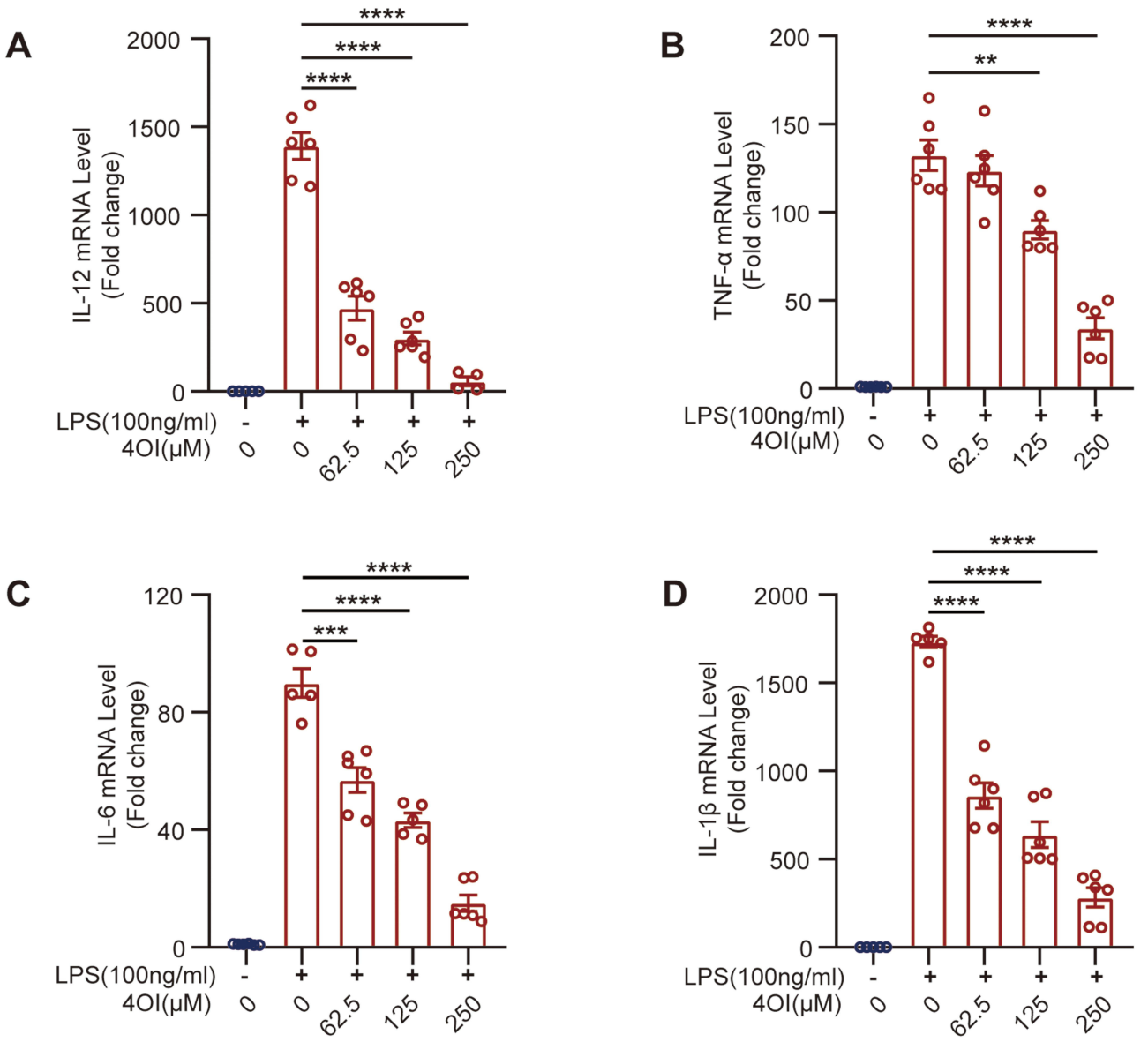

Figure 2 4OI inhibits the LPS-activated pro-inflammatory cytokines production in BMDCs. (**A**–**D**) BMDCs were pretreated with 4OI (250μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) for 3 hours. (**A**). RT-qPCR results showing mRNA expression of IL-12a. (**B**). RT-qPCR results showing mRNA expression of TNF-α. (**C**). RT-qPCR results showing mRNA expression of IL-6. (**D**). RT-qPCR results showing mRNA expression of IL-1β. Data are shown as mean ± s.e.m from three independent experiments (n=6). $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

4OI Represses BMDCs-Induced Antitumor T Cell Responses

Given the attenuated capacity of 4OI-treated DCs to inhibit tumor growth, we hypothesized that 4OI-treated BMDCs-immunized tumor-bearing mice would elicit weaker immune responses in tumor-bearing mice. To test this hypothesis, we intravenously transferred 4OI-pretreated or untreated BMDCs pulsed with OVA protein to WT mice. Seven days later, the mice were subcutaneously injected with B16-OVA cells. The tumor-bearing mice were euthanized 19 days post tumor inoculation followed by analysis of the percentages and absolute numbers of T cells that harvested from tumors, tumor-draining lymph nodes (DLNs), and spleens. As shown in Figure 6A, compared to the untreated BMDC group, there was a significant decrease in both the percentages and counts of $CD4^+$ and $CD8^+$ T cells in tumors and DLNs, but not in spleen tissues.

Next, we examined the effect of 4OI-treated BMDCs immunization on the functional activity of T cells in tumors, DLNs and spleens. On day 19 after B16-OVA cells inoculation, cells derived from tumors, DLNs, and spleens underwent short-term ex vivo activation by a pre-mixed leukocyte activation cocktail with phorbol myristate acetate, ionomycin, and

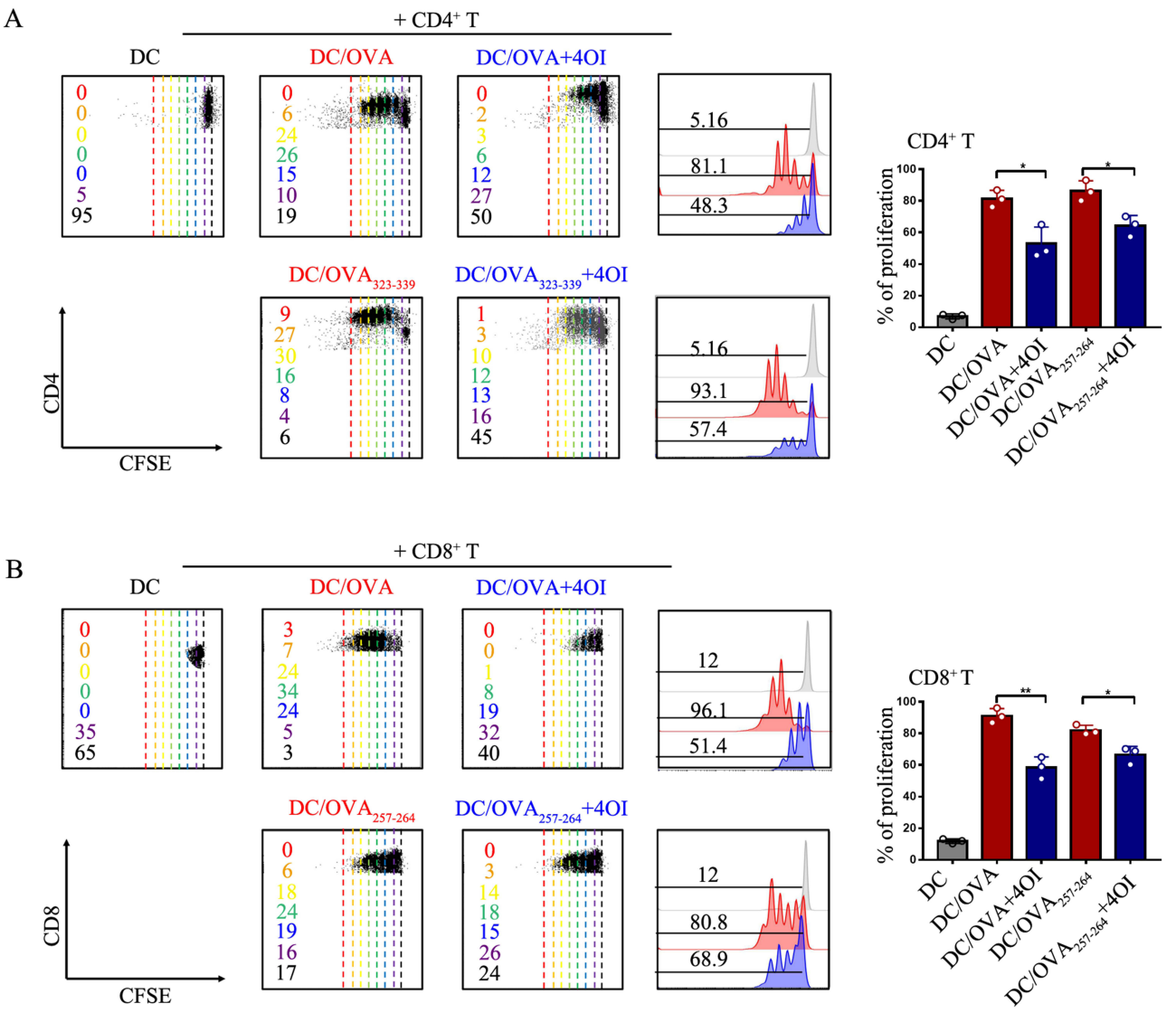

Figure 3 4OI inhibits BMDCs-induced T-cells proliferation. (**A** and **B**) BMDCs were pretreated with 4OI (250μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) for 3 hours, washed and pulsed with OVA (50μg/mL) or OVA peptide ($OVA_{323-339}$/$OVA_{257-264}$) for 5 hours, and then 4OI and OVA treated BMDCs were collected to coculture with purified carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD4^+$ OT-II or $CD8^+$ OT-I T cells. After a co-culture period of 72 hours (**A**) or 48 hours (**B**), $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry for proliferation as measured by CFSE dilution. Data are shown as mean ± s.e.m from three independent experiments (n=3). *$p < 0.05$; **$p < 0.01$.

brefeldin A, and were then assessed for T cell cytokine production. The percentages and numbers of TNF-α and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells from tumors, DLNs, and spleens of tumor-bearing mice were decreased compared with immunized with untreated BMDCs (Figure 6B and C). Together, these data suggest that 4OI represses BMDCs-induced T-cell responses in tumor-bearing mice.

4OI Represses Secretion of Pro-Inflammatory Cytokines Through Increasing and Activating NRF2

To explore the molecular basis of how 4OI modulates the function of DCs, we analyzed differential gene expression in BMDCs treated with 4OI using RNA sequencing. We identified that a subset of genes manipulated by NRF2 (including Nqo1, Gclc, Gclm and Hmox1), which were involved in antioxidant responses and cell metabolism, were significantly up-regulated in 4OI-treated BMDCs (Figure 7A). Our RNA sequencing data also revealed that the 4OI treatment impeded the expression of several cytokines, such as Il-12a and Il-12b (Figure 7A). The findings indicate that the

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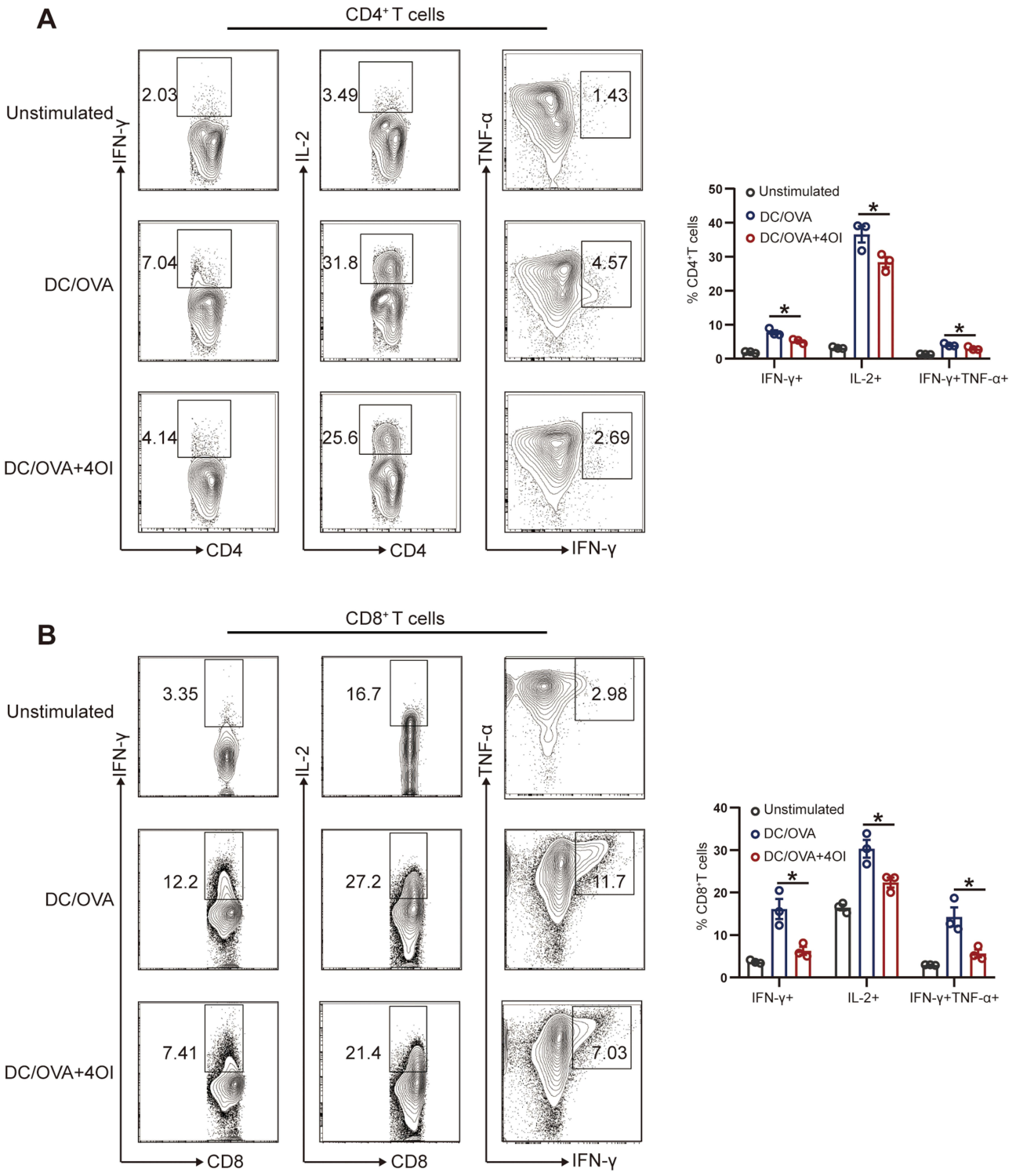

Figure 4 4OI represses BMDCs-induced T-cells cytokines production. BMDCs were pretreated with 4OI (250μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) for 3 hours, washed and pulsed with OVA (50μg/mL) for 5 hours, and then 4OI and OVA treated BMDCs were collected to coculture with purified $CD4^+$ OT-II or $CD8^+$ OT-I T cells. After a co-culture period of 36 hours, the OT-II (**A**) and OT-I (**B**) T cells were analyzed by flow cytometry for IFN-γ, IL-2, and TNF-α production. Data are shown as mean ± s.e.m from three independent experiments (n=3). *$p < 0.05$.

induction of inflammatory cytokines was significantly abrogated and expression of antioxidant and metabolism related genes was enhanced in 4OI treatment. This suggests that 4OI may be involved in the KEAP1/NRF2 pathway. As expected, functional enrichment analysis of Molecular Function Gene Ontology (MFGO) and Kyoto Encyclopedia of

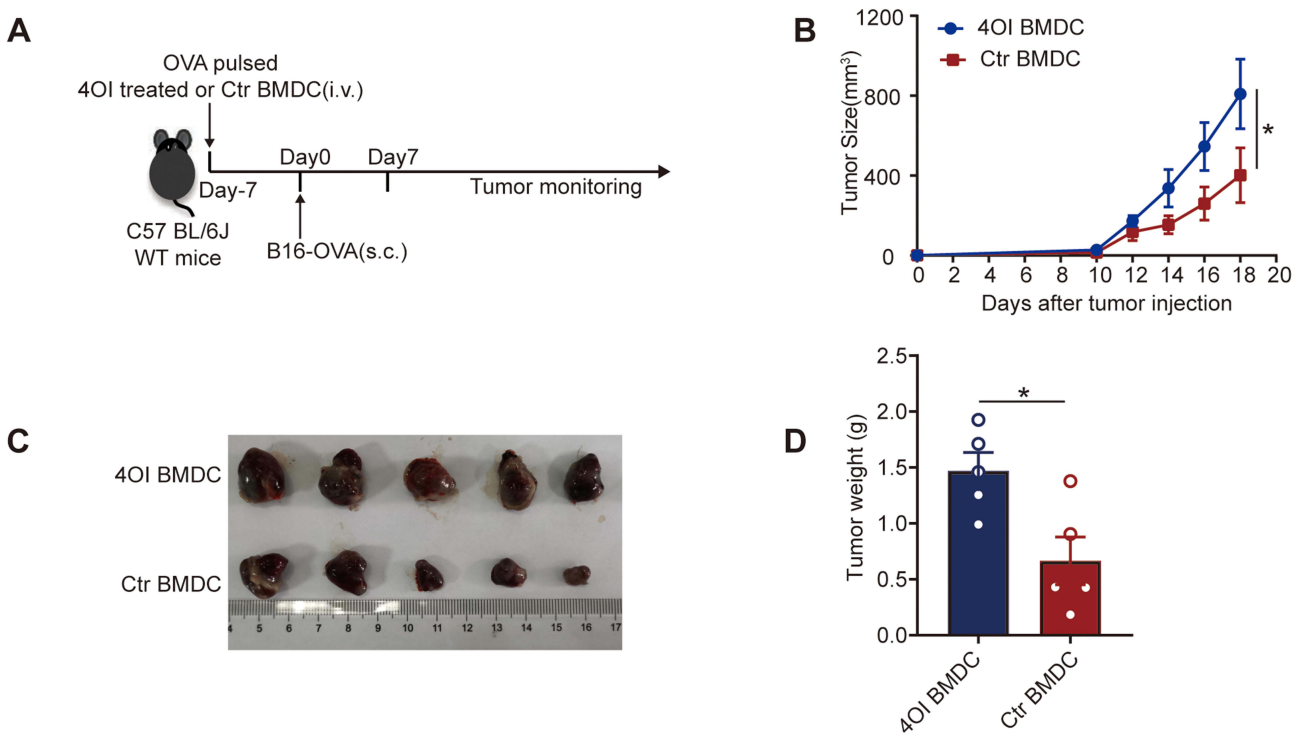

Figure 5 4OI attenuates the antitumor function of BMDCs. (**A**–**D**). WT mice were immunized OVA pulsed 4OI treated and Ctrl BMDCs and then were subcutaneously transplanted with B16-OVA cells. Tumor volumes (**B**) were monitored every 2–3 days, and the mass of tumors was pictured (**C**) and weighted (**D**) upon the sacrifice of mice on Day 19. Data are from one experiment representative of two independent experiments with similar results (n=5) (mean + s.e.m.). *$p < 0.05$.

Genes and Genomes (KEGG) revealed that 4OI not only significantly affected cytokine activity and TLR pathway, but also influenced antioxidant activity, glutathione metabolism, and xenobiotic metabolism in BMDCs (Figure 7B and C), further suggesting that 4OI could regulate NRF2 pathway. Differential gene expression analysis revealed that a significant subset of NRF2 downstream target genes was upregulated in BMDCs treated with 4OI (Figure 7D). To further validate the RNA sequencing results, we conducted RT-qPCR, confirming that the NRF2 target genes tested were also upregulated in 4OI-treated BMDCs (Figure 7E). As shown in Figure 7F, itaconate features an electrophilic α, β-unsaturated carboxylic acid that has the potential to alkylate cysteine residues in proteins through a Michael addition, resulting in the formation of a 2.3-dicarboxypropyl adduct. KEAP1, important in the antioxidant response, is a prominent candidate for cysteine alkylation. Under normal conditions, KEAP1 interacts with and facilitates the degradation of NRF2. Nonetheless, when key cysteine residues in KEAP1 are alkylated, newly produced Nrf2 can accumulate, translocate to the nucleus, and prompt the transcription machinery for enhancing antioxidant and anti-inflammatory responses. To verify whether 4OI could regulate KEAP1/NRF2 pathway, 4OI was used to pretreat BMDCs prior to LPS stimulation, and the protein level of NRF2 was analyzed by immunoblotting. The results demonstrated that 4OI significantly enhanced LPS-induced NRF2 accumulation (Figure 7G), indicating that 4OI promotes NRF2 activation. To investigate whether the inhibitory effect of 4OI on DC function is dependent on NRF2, we employed a targeted siRNA approach to knockdown NRF2 expression in BMDCs. Following NRF2 silencing, BMDCs were pretreated with 4OI and subsequently stimulated with LPS. RT-qPCR analysis was performed to evaluate the expression of inflammatory cytokines. The results revealed that genetic ablation of NRF2 significantly attenuated the 4OI-mediated suppression of pro-inflammatory cytokine production in BMDCs (Figure 7H). These findings provide compelling evidence that the immunomodulatory effects of 4OI on DC function are mechanistically dependent on the NRF2.

Discussion

The initiation of adaptive immune responses, essential for stopping tumor cells from escaping immunosurveillance, relies heavily on DCs.[3,4] For effective immune responses against tumors, the maturation and activation of DCs are crucial.[31]

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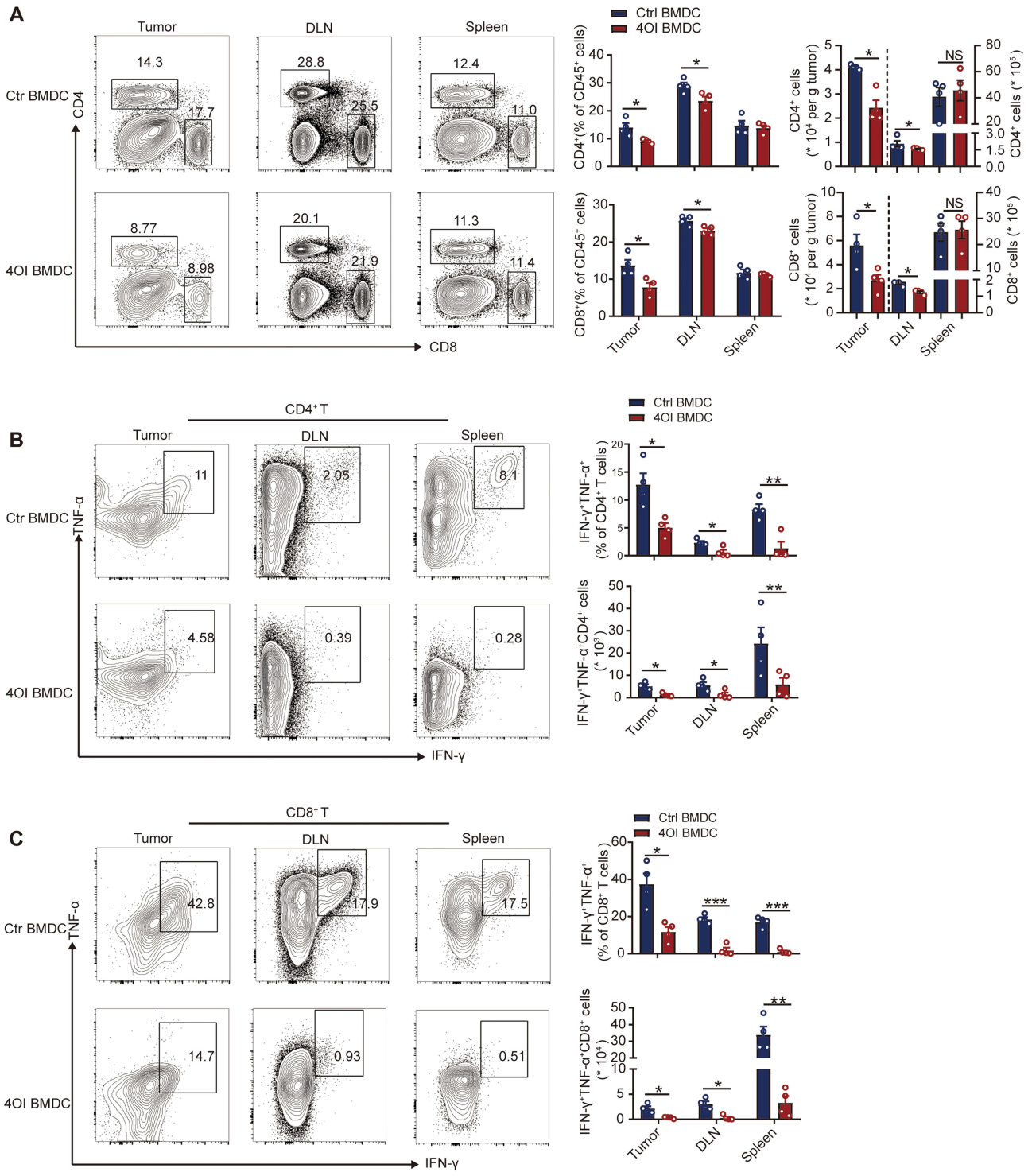

Figure 6 4OI represses BMDCs-induced antitumor T cell responses. (**A**–**C**) WT mice were immunized OVA pulsed 4OI treated and Ctrl BMDCs and then were subcutaneously transplanted with B16-OVA cells. Cells in the tumor, DLN, and spleen were isolated and stained with antibodies as indicated. Representative flow cytometric dot-plots showing the percentages of indicated cell subsets in the tumor, DLN, and spleen in control (Ctrl) vs 4OI treated BMDCs group. Bar graphs showing the percentages (middle panels) and absolute numbers (right panels) of indicated cells in the tumor, DLN, and spleen. Data are from one experiment representative of two independent experiments with similar results (n=4) (mean + s.e.m.). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS, no significance.

However, DCs within the TME commonly reside in a state of quiescence, imposing constraints on the elicitation of a potent adaptive immunity against the burgeoning tumor.[3,4] Extensive studies have described metabolites in the TME that are recognized for inhibiting DC activation and causing changes in the DC phenotype.[10,11,32,33] Tumor-derived lactate

signaling causes DCs to transition their metabolism from glycolysis to fatty acid oxidation, programming them into a regulatory state.[10] Similar to lactate, another metabolite, Alpha-Ketoglutarate (α-KG) promote an immunosuppressive cellular phenotype of DCs.[33] Therefore, exploring the immunomodulatory effects of metabolites on DCs in TME will provide new strategies for tumor immunotherapy. In this study, we discovered that itaconate, possessing immunomodulatory abilities, significantly disrupts the development and activity of DCs in vitro, encouraging an immature DC phenotype with decreased levels of CD40, CD80, CD86, and MHC-II, and ultimately facilitating tumor progression. However, it is essential to determine the optimal dosage of 4OI for its therapeutic application.

Due to its powerful immune modulation properties, itaconate is associated with a range of inflammatory diseases.[12,13] The mechanisms by which itaconate influences immune responses have been the subject of extensive investigation.[13] Growing evidence indicates that itaconate influences both innate and adaptive immune cells.[34–37] So far, activated macrophages and myeloid cells are the sole cells identified to generate significant amounts of itaconate.[13] Besides, Weiss, J.M. et al demonstrated that itaconate was greatly upregulated in macrophages isolated from the peritoneal cavity of mice with tumors compared to naïve mice.[21] Itaconate exhibits both anti-inflammatory and tumor-promoting effects in macrophages.[22,23] Recently, it has been reported that tumor cells stimulate IRG1 expression in tumor-associated

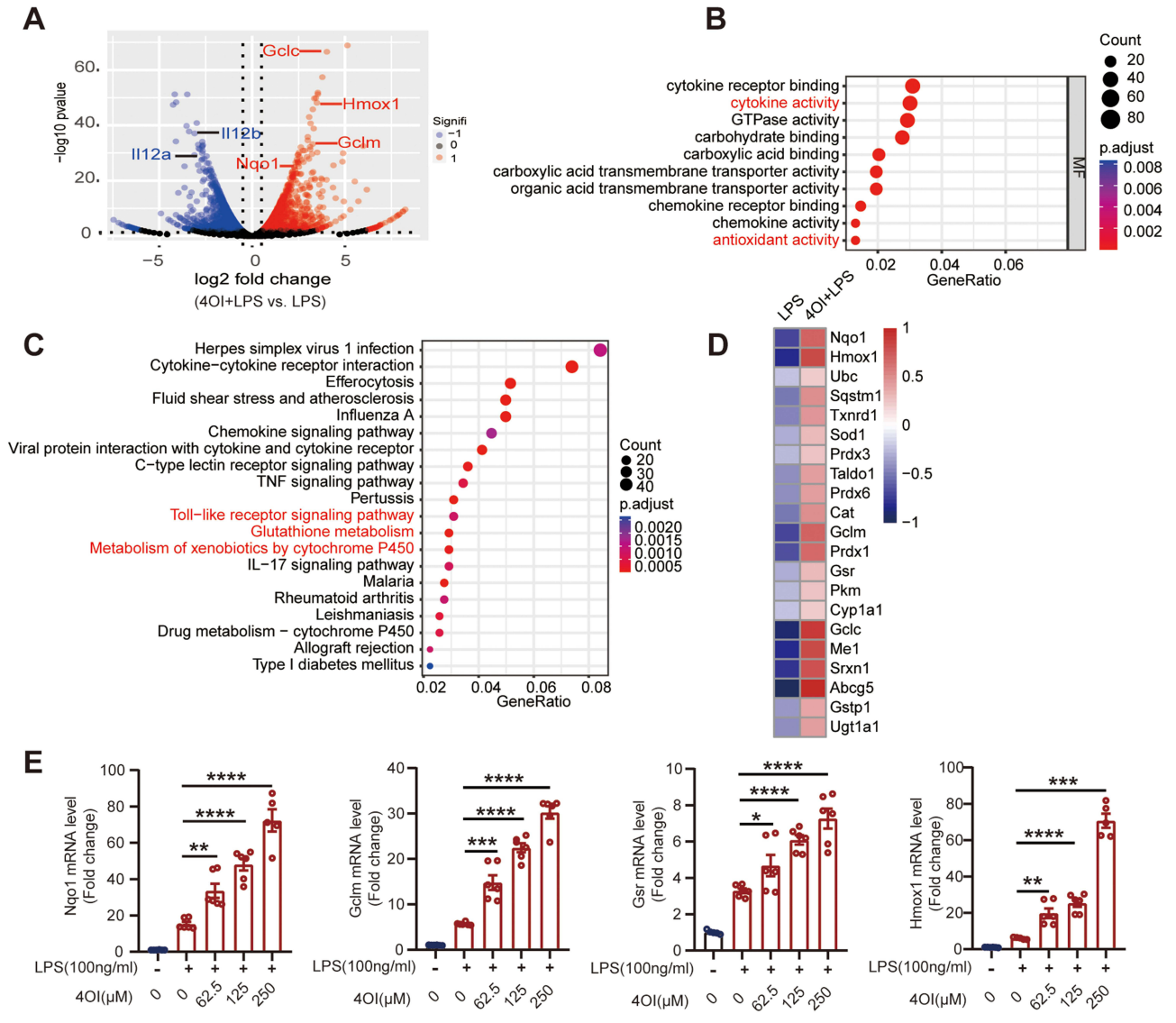

Figure 7 Continued.

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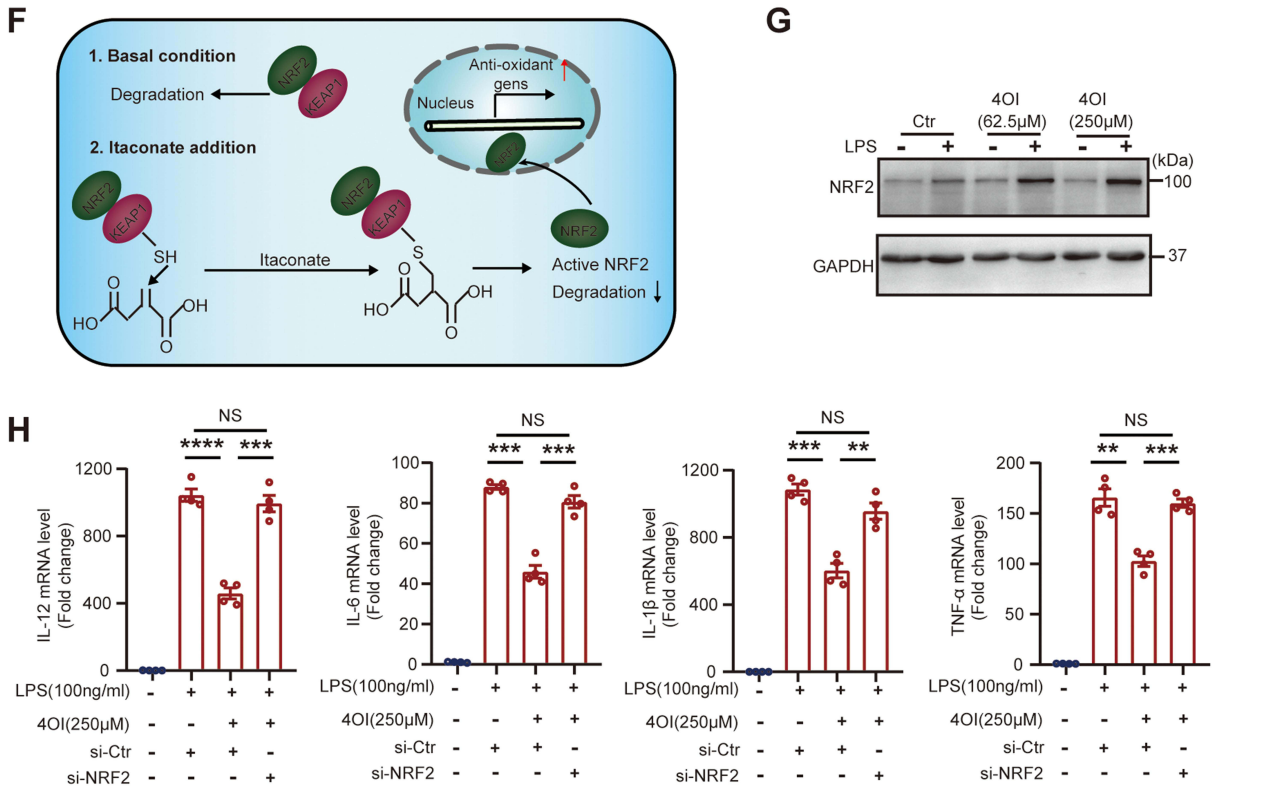

Figure 7 4OI represses production of pro-inflammatory cytokines through increasing and activating NRF2. (**A**–**D**) RNA-sequencing analysis in BMDCs were pretreated with 4OI (250μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100ng/mL) for 3 hours. A total of 738 genes were significantly down-regulated (blue) and 737 genes were significantly up-regulated (red) in the 4OI+LPS group, comparing with the LPS group. Differential expressed genes were defined as the genes with the changes ≥2 folds and *p* value <0.05 between two groups (**A**). Top 10 MFGO terms that are enriched in (**A**) were analyzed using the R package cluster Profiler (**B**). Top 20 KEGG pathways significantly changed in (4OI+LPS)-treated BMDCs compared to LPS-treated BMDCs (**C**). A heatmap of up-regulated NRF target genes in (4OI+LPS)-treated BMDCs relative to that in LPS-treated BMDCs (**D**). RT-qPCR analysis of the indicated NRF2 target genes in the BMDCs with or without LPS (3h) and 4OI (**E**). Reactivity of itaconate with KEAP1 thiol group (**F**). Immunoblot analysis was performed to assess the NRF2 protein levels in whole-cell lysates of BMDCs that were pretreated with 4-OI for 2 hours, followed by stimulation with LPS (100ng/mL) for 24 hours (**G**). By transfecting siRNA (si-NRF2), NRF2 was knocked down in BMDCs. Then, the BMDCs were pretreated with 4OI (250 μM) or DMSO (Ctrl) for 2 hours, followed by stimulation with LPS (100 ng/mL) for 3 hours. RT-qPCR results showing mRNA expression of IL-12a, IL-6, IL-1β and TNF-α (**H**). Data are from one experiment representative of two independent experiments with similar results (mean + s.e.m.). **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001; NS, no significance.

macrophages, and itaconate produced by IRG1/ACOD1 inhibits the expression of inflammatory genes.[22] Besides the ability to modulate macrophages, Zhao H. et al demonstrate that myeloid-derived suppressor cells (MDSCs) release itaconate, which is absorbed by $CD8^+$ T cells, then inhibiting growth, cytokine secretion, and cytolytic function.[23] Recent reports indicate that tumor cell-intrinsic itaconate enhances tumor immunogenicity,[38] suggesting that itaconate possesses a dual role in tumors. Specifically, tumor cell-intrinsic itaconate appears to exert anti-tumor effects, while immune cell-intrinsic itaconate may promote tumor progression. Here, we observed the regulation of exogenous itaconate on DCs. We found that itaconate derivative, 4OI, was capable of directly altering the maturation and activity of DCs. Following 4OI treatment, DCs experienced a significant inhibition in function, with decreased levels of proinflammatory cytokines. Additionally, the expressions of CD40, CD80, CD86, and MHC-II were reduced. The evidence indicates that 4OI could easily impair the maturation and activity of DCs and then accelerate the progression of tumors. However, due to the considerable complexity and heterogeneity of the TME, further investigation is necessary to ascertain whether itaconate exerts similar immunomodulatory effects on DCs within this context. Additionally, the processes by which DCs absorb itaconate in the TME are not yet completely understood. Current literature suggests potential intracellular uptake through receptors such as OXGR1 and SLC13A3;[39,40] however, it remains unclear whether itaconate is internalized via these pathways or through alternative novel mechanisms, warranting further research.

Itaconate exerts its immunomodulatory properties through a multitude of mechanisms.[13] These mechanisms encompass the suppression of succinate dehydrogenase, which can affect the level of succinate with diverse functions in inflammatory processes,[41,42] the restraint of glycolysis at various levels (thus curtailing inflammation),[43,44] stimulation of anti-inflammatory transcription factors NRF2 and ATF3,[18] and the repression of the NLRP3 inflammasome.[45,46] Recent studies have reported that itaconate can also exert immunomodulatory roles through epigenetic reprogramming and post-translational modifications.[47,48] Chen LL. et al demonstrate that itaconate selectively inhibits TET DNA dioxygenases to dampen the inflammatory responses.[47] Furthermore, itaconate was also found to suppress inflammation via alkylation of STING.[48]

In this study, we observed that exogenous itaconate derivative, 4OI, could significantly suppress the maturation and function of DCs in vitro. Mechanistically, 4OI activates KEAP1/NRF2 pathway in DCs. It has been previously reported that metabolites in the tumor TME, such as lactate, interact with GPR81 expressed on DCs, thereby inhibiting their maturation and antitumor functions.[11] Furthermore, products of lipid peroxidation in the TME cause endoplasmic reticulum stress in DCs, resulting in lipid buildup and the creation of cytosolic lipid droplets. This process subsequently disrupts the transport of antigen peptide-MHC-I complexes.[7] In this study we propose a new metabolite, itaconate, and a new mechanism to inhibit DCs's anti-tumor immunity. However, it remains unclear whether 4OI inhibits the maturation and function of DCs in a NRF2-dependent manner, and whether endogenous IRG1/itaconate play similar regulatory roles in modulating DCs function. In addition, NRF2 is known to exert pleiotropic effects, which may manifest as both pro-inflammatory and anti-inflammatory outcomes depending on the specific context.[49] Therefore, the dual role of NRF2 in DCs maturation and cytokine production warrants further investigation.

In summary, our study suggests that itaconate negatively affects the maturation and functionality of DCs, which in turn accelerates tumor development. This study reveals a previously unknown mechanism through which itaconate fosters tumor growth by inducing immune tolerance of DCs. These insights provide a compelling rationale for targeting IRG1/itaconate to enhance the effectiveness of tumor immunotherapy.

Abbreviations

DCs, dendritic cells; BMDCs, bone marrow-derived dendritic cells; 4OI, 4-octyl itaconate; NRF2, nuclear factor, erythroid 2p45-related factor 2; KEAP1, Kelch-like ECH-associated protein 1; TME, tumor microenvironment; CTLA4, cytotoxic T lymphocyte antigen 4; IRG1, immune-responsive gene 1; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; WT, wild-type; OVA, ovalbumin; DLNs, draining lymph nodes; MFGO, Molecular Function Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; α-KG, Alpha-Ketoglutarate; MDSCs, myeloid-derived suppressor cells.

Ethical Statement

All mouse experiments were approved by the Committee on the Use of Live Animals in Research and Teaching of Jiangsu University (UJS-IACUC-AP-20190307016). All animal experiments followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.5–23, revised 1996; NIH Bethesda, MD, USA).

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Disclosure

All authors declare no conflicts of interest.

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