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Cell Metabolism

Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation

Graphical Abstract

Highlights

- Exogenous itaconate exerts anti-inflammatory effects in vitro and in vivo
- Itaconate inhibits Sdh and regulates succinate levels in LPS-activated macrophages
- Itaconate controls mitochondrial respiration changes in inflammatory macrophages
- Itaconate limits IL-1β, IL-18, IL-6, IL-12, NO, and HIF-1α, but not TNF-α, production

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In Brief

Macrophage activation is accompanied by TCA cycle remodeling, resulting in endogenous metabolites moonlighting as regulatory mediators of the inflammatory response. Lampropoulou et al. investigate the role of itaconate, one of the most highly induced metabolites in activated macrophages, and show that itaconate regulates succinate levels, mitochondrial respiration, and cytokine production.

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Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation

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SUMMARY

Remodeling of the tricarboxylic acid (TCA) cycle is a metabolic adaptation accompanying inflammatory macrophage activation. During this process, endogenous metabolites can adopt regulatory roles that govern specific aspects of inflammatory response, as recently shown for succinate, which regulates the pro-inflammatory IL-1β-HIF-1α axis. Itaconate is one of the most highly induced metabolites in activated macrophages, yet its functional significance remains unknown. Here, we show that itaconate modulates macrophage metabolism and effector functions by inhibiting succinate dehydrogenase-mediated oxidation of succinate. Through this action, itaconate exerts anti-inflammatory effects when administered in vitro and in vivo during macrophage activation and ischemia-reperfusion injury. Using newly generated Irg1−/− mice, which lack the ability to produce itaconate, we show that endogenous itaconate regulates succinate levels and function, mitochondrial respiration, and inflammatory cytokine production during macrophage activation. These studies highlight itaconate as a major physiological regulator of the global metabolic rewiring and effector functions of inflammatory macrophages.

INTRODUCTION

Macrophage activation by lipopolysaccharide (LPS) is accompanied by marked metabolic changes including upregulation of aerobic glycolysis (Everts et al., 2014), impaired mitochondrial respiration, disruption of the TCA cycle (Jha et al., 2015), and accumulation of succinate (Kelly and O’Neill, 2015). While a significant portion of this metabolic adaptation is transcriptionally controlled (e.g., via modulation of Nos2 and Cox2), succinate represents an example of a metabolite that affects major inflammatory pathways in both immune and non-immune cells by controlling IL-1β expression, HIF-1α activity, and ROS production (Kelly and O’Neill, 2015). However, the mechanism(s) regulating succinate levels in inflammatory macrophages have remained unknown.

Itaconate has been recently shown to be made by macrophages in response to LPS (Streiko et al., 2011) and certain infections (Michelucci et al., 2013) and is generated by the mitochondria-associated enzyme (Degrandi et al., 2009) immune responsive gene 1 (Irg1). Iaconate is believed to have anti-bacterial function due to its ability to inhibit isocitrate lyase, a bacterial glyoxylate shunt enzyme and due to its bactericidal effect when added at supraphysiological concentrations to macrophage-free S. enterica, M. tuberculosis, and L. pneumonophila cultures (Michelucci et al., 2013) (Naujoks et al., 2016). An effect of itaconate on mammalian enzymes also was reported in the context of Sdh inhibition (Ackermann and Potter, 1949) (Németh et al., 2016). It is unknown, however, whether this biochemical effect of itaconate translates into macrophage metabolism and function. Here, using exogenous itaconate as well as newly generated Irg1−/− mice, we report that itaconate...
potently modulates macrophage activation and inflammatory responses by controlling TCA cycle remodeling.

RESULTS AND DISCUSSION

Itaconate Treatment Has Anti-inflammatory Effects during Macrophage Activation

Itaconate production is one of the hallmarks of macrophage activation on both the transcriptional and metabolic levels: Irg1 is one of the most highly induced enzymes in activated macrophages, and itaconate accumulates at high levels within cells (Figure 1A). Metabolomic profiling of culture supernatants from activated bone marrow-derived macrophages (BMDMs) revealed that itaconate was also secreted, as suggested previously (Strelko et al., 2011) (Figure 1B). The magnitude of itaconate production prompted us to investigate its potential regulatory role.

We first tested the effect of exogenously added itaconate on the inflammatory response induced after LPS or LPS + IFN-γ.
stimulation. We treated mouse BMDM with physiologically relevant doses of dimethyl itaconate (DI) (Figure S1A), a membrane-permeable non-ionic form of itaconate. Pretreatment with DI suppressed iNOS protein expression (Figure 1C) and IL-12p70 and IL-6 secretion (Figures 1D and 1E), thereby interfering with activation of pro-inflammatory macrophages. In contrast, TNF-α levels remained unchanged (Figure 1E), indicating that the effects of DI-treatment were not due to global inhibition of NF-kB-dependent gene expression. To determine the specific pathways affected by itaconate, we performed global transcriptional profiling by RNA-seq on BMDM pretreated with DI or vehicle and then stimulated with LPS. Differential gene expression confirmed that DI-treatment led to downregulation of a spectrum of pro-inflammatory transcripts (Figures 1F and S1B), including Nos2, Il1b, and Il12b.

The RNA-seq analysis also revealed that DI-pretreatment modulated the expression of several LPS-regulated genes involved in inflammasome activation and function (Figure 1F), including Il1b, Il18, P2rx7, Casp1, and Pycard (ASC). Indeed, DI potently inhibited production of mature IL-1β and IL-18 induced under prototypical NLRP3-activating conditions, namely LPS-driven priming (signal 1) followed by signal 2 cytokines ATP, nigericin, and monosodium urate crystals (Figures 1G and 1H), whereas it affected inflammasome-induced cytotoxicity only moderately (Figure S1C). DI-treated BMDM also had impaired IL-1β production upon AIM-2-dependent inflammasome activation (Figure S1D), suggesting a broader regulatory effect on the inflammasome. The decreased protein expression of pro-IL-1β, ASC, and NLRP3 in DI-treated cells (Figure 1I) indicated that itaconate-mediated inhibition of inflammasome function was due primarily to a defective priming phase.

In view of this anti-inflammatory action of itaconate, and given its known cidal ability on extracellular bacteria, we next determined its effect on intracellular bacteria during macrophage infection. To this end, we infected BMDMs with Salmonella typhimurium, an intracellular Gram-negative bacterium that triggers TLR4 signaling and NLRP3 inflammasome activation (Broz et al., 2010) in the presence or absence of DI pretreatment. Consistent with our previous findings, infection-induced IL-1β, IL-6, and nitric oxide (NO), but not TNF-α, were abrogated in DI-treated cells (Figure S1E), whereas the number of intracellular bacteria was comparable between DI-treated and control BMDM (Figure S1F), indicating that the anti-inflammatory effects of itaconate at non-cytotoxic concentrations did not result from direct bactericidal activity. Rather, itaconate likely has a regulatory role, as supported by lrg1 induction in the viral infection context (Cho et al., 2013) (Figure S1G), indicating it has regulatory functions that are not specific to anti-bacterial response.

**Exogenous Itaconate Inhibits Succinate Dehydrogenase In Vitro and In Vivo**

As perturbations in cellular metabolism can lead to transcriptional defects in IL-1β production and inflammasome activation (Moon et al., 2015), we hypothesized that itaconate exerts its anti-inflammatory action, in part, by interfering with cellular metabolism. Using computational analysis of transcriptional data (Becker and Palsson, 2008), we investigated the possible rewiring of the metabolic flux triggered by itaconate in the absence of LPS. Using a flux balance analysis framework, we extended the metabolic model originally formulated for RAW 264.7 macrophage cell line (Bordbar et al., 2012) by including several reactions and enzymes that were absent in the original model (e.g., lrg1 and itaconate, see Experimental Procedures for details). We searched for fluxes in untreated and itaconate-treated conditions that were most consistent with the RNA-seq data (see Experimental Procedures). A comparative network highlights three types of metabolic flux change in response to itaconate treatment (Figures 2A and S2A): decreased metabolic flux (blue edges), increased metabolic flux (red), and reactions insensitive to itaconate addition (gray).

Two features of the computational analysis were apparent. First, itaconate addition was predicted to increase lactate dehydrogenase (Ldh) production. We confirmed this experimentally using Seahorse technology on unstimulated BMDM with and without DI treatment. We observed that extracellular acidification rate (ECAR), which occurs as a consequence of lactate accumulation in the medium, was increased in the presence of itaconate (Figure 2B). The second prediction was that itaconate addition should decrease the metabolic flux through Sdh. This suggested that itaconate might inhibit Sdh competitively, conceivably due the structural similarity between itaconate, succinate, and malonate, the latter a known Sdh inhibitor (Figure 2C). Indeed, 67 years ago, an inhibitory effect of itaconate on Sdh was postulated based on similar effects of malonate and itaconate on mitochondrial function (Ackermann and Potter, 1949). To evaluate this hypothesis, we compared the activity of purified Sdh in the presence or absence of itaconate. Notably, itaconate dose-dependently blocked the activity of Sdh when succinate was used near physiological concentration 1 mM (Bennett et al., 2009) (Figure S2B), and itaconate treatment of resting BMDM led to increased succinate levels (Figure 2D). Kinetic analysis confirmed the competitive mode of inhibition of Sdh by itaconate (Figure 2E, left), and Dixon plot analysis showed Ki for itaconate 0.22 mM (Figure 2E, right). The calculated Km value for succinate was 0.29 ± 0.8 mM. These results are in line with the molar amounts of intracellular succinate and itaconate present in LPS-activated macrophages at 24 hr (Figure S2C). These data suggest that the anti-inflammatory effects of itaconate in BMDM were likely due to inhibition of Sdh. Supporting this notion, pretreatment with dimethyl malonate also inhibited IL-1β production after LPS + ATP stimulation (Figure S2D).

We next evaluated whether itaconate inhibits Sdh activity in vivo. A recent study (Chouchani et al., 2014) highlighted the proinflammatory role of succinate oxidation in the context of cardiac ischemia-reperfusion (IR) injury. mROS generation during IR injury was proposed to occur in the mitochondria as a result of reverse electron transport (RET), whereby Sdh was fueled by the succinate that accumulated during ischemia. Accordingly, inhibition of Sdh by dimethyl malonate limited IR injury and mROS levels (Chouchani et al., 2014). We used this model to test whether itaconate would inhibit Sdh-mediated oxidation in vivo. Intravenous infusion of DI during ischemia markedly reduced myocardial infarct size (Figures 2F and 2G). While the area-at-risk was similar between the two groups (Figure 2H), the 42% reduction in infarct size with DI treatment was comparable to that seen after dimethyl malonate administration (Chouchani et al., 2014), suggesting a common mechanism of action.
To evaluate this hypothesis, we used an in vitro assay that mimics myocardial infarction injury by subjecting neonatal cardiomyocytes to hypoxic insult (Ma et al., 2012). Pretreatment with DI attenuated the hypoxia-induced increase in ROS generation (Figure 2I) and conferred dose-dependent protection from hypoxia-induced cell death (Figure 2J).

Therefore, we assessed whether itaconate-mediated Sdh inhibition influenced mROS generation. Pretreatment with DI...
impaired the ability of BMDM to upregulate mROS in response to LPS (Figures 2K and 2L). As interfering with mROS can affect inflammasome priming (Bauernfeind et al., 2011), the blunted mROS response mechanistically links Sdh inhibition and the anti-inflammatory effects of itaconate on IL-1β and IL-18 production (Figure 1G). Furthermore, it suggests that succinate processing rather than accumulation is important for the inflammatory rewiring of macrophages.

**Endogenous Itaconate Regulates Metabolic Remodeling, Succinate Levels, and Mitochondrial Respiration in Inflammatory Macrophages**

To test the physiological relevance of endogenous itaconate, we generated mice with a targeted disruption of the Irg1 gene (Figure S3). As BMDMs from Irg1−/− mice failed to produce or secrete itaconate (Figure 3A) after stimulation with LPS and IFN-γ, we conclude that Irg1 is the only enzyme carrying out itaconate synthesis under these conditions. LPS-activated WT macrophages show increased concentration of fumarate and malate due to an active aspartate-argininosuccinate shunt (Jha et al., 2015), as well as accumulation of succinate, presumably due to itaconate inhibition. To confirm this, we metabolically profiled Irg1−/− BMDMs and observed marked changes indicative of altered Sdh activity. Lack of Irg1 expression led to abrogation of succinate accumulation, whereas fumarate and malate concentrations were yet increased (Figure 3B). Thus, in the absence of endogenous itaconate, Sdh remained active and oxidized succinate to fumarate, which was rapidly converted to malate (Figure 3C).

Besides its role in the TCA cycle, Sdh is also part (as complex II) of the mitochondrial electron transport system. In LPS-activated macrophages, mitochondrial respiration decreases significantly (Kelly and O’Neill, 2015). Thus, we tested whether itaconate-mediated inhibition of Sdh influenced mitochondrial function by measuring oxygen consumption rates (OCR). Remarkably, contrary to WT cells, Irg1−/− BMDMs showed increased OCR at 24 hr post activation (Figure 3D), demonstrating that, by inhibiting Sdh, endogenous itaconate modulates mitochondrial respiration, as recently proposed for exogenous itaconate (Németh et al., 2016).

**Endogenous Itaconate Regulates Macrophage Inflammatory Responses**

We next assessed macrophage activation in the absence of endogenous itaconate. We used RNA-seq to profile differences in gene expression of LPS-activated wild-type (WT) and Irg1−/− BMDMs. Notably, the transcriptional signature in Irg1−/− cells was essentially inversely correlated with itaconate-treated WT cells: genes upregulated in Irg1−/− cells were downregulated in itaconate-treated WT BMDMs (Figure 4A), providing additional evidence that endogenous itaconate functions in a manner similar to exogenously added DI. Consistent with these observations, LPS-activated Irg1−/− BMDM produced more IL-12, NO,
Figure 4. LPS-Activated \(\text{Irg}^1^{-/-}\) Macrophages, which Lack Itaconate, Show Augmented Inflammatory Responses

(A) Transcriptional signatures of activated \(\text{Irg}^1^{-/-}\) and DI-treated activated WT BMDM are inversely related.
(B) IL-12 and NO levels in supernatants of WT and \(\text{Irg}^1^{-/-}\) BMDM stimulated with LPS + IFN-\(\gamma\) (24 hr).
(C) IL-6 and TNF-\(\alpha\) levels in supernatants of LPS-activated (24 hr) WT and \(\text{Irg}^1^{-/-}\) BMDM.
(D) Mature IL-1\(\beta\) and IL-18 levels in supernatants of WT and \(\text{Irg}^1^{-/-}\) BMDM stimulated with LPS (4 hr) and ATP.
(E) Relative \(\text{hif}1\alpha\) mRNA expression in LPS-activated WT and \(\text{Irg}^1^{-/-}\) BMDM (4 hr).
(F) Western blot analysis of HIF-1\(\alpha\) in lysates of WT and \(\text{Irg}^1^{-/-}\) BMDM activated as in (E); \(\alpha\)-tubulin was used as a loading control. Blot shown is representative of two independent experiments.

Data in (B)–(E) are shown as mean ± SEM (n = 3/group, each in 2–3 replicates). \(p\) values were calculated with two-tailed Student’s t test. \(*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.\)
and IL-6, but similar amounts of TNF-α, compared to WT cells (Figures 4B and 4C). Irg1<sup>−/−</sup> BMDM also sustained higher expression of mature IL-1β and IL-1β under conditions that stimulate NLPR3 (Figure 4D). Consistent with the known IL-1β-promoting effect of HIF-1α (Tannahill et al., 2013), we observed increased HIF-1α mRNA and protein levels in Irg1<sup>−/−</sup> cells and, reciprocally, suppression of HIF-1α expression in DI-treated BMDM (Figures 4E–4G). Thus, changes in HIF-1α and IL-1β production correlate with efficiency of succinate oxidation by Sdh. These observations raise the possibility that the HIF-1α axis is linked to the efficiency and directionality of the electron transport chain in activated macrophages rather than direct signaling through succinate accumulation.

In summary, our work identifies itaconate as part of a posttranscriptional mechanism that governs TCA cycle remodeling and macrophage activation via its inhibitory effect on Sdh and regulation of succinate levels. Our results expand the physiological roles of itaconate beyond a direct anti-bacterial action, to include regulation of TLR-mediated inflammatory cytokine production, and provide a physiological regulatory mechanism to control electron transport chain flow, succinate levels, ROS production, and tissue inflammation.

**EXPERIMENTAL PROCEDURES**

**Mice**

Irg1<sup>−/−</sup> (MGI:103206) mice were generated at Washington University after receiving embryonic stem cells (ESCs) (Irg1<sup>tm1Tok</sup>/KOMP2flox<sup>lacZ</sup>) from the Knockout Mouse Project Repository (KOMP, University of California, Davis) containing an insertion cassette between exons 3 and 4. This cassette prevents transcription of downstream exons 4 and 5 and production of mature protein. Irg1<sup>−/−</sup> C57BL/6N ESCs were microinjected into Cgju-Tyrc2J/J albino recipient female C57BL/6 mouse blastocystos. Chimeric mice with black coat color were selected and bred to wild-type C57BL/6N mice. Homozygous Irg1<sup>−/−</sup> mice were generated by intercrossing the heterozygous animals and confirmed by PCR. Irg1<sup>−/−</sup> mice were fertile and exhibited normal Mendelian frequencies, and BMDM from both sexes were used.

C57BL/6N WT mice from Charles River Laboratories were used as age-matched controls. Mice were maintained at Washington University under specific pathogen-free conditions in accordance with Federal and University guidelines and protocols approved by the Animal Studies Committee of Washington University.

**Differential and Activation of Macrophages**

BMDM were prepared from 6- to 8-week-old mice as described (Jha et al., 2015) and seeded at 10<sup>5</sup> cells/well in 96-well tissue-culture plates in RMPI-1640 medium (Thermo Fisher Scientific), and 100 U/mL penicillin-streptomycin (Thermo Scientific). Cells were treated or not with DI (0.25 mM, 12 hr; Sigma) or monosodium urate crystals (250 μg/mL; 4 hr; Sigma) and subjected to hypoxia in an oxygen control cabinet (Bordbar et al., 2012). Cells were subjected to hypoxia in an oxygen control cabinet (Bordbar et al., 2012). Cells were subjected to hypoxia in an oxygen control cabinet (Bordbar et al., 2012). Cells were subjected to hypoxia in an oxygen control cabinet (Bordbar et al., 2012). Cells were subjected to hypoxia in an oxygen control cabinet (Bordbar et al., 2012). Cells were subjected to hypoxia in an oxygen control cabinet (Bordbar et al., 2012).

**Quantification of Cytokines and Nitric Oxide**

Cytokines were quantified using DuoSet kits for IL-1β/IL-1F2, TNF-α, and IL-6 (all R&D systems), IL-12 ELISA MAX Deluxe Set (BioLegend), and IL-18 ELISA kit (Medical & Biological Laboratories). Nitric oxide was detected with Griess Reagent System (Promega).

**Western Blotting**

Cells were lysed in RIPA Lysis Buffer (Santa Cruz) and heat-denatured in reducing sample buffer (Thermo Fisher Scientific). Proteins were separated on 4%-20% polyacrylamide gradient gels (BioRad) and transferred onto PVDF membranes. Non-specific binding was blocked with 5% skim milk, and membranes were probed with primary antibodies to IL-1β (1:1,000; 125075, Cell Signaling), Nlrp3 (1:500; NB2P-12446, Novus), HIF-1α (1:500; NB100-449, Novus), α-tubulin (1:12,000; 212SS, Cell Signaling), and ASC (1:1,000; sc-22514-R, Santa Cruz), followed by incubation with anti-rabbit-HRP (1:10,000; sc-2030, Santa Cruz) and Clarity western ECL substrate (Bio-Rad).

**Extracellular Flux Analysis**

Real-time extracellular acidification and oxygen consumption rates were measured using Seahorse technology as described (Huang et al., 2014).

**Metabolite Profiling by GC-MS**

Cellular metabolites were extracted from an equal number of cells per sample and analyzed by GC-MS as described (Vincent et al., 2015). Briefly, intracellular metabolism was quenched with 800 μl of 80% methanol. To analyze secreted metabolites, 20 μl supernatants were added to 800 μl of 80% methanol. D-myristic acid (750 ng/sample) was used as internal standard. Extracts were dried by vacuum centrifuge and pellets resuspended in 30 μl pyridine-containing 10 mg/ml methoxamine hydrochloride, before being derivatized using N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide. Metabolite abundance was expressed relative to the internal standard.

**RNA-Seq Analysis**

mRNA was extracted with oligo-dT beads (Invitrogen), and libraries were prepared and quantified as described (Vincent et al., 2015).

**Flux Balance Analysis**

To investigate possible rewiring of the metabolic fluxes, we used flux balance analysis framework (FBA). Using the RAW 264.7 macrophage cell line metabolic model (Jordar et al., 2012) and an algorithm similar to GIMME (Becker and Paterson, 2009) and MADE (Jensen and Papin, 2011), we simulated fluxes in untreated and DI-treated conditions based on their consistency with the RNA-seq data for each condition. See Supplemental Information for detailed analysis.

**Myocardial Ischemia-Reperfusion Model**

In vivo ischemia-reperfusion modeling was done as described (Ma et al., 2012). 8- to 10-week-old mice were anesthetized and subjected to open chest procedure of reversible left anterior descending artery ligation for 30 min and subsequent reperfusion for 2 hr. Saline or DI (4 mg/kg/min) was infused intravenously 10 min before and during ischemia. A cardioplegic solution followed by Evans Blue (after reocclusion of the LAD) was injected in a retrograde manner through the aorta in situ. The left ventricle was then sectioned into five slices, incubated in TTC (triphenyltetrazolium chloride) at 37°C (30 min), and images analyzed with ImageJ (NIH).

**Hypoxia Modeling in Neonatal Rat Cardiac Myocytes**

Neonatal rat cardiac myocytes were isolated and cultured as described (Ma et al., 2012). Cells were subjected to hypoxia in an oxygen control cabinet.
(Coy Laboratories) mounted within an incubator and equipped with oxygen controller and sensor for continuous oxygen level monitoring. A mixture of 95% nitrogen and 5% CO2 was utilized to create hypoxia, and oxygen levels in the chamber were monitored and maintained at <1%. Cell death was assessed with the Live-Dead Cytotoxicity Viability kit for Mammalian cells (Invitrogen) and ROS detected by flow cytometry with fluorescent carboxy-H2DCFDA (after incubation in 10 μmol/L for 30 min) (Ma et al., 2012).

**mROS Measurement**

Cells were treated with DI (0.25 mM, 12 hr) or vehicle, loaded with 5 μM MitoSOX (Invitrogen) at 37°C for 30 min in HBSS supplemented with 0.1% BSA, and rinsed with warm culture medium, before stimulation with LPS 1 hr later (for 3 hr) and analysis on Canto II flow cytometer (Becton Dickinson).

**Statistical Analysis**

Statistical analyses were performed in GraphPad Prism 6 software using statistical tests indicated for each experiment.

**ACCESSION NUMBERS**

The accession number for the RNA sequencing data reported in this paper is GEO: GSE82043.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.06.004.

**AUTHOR CONTRIBUTIONS**

V.L. and M.N.A. conceived and designed the study. V.L. performed many of the immunological experiments, flow cytometry, Seahorse experiments, and data analysis. A.S. performed computational analysis of RNA-seq data and flux balance analysis. M.B. performed western blots, sdh activity assay, and mROS measurement in macrophages. E.L. performed genomics analysis and helped with experiments. S.N. and M.S.D. generated the Irg1 targeting allele. L.C.-B provided Salmonella typhimurium cultures. C.J.W., X.M., and V.L. performed the ischemia-reperfusion model experiments and data analysis, under the supervision of A.D. S.C.-H. helped with Seahorse experiments. E.J.D., S.K., G.J.R., and M.S.D. contributed to study design and critical editing of the manuscript. V.L. and M.N.A. wrote the initial draft of the manuscript.

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**REFERENCES**


substrate-level phosphorylation by itaconic acid produced by LPS-induced Irg1 expression in cells of murine macrophage lineage. FASEB J. 30, 286–300.

