Endogenous itaconate is not required for particulate matter-induced NRF2 expression or inflammatory response

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ABSTRACT

Particulate matter (PM) air pollution causing significant cardiopulmonary mortality via macrophage-driven lung inflammation; however, the mechanisms are not completely understood. RNA-sequencing demonstrated *Acod1* (*Aconitate decarboxylase 1*) as one of the top genes induced by PM in macrophages. *Acod1* encodes a mitochondrial enzyme that produces itaconate, which has been shown to exert anti-inflammatory effects via NRF2 after LPS. Here, we demonstrate that PM induces Acod1 and itaconate, which reduced mitochondrial respiration via complex II inhibition. Using *Acod1*$^{-/-}$ macrophages, we found that Acod1/endogenous itaconate was not required for PM-induced inflammation or NRF2 activation. In contrast to endogenous itaconate, exogenous cell permeable form of itaconate (4-octyl itaconate (OI)) attenuated the PM-induced inflammation and activated NRF2 but NRF2 was not required for the anti-inflammatory effects of OI. We conclude that the effects of itaconate production on inflammation are stimulus-dependent, and that there are important differences between endogenous and exogenously-applied itaconate.

INTRODUCTION

Exposure to particulate matter (PM) air pollution is associated with significant morbidity and mortality and is one of the top preventable causes of death in the world (McGlade and Landrigan 2019). The World Health Organization estimates that exposure to air pollution is responsible for 4.2 million premature deaths worldwide every year (Organization 2018). Air pollution has been identified as a leading cause of global disease burden, and as the fifth mortality risk factor particularly in low- and middle-income countries (Cohen, Brauer et al. 2017). The majority of PM-associated morbidity and mortality is due to cardiopulmonary diseases including asthma, chronic obstructive lung disease, lung cancer, congestive heart failure and ischemic/thrombotic cardiovascular disease (myocardial infarction, ischemic stroke) (Cohen, Brauer et al. 2017, Hamanaka and Mutlu 2018).

We have previously shown that lung macrophages are required for PM-induced lung inflammation and consequently acute thrombotic events (Mutlu, Green et al. 2007, Chiarella, Soberanes et al. 2014, Soberanes, Misharin et al. 2019). PM induces the release of pro-inflammatory cytokines including interleukin-6 (IL-6), which is required for the PM-induced prothrombotic state and resultant acceleration of vascular thrombosis (Mutlu, Green et al. 2007, Chiarella, Soberanes et al. 2014). Furthermore, we found that PM affects mitochondrial function in lung macrophages characterized by increased oxygen consumption rate and generation of reactive oxygen species (ROS), which are both required for PM-induced IL-6 production (Soberanes, Misharin et al. 2019).

Despite significant improvement in our understanding about the mechanisms by which PM induces pro-inflammatory cytokines such as IL-6, the mechanisms that regulate PM-induced lung inflammation are not completely known. To better understand these mechanisms, we performed RNA-sequencing in macrophages following PM exposure. Here, we demonstrate that PM regulates the expression of genes that have not been previously reported. One of the top genes induced by PM was *Irg1* (Immune-responsive Gene 1) or *Acod1* (Aconitate

decarboxylase 1), which encodes a mitochondrial enzyme that produces itaconate from the TCA cycle metabolite cis-aconitate (Strelko, Lu et al. 2011, Michelucci, Cordes et al. 2013). Itaconate has been reported to be produced in macrophages following LPS stimulation and to have anti-inflammatory effects (Mills, Ryan et al. 2018). We confirmed that PM induces both mRNA and protein expression of Acod1 in macrophages, and increases intracellular and media levels of itaconate. Our metabolic analysis showed that itaconate inhibits mitochondrial complex II (succinate dehydrogenase) and that Acod1 is an important regulator of cellular respiration following PM exposure. Treatment of macrophages with a cell-permeable itaconate derivative, 4-octyl itaconate (OI) attenuated PM- and LPS-induced cytokine production. *Acod1*$^{-/-}$ cells, which lack endogenous itaconate production, exhibited exaggerated IL-β production following LPS exposure; however, no effect on PM-induced inflammation was observed. As recently described, OI induced NRF2 protein and expression of its target genes such as *Nqo1* and *Hmox1* (Mills, Ryan et al. 2018); however, we found that NRF2 was not required for the anti-inflammatory effects of OI. Furthermore, using *Acod1*$^{-/-}$ cells, we found that endogenous itaconate production is not required for NRF2 protein or its target gene expression following treatment with either PM or LPS. These results suggest that recently reported anti-inflammatory and NRF2-inducing effects of exogenous itaconate (OI) may not represent the effects of endogenously produced itaconate and therefore should not be used as a surrogate for Acod1 and endogenous itaconate. Collectively, our results suggest that stimulus and location of itaconate production play major roles in governing the effect of itaconate on inflammation in macrophages. Furthermore, in contrast to recent studies, we suggest that NRF2 is not a major regulator of the anti-inflammatory effects of itaconate.

RESULTS

Particulate matter induces aconitate decarboxylase 1 (*Acod1*) and production of itaconate in macrophages. Previous studies that evaluated the effect of PM on macrophages have evaluated a limited number of regulated genes, primarily focusing on pro-inflammatory cytokines. To gain a non-biased understanding of the mechanisms by which PM induces lung inflammation, we treated BMDMs from C57BL/6 mice with PM or vehicle (media) for 24 hours and performed RNA-Seq to analyze PM-induced changes in the transcriptome. Differentially expressed gene (DEG) analysis revealed 370 unique genes significantly regulated by PM. Gene ontology analysis of RNA-seq data showed upregulation of pathways relating to inflammatory responses, metabolism, and cytokine stimulation (Fig. 1A). The most highly upregulated gene induced by PM exposure was *Acod1* (Fig. 1B and source data for Figure 1). Other highly upregulated genes included proinflammatory cytokine genes such as *Tnfa, Il1b* as well as *Nqo1,* transcriptional target of Nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Fig. 1B).

Acod1 encodes a mitochondrial enzyme, which catalyzes the conversion of cis-aconitate to itaconate, a mitochondrial metabolite that has recently been shown to regulate inflammatory responses (Michelucci, Cordes et al. 2013). To confirm that PM upregulates mRNA and protein expression of Acod1, we first treated BMDMs with PM for 4, 8 or 24 hours, and then assessed the expression of *Acod1* through qPCR and western blot over time. PM induced *Acod1* gene expression as early as 4 hours after treatment, peaking at 8 hours (Fig. 1C). Western blot analysis showed expression of ACOD1 protein was delayed compared to mRNA expression and was detectable beginning 24 hours after PM treatment (Fig. 1D). To determine whether Acod1 protein induction was associated with increased cellular levels of its metabolic product, we measured intracellular levels of itaconate in BMDMs 24 hours after treatment with PM using capillary electrophoresis–mass spectrometry (CE-MS) (Human Metabolome Technologies, Boston, MA). We found that PM caused a significant increase in intracellular itaconate levels (Fig. 1E), correlating with the upregulation of ACOD1.

Furthermore, we detected increased concentrations of itaconate in the medium of macrophages treated with PM, indicating that itaconate is released from cells (Fig. 1F). Collectively, these findings demonstrate that PM causes a time-dependent expression of ACOD1 and production of itaconate, prompting us to further study whether itaconate plays a role in the regulation of PM-induced inflammation.

Itaconate decreases mitochondrial oxygen consumption by inhibiting succinate dehydrogenase/complex II in macrophages. Since itaconate has been shown to be a weak inhibitor of complex II, succinate dehydrogenase (SDH) (Cordes, Wallace et al. 2016, Lampropoulou, Sergushichev et al. 2016), we hypothesized that PM exposure may regulate mitochondrial respiration in macrophages through induction of Acod1 and production of itaconate. We first confirmed that itaconate inhibits SDH in BMDMs using the Seahorse XF Plasma Membrane Permeabilizer to assess the effect of itaconate on individual mitochondrial respiratory complexes. To measure the effect of itaconate on complex II/SDH activity, we permeabilized cells in the presence of rotenone (to eliminate the contribution of complex I), ADP, and succinate as substrate. Measurement of oxygen consumption rate (OCR) showed an immediate decrease in OCR following injection of either itaconate or malonate, a known SDH inhibitor used as a positive control. Addition of oligomycin did not cause a further reduction in OCR (Fig. 2A). These data suggest that itaconate, like malonate, is indeed an SDH inhibitor. When cells were permeabilized in media containing only the complex I substrates pyruvate/malate, injection of itaconate caused a smaller decrease in OCR relative to that seen during complex II-dependent respiration. This reduction was again similar to what was observed with malonate. Injection of oligomycin resulted in a further decrease in OCR, consistent with itaconate mediating its inhibitory effects on respiration via complex II (Fig. 2B).

Because there are no known mechanisms of transporting itaconate into the cell, studies have used different forms of itaconate that are cell membrane permeable to examine the

role of itaconate *in vitro*. 4-octyl itaconate (OI) has been shown to cross the plasma membrane and increase intracellular concentrations of itaconate (Mills, Ryan et al. 2018). We thus measured OCR in intact cells in the presence or absence of OI (0.25mM, a dose shown by Mills *et al* to reduce LPS-induced inflammation (Mills, Ryan et al. 2018)). OI injection caused a small reduction in basal OCR (Fig. 2C, 2D). Oligomycin then decreased the OCR of both groups to the same level consistent with OI-induced reduction in coupled respiration. Maximal OCR determined following FCCP, an uncoupler, was lower in OI-treated BMDMs compared to control BMDMs (Figure 2E). Together, these results suggest that itaconate is sufficient to reduce mitochondrial OCR via inhibition of complex II/SDH.

PM causes time-dependent effects on mitochondrial oxygen consumption: An initial increase OCR is followed by a late reduction in OCR. Since itaconate inhibits mitochondrial respiration and ACOD1 is induced late following PM exposure, we sought to determine whether PM exposure exerts time-dependent changes in mitochondrial oxygen consumption rate in macrophages. We thus measured OCR in BMDMs following PM treatment at 1 hour and 24 hours, when ACOD1 expression is undetectable and present, respectively. As we have previously shown (Soberanes, Misharin et al. 2019), after 1 hour of exposure, cells treated with PM exhibited increased basal oxygen consumption rate compared with control BMDMs (Fig. 3A). Interestingly, after 24 hours, PM-treated macrophages exhibited decreased both basal and maximal OCR relative to control-treated cells. (Fig. 3B). This decrease in OCR following 24 hour PM treatment was similar to the decrease in OCR after itaconate treatment (Figure 2C) suggesting that ACOD1 induction and itaconate production are important regulators of macrophage metabolism following PM treatment.

Endogenous itaconate production is required for PM-induced reduction in mitochondrial respiration and metabolic reprogramming in macrophages. We next sought to determine whether PM-induced ACOD1 expression and endogenous itaconate

production is required for PM-induced changes in mitochondrial metabolism at 24 hours. In order to answer this question, we used BMDMs from *Acod1*$^{-/-}$ mice, which lack the ability to produce itaconate. We first measured TCA cycle intermediates in WT and *Acod1*$^{-/-}$ BMDMs at 24 hours following treatment with PM or vehicle. PM-induced production of itaconate was detectable only in WT but not in *Acod1*$^{-/-}$ BMDMs, confirming that ACOD1 is required for itaconate production (Fig. 4A, B). Consistent with the role of itaconate as an inhibitor of SDH, succinate accumulated in WT BMDMs, but not *Acod1*$^{-/-}$ cells following PM treatment (Fig. 4A, C). This finding provides further support for endogenously produced itaconate inhibiting SDH and leading to accumulation of succinate, as SDH catalyzes the oxidation of succinate to fumarate. In contrast, *Acod1*$^{-/-}$ BMDMs had higher levels of TCA metabolites downstream of succinate (fumarate, malate) (Fig. 4A). This is consistent with previous reports using LPS as a stimulus (Lampropoulou, Sergushichev et al. 2016).

To determine whether PM-induced ACOD1 expression and production of itaconate are required for the reduction of OCR at 24 hours following PM, we performed a mitochondrial stress test in WT and *Acod1*$^{-/-}$ BMDMs. While WT BMDMs exhibited significantly reduced OCR following PM, *Acod1*$^{-/-}$ cells treated with PM for 24 hours did not show any reduction in OCR compared to control treatment (Fig. 4D). Collectively, these results suggest that endogenously produced itaconate is required for PM-induced reduction in OCR.

Exogenous, but not endogenous itaconate attenuates the PM-induced inflammatory response in macrophages. We have previously shown that PM induces an inflammatory response in macrophages, including the release of proinflammatory cytokines such as IL-6 and TNFα (Mutlu, Green et al. 2007, Chiarella, Soberanes et al. 2014). Since the expression of ACOD1 was time-dependent, we first sought to determine whether the effect of PM on cytokine expression was also time-dependent. We thus treated BMDMs with PM for 4, 8 or 24 hours and then analyzed the pro-inflammatory cytokine mRNA expression. We found that the mRNA expression *Il6, Tnfa* and *Il1b* increased at 4 and 8 hours, and then decreased at

24 hours suggesting that PM-induced expression of cytokines is time-dependent similar to the expression of ACOD1 (Fig. 5A).

Since the reduction in inflammatory cytokine mRNA coincided with the induction of ACOD1 and itaconate production, we hypothesized that itaconate may play a role in the reduction in cytokine expression observed at 24 hours. To test this hypothesis, we first evaluated the effect of exogenous itaconate on the early PM-induced inflammatory response. We pre-treated BMDMs with OI for 2 hours before treating with PM for 4 hours and measured mRNA expression of pro-inflammatory cytokines by qPCR. Pretreatment with OI attenuated the PM-induced mRNA expression of *Tnfa*, *Il6* and *Il1b*, suggesting that accumulation of itaconate with the induction of ACOD1 may be responsible for reduced cytokine mRNA expression at 24 hours following PM treatment (Fig. 5B).

To determine whether absence of itaconate accumulation would exaggerate PM-induced inflammation at later timepoints, we measured cytokine mRNA and protein level in both wild-type and *Acod1*$^{-/-}$ BMDMs 24 hours after exposure to PM. Interestingly, loss of itaconate accumulation did not result in increased mRNA expression of *Il6, Tnfa* or *Il1b* or protein level in media in *Acod1*$^{-/-}$ BMDMs (Fig. 5C, D). While loss of Acod1/itaconate did not affect PM-induced IL-6 protein level, there was a reduction in TNFα protein level in media (Fig. 5D). Overall, these results suggest that endogenous itaconate does not affect PM-induced inflammatory response.

This was a surprising finding as Acod1/itaconate deficiency has been shown to augment inflammatory cytokine expression in response to LPS (Lampropoulou, Sergushichev et al. 2016). To ensure that our findings were not the result of experimental differences other than stimulus, we treated wild-type and *Acod1*$^{-/-}$ BMDMs with LPS for 24 hours and measured *Il6, Tnfa* or *Il1b* mRNA expression. In contrast with PM treatment, *Acod1*$^{-/-}$ cells exhibited augmented *IL6* and *Il1b* mRNA expression after LPS treatment (Fig. 5D), consistent with

previously published results (Lampropoulou, Sergushichev et al. 2016). There was no effect of *Acod1* deletion on LPS-induced *Tnfa* expression (Fig. 5E) , also consistent with previous findings (Lampropoulou, Sergushichev et al. 2016). While the increase in *Il6* mRNA did not lead to an increase in IL-6 protein in media, loss of Acod1/itaconate resulted in increased IL-1β protein level in media (Fig. 5F). These findings suggest that the effect of itaconate on inflammation is stimulus-dependent. Importantly, these results also suggest that the effect of endogenously-produced itaconate on inflammation is different than that of exogenously-applied itaconate.

Differential effects of endogenous versus exogenous itaconate on the PM-induced transcriptomic response. To better understand the role of endogenous and exogenously applied itaconate on PM-induced response in macrophages, we performed RNA-sequencing in *Acod1*$^{-/-}$ and WT BMDMs exposed to PM (Fig. 6A and source data for Figure 6A). We found only 51 DEGs between *Acod1*$^{-/-}$ and WT BMDMs following PM (Fig. 6B and source data for Figure 6B). Both inflammatory genes, including *Il1b* and *Tnfa*, and NRF2 target genes, including *Nqo1* and *Gclm*, were not significantly different between *Acod1*$^{-/-}$ and WT. Next, to determine the effect of exogenous itaconate on PM-induced gene expression, we performed RNA-sequencing in WT BMDMs exposed to PM following 2 hours pretreatment with vehicle or OI. Transcriptomic analysis showed 1,030 DEGs between groups with and without OI (Fig. 6C and source data for Figure 6C). OI pretreatment significantly downregulated inflammatory gene expression in PM-treated BMDMs and upregulated NRF2 target genes. The effect of OI on these genes was not genes was not significantly different between WT and *Acod1*$^{-/-}$ *cells.* These results suggest that the effect of exogenous itaconate on the PM-induced transcriptomic response is markedly different from the effect of endogenous itaconate (Fig. 6A).

Endogenous itaconate production is not required for PM- or LPS-induced NRF2 expression. Recent studies suggest that itaconate exerts its inhibitory effects on LPS-induced inflammation via activation of NRF2, a transcriptional factor that plays a key role in antioxidant defense (Mills, Ryan et al. 2018). Both PM and LPS induce mitochondrial production of reactive oxygen species which promote inflammatory gene expression (Hsu and Wen 2002, Soberanes, Gonzalez et al. 2012); however, PM contains a mixture of metals and other compounds which have their own redox-modulating properties (Jeng 2010). We thus hypothesized that differential effects of PM and LPS on NRF2 activation may explain the lack of effect of endogenous itaconate on inflammation in PM-treated *Acod1*$^{-/-}$ BMDMs. We found that PM upregulated NRF2 and its target genes (*Nqo1, Gclm, Hmox1*) equally in WT and *Acod1*$^{-/-}$ BMDM (Fig. 7A, B). These results suggested that itaconate-independent activation of NRF2 might explain the fact that no augmented inflammatory gene expression was observed in *Acod1*$^{-/-}$ cells after PM exposure.

As our experiments in *Acod1*$^{-/-}$ BMDMs showed that Acod1 is not required for PM-induced NRF2 activation, we sought to determine whether endogenous itaconate production is required for LPS-induced NRF2 activation. Surprisingly, we found that similar to PM-induced NRF2 activation, Acod1 expression was not required for LPS-mediated induction of NRF2 protein or target gene expression (Fig. 7C, D). NRF2 protein levels were similarly upregulated in both LPS-treated WT and *Acod1*$^{-/-}$ cells (Fig. 7C). While *Gclm* expression was slightly reduced in *Acod1*$^{-/-}$ cells, *Hmox1* induction was not affected by *Acod1* deficiency and *Nqo1* was more highly induced by LPS in *Acod1*$^{-/-}$ cells (Fig 7D).

Furthermore, we found that NRF2 induction after LPS treatment occurs prior to Acod1 induction (Fig. 7E). Treatment with LPS induced a time-dependent expression of ACOD1 and NRF2. Interestingly, expression of NRF2 occurred at an earlier time point compared to ACOD1 (4 hours vs. 8 hours). Furthermore, maximal expression of NRF2 also preceded the maximal expression of ACOD1 (8 hours vs 24 hours).Taken together, our results suggest

that although NRF2 activation has been proposed to be the mechanism by which itaconate exerts its inflammatory effect, NRF2 induction occurs prior to ACOD1, and furthermore, ACOD1 is not required for NRF2 activation downstream of either PM or LPS.

NRF2 is not required for the anti-inflammatory effects of exogenous itaconate. To date, the majority of studies investigating the role of Acod1 and itaconate on inflammation, including those linking NRF2 to the mechanisms by which itaconate may regulate inflammation, have largely focused on the effects of exogenously administered cell membrane-permeable forms of itaconate (e.g., dimethyl itaconate and OI) (Lampropoulou, Sergushichev et al. 2016, Bambouskova, Gorvel et al. 2018, Zhao, Jiang et al. 2019). NRF2 has been suggested to regulate the effect of OI on IL1β expression; however, as we saw that OI reduced PM and LPS-induced expression of other cytokines, including TNFα, the expression of which is not regulated by endogenous itaconate, we examined whether the effect of OI on these cytokines was regulated by NRF2. We found that, consistent with previous findings (Mills, Ryan et al. 2018), OI treatment was sufficient to induce NRF2 protein and expression of NRF2 target genes (*Nqo1*, *Gclm*, and *Hmox1*) (Fig. 8A, B). OI induced NRF2 protein expression to a similar extent as PM, and the combination of OI and PM caused a further increase in NRF2 protein levels and expression of its target gene, *Nqo1* (Fig. 8A, B). To determine whether NRF2 is required for the OI-mediated attenuation of inflammatory cytokine expression, we transfected BMDMs with 2 independent siRNAs targeting *Nrf2*, or a non-targeting control. NRF2 protein was confirmed to be eliminated by Western blot analysis, as *Nrf2* siRNA-transfected cells did not upregulate NRF2 protein following 4 hours of PM treatment (Fig. 8C and Supplemental Figure).

Despite the loss of NRF2, we found that OI still exhibited its anti-inflammatory effects on PM-induced inflammation. OI attenuated PM-induced proinflammatory cytokine (*Tnfa*, *Il6*, *Il1b*) expression in both control and NRF2 knockdown cells (Fig. 8D). These data suggest that NRF2 is not required for exogenous itaconate to attenuate PM-induced inflammation and are

in contrast to recent results suggesting that OI exerts its anti-inflammatory effect on LPS-induced inflammation by upregulating NRF2 (Mills, Ryan et al. 2018).

Because of the differences between PM and LPS, we then looked at the effect of OI on LPS-induced inflammation. While OI similarly decreased LPS-induced inflammation (Fig. 9A), in contrast to PM treatment, LPS alone did not induce significant NRF2 target gene activation after 4 hours. No additional effect was seen on NRF2 target gene expression with the combination of LPS and OI (Fig 9B). Furthermore, consistent with our findings with PM, OI reduced inflammatory gene expression in response to LPS independent of NRF2 induction (Fig. 9C). Taken together, our results suggest that although NRF2 is induced by OI, this activation is not required for the anti-inflammatory effect of exogenous itaconate.

MATERIALS AND METHODS

Cell Isolation and Culture

All animal experiments and procedures were performed according to the protocols approved by the Institutional Animal Care and Use Committee at the University of Chicago. We used primary murine cells (bone marrow-derived macrophages (BMDMs), which we isolated as we have recently reported (Woods, Kimmig et al. 2019). Hematopoietic cells were isolated from bone marrow of C57BL/6NJ (Stock No: 005304) and *Acod1*$^{-/-}$ (Stock No: 029340) (both from Jackson Laboratory) mice and cultured with M-CSF (20 µg/L, BioLegend, catalog number 576408) *in vitro* for 8-10 days to generate BMDMs. For all experiments, cells were cultured in complete medium containing RPMI (Gibco, catalog number A10491), supplemented with 10% heat-inactivated FBS (Gemini, catalog number 100-106) and 1% penicillin-streptomycin (Gemini, 400-109).

Reagents were purchased from Sigma-Aldrich, including: 4-Octyl itaconate (catalog number SML2338), Itaconic Acid (catalog number I29204), Dimethyl Malonate (136441), Malonic Acid (M1296), *N*-Acetyl-L-cysteine (A7250). Particulate matter (SRM 1649a, Urban Dust) was from National Institute of Standards Technology (NIST). Lipopolysaccharide was purchased from Santa Cruz (sc-3535).

Cell Lysis and Western Blotting

Cells were scraped into RIPA buffer (Thermo-Scientific, catalog number 89900) with protease and phosphatase inhibitors (Thermo-Scientific, 1861284), sonicated for 10s on a Fisher Scientific 100 model at speed setting 2. Samples were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred to nitrocellulose (Bio-Rad, catalog number 1620167). Primary antibodies used were mouse anti-β-actin monoclonal antibody (Sigma, catalog number A5441; lot number 037K488; 1:10,000), ACOD1 polyclonal antibody (Invitrogen, catalog number PA5-49094), NRF2 monoclonal antibody (Abcam, catalog number ab62352), IL- 1β monoclonal antibody (Cell Signaling Technology, catalog number

12242). Secondary antibodies used were anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, catalog number 7074S) and anti-mouse IgG HRP-linked antibody (Cell Signaling Technology, catalog number 7076S).

Quantitative PCR

Total RNA was extracted with TRI Reagent (Zymo Research, R2050-1-200). RNA was then isolated using the Zymo Direct-zol RNA Miniprep Kit (Zymo Research, catalog number R2053) and reverse-transcribed using Bio-Rad iScript Reverse Transcription Supermix (Bio-Rad, catalog number 1708841) in a Bio-Rad C1000 Touch Thermal Cycler. Quantitative mRNA expression was determined by real-time qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad, catalog number 172-5121).

qRT-PCR primer sequences used are as follows:

Rpl13a (control) (5'-GAGGTCGGGTGGAAGTACCA-3', TGCATCTTGGCCTTTTCCTT-3'),

Acod1 (5'- TTTGGGGTCGACCAGACTTC-3', 5'-CCATGGAGTGAACAGCAACAC-3'),

Il6 (5'-TCCTCTCTGCAAGAGACTTCC-3', 5'-AGTCTCCTCTCCGGACTTGT-3'),

Tnfa (5'-ATGGCCTCCCTCTCATCAGT-3', 5'-TGGTTTGCTACGACGTGGG-3'),

Il1b (5'-GCCACCTTTTGACAGTGATGA-3',5'- GACAGCCCAGGTCAAAGGTT-3'),

Nqo1 (5'-GGTAGCGGCTCCATGTACTC-3', 5'-CGCAGGATGCCACTCTGAAT-3'),

Gclm (5'- AGTTGACATGGCATGCTCCG -3', 5'- CCATCTTCAATCGGAGGCGA-3'),

Hmox1 (5'- GAGCAGAACCAGCCTGAACT -3', 5'- AAATCCTGGGGCATGCTGTC-3').

ELISA

Cells were treated in complete medium for 4 hours, and the media was collected. IL-6 and TNFα cytokine levels in the media were then measured with DuoSet ELISA kits (R&D systems, catalog number DY406 and DY410) according to manufacturer's protocol.

RNA Sequencing

RNA was isolated and submitted for sequencing (50bp SE). Low expression genes were removed at cpm=1.5, and differentially expressed genes (DEGs) were identified using DESeq2 analysis at FC > 2 and FDR adjusted p-value $p < 0.05$.

Metabolomics

BMDMs were plated at 3×10^5 cells on 60mm tissue culture plates for 2 hours, then treated with PM ($20\mu g/cm^2$) for 24 hours prior to metabolite extraction. Cells were washed with 5% mannitol solution and metabolites were extracted with 400µl methanol. 275µl internal standard was added, then the extracts were centrifuged at 2,300 × g for 5 minutes. The supernatant was transferred to pre-washed centrifugal filter units (HMT, Human Metabolome Technologies, Boston, MA) and centrifuged at 9,100 × g at 4 °C for 2 hours. Centrifuged samples were sent to HMT for processing and analysis.

siRNA Knockdowns

BMDMs (1×10^6 cells) were transfected with siRNA (250 pmol) and Amaxa Mouse Macrophage Nucleofector Kit (Lonza, catalog number VPA-1009) using a Lonza Nucleofector 2b device (Lonza, #AAB-1001) on the mouse macrophage (Y-001) setting. Cells were cultured for 2 days post transfection before treatment. Success of siRNA transfections were confirmed with western blots and qRT-PCR. siRNAs were purchased from Dharmacon: D-001810-01-05 (non-targeting siRNA); J-040766-08-0002 (NRF2 #1), J-040766-06-0002 (NRF2 #2).

Seahorse Analysis

The Seahorse XF^e24 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA), was used to measure oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) as we have previously described (Nigdelioglu, Hamanaka et al. 2016, Hamanaka, O'Leary et al. 2019, Soberanes, Misharin et al. 2019, Woods, Kimmig et al.

2019).Macrophages were seeded at a density of 4×10^4 per well on a Seahorse XF24 Cell Culture Microplate (Woods, Kimmig et al. 2019).

Mitochondrial stress tests were performed according to manufacturer's protocol in XF DMEM base medium (Agilent 103334-100) containing Glutamine (2mM), Sodium Pyruvate (1mM), and Glucose (25mM). Compounds of interest were injected, followed by sequential injections of Oligomycin (1.5μM), FCCP (1.5μM) and Antimycin A/Rotenone (1.25μM).

Membrane permeabilization assays were performed in 1x MAS buffer containing mannitol (220mM), sucrose (70mM), monopotassium phosphate (10mM), magnesium chloride (5mM), HEPES (2mM), EGTA (1mM), with the addition of Seahorse XF Plasma Membrane Permeabilizer (Agilent, catalog number 102504-100) and Fatty Acid Free BSA (0.2%).

Statistical analysis. Data were analyzed using Prism 7 (GraphPad Software, Inc). All data are shown as mean ± standard error of the mean (SEM). Significance was determined by unpaired two-tailed Student's t test (for comparisons between two samples), or by one-way ANOVA using Bonferroni correction for multiple comparisons. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

Exposure to PM air pollution is associated with significant morbidity and mortality (Hamanaka and Mutlu 2018). Air pollution exposure is one of the top preventable causes of death in the world (McGlade and Landrigan 2019). It is estimated that exposure to air pollution causes 4.2 million premature deaths worldwide every year according to the World Health Organization (Hamanaka and Mutlu 2018, Organization 2018)). Since limiting exposure to PM cannot be achieved immediately, a better understanding of the mechanisms by which PM causes morbidity and mortality is required in order to combat the effects of air pollution on public health.

As lungs are the entry site for PM, it is not surprising that lung inflammation plays an important role of PM-induced adverse health effects, which are predominantly cardiopulmonary diseases. We have previously reported that macrophages, and in particular, their production of IL-6, are required for PM-induced lung inflammation and resultant acute thrombotic cardiovascular events (Mutlu, Green et al. 2007, Chiarella, Soberanes et al. 2014, Soberanes, Misharin et al. 2019). However, the mechanisms regulating PM-induced inflammation in the lung are not completely understood.

In this study, using an unbiased approach with RNA-seq, we first discovered *Acod1* as the highest-induced gene by PM in macrophages. In a time-dependent fashion, PM induced the mRNA and protein expression of ACOD1, which was not evident at earlier time points (4, 8 hours) but was detected at 24 hours. Confirming a functional role, PM-induced expression of ACOD1 led to production of itaconate in our metabolomics measurements.

Assessment of the effect of PM on mitochondrial function showed that PM has a time-dependent effect on mitochondrial respiration. While PM increased mitochondrial OCR at 1 hour, it reduced OCR at 24 hours. Similarities in the time dependency between the effects of PM on ACOD1/itaconate and mitochondrial respiration led us to explore whether itaconate is

responsible for the late reduction of OCR following PM exposure. Using *Acod1*$^{-/-}$ macrophages, we found that itaconate is required for PM-induced reduction in mitochondrial OCR at 24 hours. Consistent with a role of itaconate as an inhibitor of succinate dehydrogenase, we found that *Acod1*$^{-/-}$ BMDMs lack succinate accumulation following PM treatment. This finding is in agreement with findings by Cordes et al., which showed loss of succinate accumulation in response to LPS stimulation in *Acod1*$^{-/-}$ macrophages (Cordes, Wallace et al. 2016). Collectively, our data support a key role for Acod1 and itaconate in the metabolic reprogramming of macrophages in response to both LPS and PM stimulation.

Interestingly, although we found that endogenous itaconate production was an important regulator of mitochondrial metabolism and respiration in PM-treated macrophages, we were unable to find an effect of endogenous itaconate on PM-induced inflammation. This was in contrast to the effect of *Acod1* deletion on LPS-induced inflammation, in which IL6 and IL1β induction was augmented in the absence of endogenous itaconate production. Our results show a stimulus dependency for the effect of itaconate that has not been previously demonstrated. Thus, although PM and LPS both promote inflammatory cytokine expression and itaconate-dependent metabolic reprogramming, the effects of itaconate on inflammation differ between the two stimuli.

To date, the majority of studies investigating the role of Acod1 and itaconate on metabolism have largely focused on the effects of exogenously administered cell membrane-permeable forms of itaconate (e.g., dimethyl itaconate and OI) (Lampropoulou, Sergushichev et al. 2016, Bambouskova, Gorvel et al. 2018, Zhao, Jiang et al. 2019) . Following the initial studies with dimethyl itaconate (Lampropoulou, Sergushichev et al. 2016, Bambouskova, Gorvel et al. 2018, Zhao, Jiang et al. 2019), a more recent study questioned whether treatment with dimethyl itaconate actually increases intracellular levels of itaconate (ElAzzouny, Tom et al. 2017). In response to this limitation of dimethyl itaconate, Mills and colleagues proposed the use of OI as a cell permeable form of itaconate and demonstrated

that OI does increase intracellular levels of itaconate (Mills, Ryan et al. 2018). Our results showing that OI reduces PM-induced cytokine production are in agreement with Mills and colleagues suggesting that exogenous cell membrane-permeable version of itaconate has anti-inflammatory effects. However, given the differing effects of endogenous itaconate production versus OI treatment on PM-treated macrophages, our results suggest additional cautions should be taken when extrapolating the effects of cell-permeable itaconate analogs to the effects of endogenously-produced itaconate. This is highlighted by the fact that while OI is sufficient to induce NRF2 activation in macrophages, Acod1 is not required for NRF2 activation in response to PM or LPS. It would be expected that if a major effect of endogenous itaconate production were NRF2 activation, this should have been absent in *Acod1*$^{-/-}$ BMDMs. This loss of function experiment was notably lacking in the previous report linking itaconate with NRF2. Our results suggest that location of itaconate plays a major role in regulating its downstream effects.

While our results agree with Mills et al that OI activates the NRF2 antioxidant pathway by upregulation of NRF2 protein and its target genes, we found that NRF2 was dispensable for the anti-inflammatory effects of OI. Mills et al assessed the anti-inflammatory effects of OI by Western blot for intracellular IL1β. Our findings examining the expression of multiple cytokines at the mRNA and secreted protein level suggest that NRF2 does not play a major role in regulating the anti-inflammatory effect of OI.

In conclusion, we found that PM induces a mitochondrial enzyme, ACOD1 and production of a mitochondrial metabolite, itaconate, which is required for PM-induced metabolic reprogramming. Although endogenous itaconate reprograms mitochondrial function in PM-treated macrophages, it does not have a major effect on PM-induced gene expression or inflammatory response. Future work will be required to determine whether endogenous itaconate regulates other macrophage functions. Our results caution about the interpretation of results achieved using exogenous cell membrane-permeable forms of itaconate as they

may not represent the effects of endogenously produced itaconate. Further, our results suggest that our understanding of the role of NRF2 in the cellular response to itaconate is far from complete. While OI is sufficient to induce NRF2 activation in macrophages, endogenous itaconate production is not required for PM or LPS-mediated NRF2 activation.

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COMPETING INTERESTS

The authors declare that no competing interests exist.

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FIGURE LEGENDS

Figure 1. Aconitate Decarboxylase 1 (Acod1) is highly upregulated by particulate matter in macrophages. (**A**) We performed RNA-seq in BMDMs treated with either PM or control vehicle (PBS) for 24 hours. Gene ontology analysis highlight the pathways involved with PM treatment. (**B**) Volcano plot of RNA-seq data representing differentially expressed genes in BMDMs treated with PM or PBS for 24 hours. *Acod1* (red point) is one of the most highly differentially expressed gene. In addition, we found induction of NRF2 target genes (i.e. *Nqo1, Gclm*; turquoise points) and inflammatory genes (i.e. *Tnf, Il1b*; orange points) following PM treatment. Black data points represent other significant genes, at FC > 2, and FDR adjusted $p < 0.05$. (**C**) qRT-PCR data of *Acod1* in BMDMs treated with PM for 4, 8 or 24 hours. Data is represented as fold change. Significance was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons. (**D**) Western blot of ACOD1 protein at 0, 4, 8, 24 and 48 hours of PM treatment. (**E**) Intracellular itaconate concentration in BMDMs treated with PM for 24 hours, as measured by metabolomics. (**F**) Extracellular itaconate in media in BMDMs treated with PM for 24 hours. Significance was determined using two-tailed unpaired student's t-test, * $p < 0.05$.

Figure 2. Itaconate decreases oxygen consumption rate via inhibition of complex II, succinate dehydrogenase (SDH). (**A**) We measured oxygen consumption rate (OCR) in permeabilized BMDMs (using XF plasma membrane permeabilizer) in the presence of ETC complex II substrate (succinate) and complex I inhibitor (rotenone), followed by injections of 1) media (control, $n = 6$), itaconate ($n = 6$) or malonate ($n = 6$), a known complex II inhibitor, 2) oligomycin, an ATP synthase inhibitor, and 3) Antimycin A, a complex III inhibitor. Both itaconate acid and malonate significantly decreased OCR. (**B**) We measured OCR in permeabilized BMDMs in the presence of ETC complex I substrates (pyruvate/malate) followed by injections of 1) itaconate or malonate, 2) oligomycin, or 3) rotenone and antimycin A. (**C**) We performed mitochondrial stress test to measure OCR in BMDMs injected with 4-octyl itaconate (OI) (0.25mM) or control vehicle ($n = 8$). OI acutely decreased

both (**D**) basal OCR and (**E**) maximal OCR measured at third time point after FCCP. Significance was determined using two-tailed unpaired student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Effect of PM on mitochondrial oxygen consumption is time dependent. (**A**) We performed mitochondrial stress test to measure OCR in BMDMs at (**A**) 1 hour or (**B**) 24 hours following treatment with PM (20μg/cm^2) or control vehicle. Oligomycin (ATP synthase inhibitor), FCCP (uncoupler), and rotenone/antimycin A (complex I/III inhibitors) were injected sequentially. Basal OCR was acutely increased at 1 hour, which is before the induction of ACOD1 protein and production of itaconate. In contrast, both basal and maximal OCR decreased at 24 hours after PM treatment, when ACOD1 protein and itaconate levels are high ($n = 8$). Significance was determined using two-tailed unpaired student's t-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Figure 5. Exogenous but not endogenous itaconate decreases PM-induced inflammation. (**A**) We treated WT BMDMs with PM for 4, 8 or 24 hours and measured mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). Significance was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons. * $p < 0.05$, $n = 3$. (**B**) We

pretreated WT BMDMs with OI or control vehicle (DMSO) for 2 hours before we treated them with PM or control (PBS) for 4 hours. We then measured mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). (**C, D**) We treated WT and *Acod1*$^{-/-}$ BMDMs with PM or PBS (control) and measured (**C**) mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). and (**D**) protein levels of IL-6 and TNFα in media (ELISA). IL-1β protein was not detectable. (**E, F**) We treated WT and *Acod1*$^{-/-}$ BMDMs with LPS or PBS (control) and measured (**E**) mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). and (**F**) protein levels of IL-6, TNFα, and IL-1β in media (ELISA). $n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6. Endogenous and exogenous Itaconate have different effects on transcriptomic changes in response to PM. (**A**) PCA plot showing top 500 of 10,250 low expression removed gene features in WT or *Acod1*$^{-/-}$ (KO) BMDMs treated with PM and/or OI for 24 hours. (**B-C**) Volcano plots showing differentially expressed genes (DEGs) in (**B**) PM-treated *Acod1*$^{-/-}$ BMDMs compared with PM-treated WT BMDMs (51 DEGs), and (**C**) OI and PM treated BMDMs compared with only PM-treated WT BMDMs (1,030 DEGs). DEGs were identified using DESeq2 at FC > 2 and FDR adjusted p-value of $p < 0.05$. Dark gray points represent significantly different genes; light gray points represent not significantly different genes. Inflammatory genes (orange) and NRF2 target genes (turquoise) were not significantly different between WT and *Acod1*$^{-/-}$ BMDMs, while OI-pretreated BMDMs significantly expressed less inflammatory genes and more NRF2 target genes compared to BMDMs without OI pretreatment.

Figure 7. Acod1 and endogenous Itaconate production is not required for PM and LPS-induced activation of NRF2 pathway. (**A, B**) We treated WT and *Acod1*$^{-/-}$ BMDMs with PM for 24 hours and measured (**A**) protein expression of NRF2 (Western blot) and (**B**) mRNA expression of NRF2 target genes *Nqo1, Hmox1* and *Gclm* (qPCR). NRF2 protein and target genes are unchanged between WT and *Acod1*$^{-/-}$ (**C**, **D**) We treated WT and *Acod1*$^{-/-}$ BMDMs with LPS (100ng/ml) for 24 hours and measured (**C**) protein expression of NRF2

(Western blot) and (**D**) mRNA expression of NRF2 target genes *Nqo1, Hmox1* and *Gclm* (qPCR). NRF2 protein is not different between WT and *Acod1*$^{-/-}$ cells. (**E**) We treated WT BMDMs with PM for 4, 8, and 24 hours and measured protein expression of NRF2 and ACOD1 (Western blot) over time. Dimethyl fumarate (DMF, 0.1mM) was used as a positive control for NRF2 expression. The expression of NRF2 precedes ACOD1 and the peak expression of NRF2 occurs before the peak expression of ACOD1. Significance of qPCR data was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 8. NRF2 is not required for the anti-inflammatory effects of exogenous itaconate (OI) on PM-induced inflammatory response. (**A**) Western blot showing upregulation of NRF2 protein following 4 hours of OI or PM treatment. The combination of OI and PM further increased NRF2 activation. (**B**) qPCR of NRF2 target genes (*Nqo1*, *Gclm*, and *Hmox1)* in WT BMDMs treated with PM for 4 hours, with or without OI pretreatment (0.25mM, for 2 hours). (**C**) BMDMs transfected with scramble control siRNA or *Nrf2* siRNA (#1), Western blot analysis of control siRNA and *Nrf2* siRNA (#1) transfected BMDMs following 4h PM treatment to induce NRF2 protein. (**D**) qPCR of pro-inflammatory cytokine genes (*Tnfa*, *Il6* and *Il1b*) in *Nrf2* siRNA transfected BMDMs treated with OI (2 hours pretreatment) and PM (4 hours). Significance of qPCR was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 9. NRF2 is not required for the anti-inflammatory effects of exogenous itaconate (OI) on LPS-induced inflammatory response. (**A-B**) qPCR of (**A**) proinflammatory cytokine (*Tnfa*, *Il6* and *Il1b*) genes and (**B**) NRF2 target genes (*Nqo1*, *Gclm*, and *Hmox1)* in WT BMDMs treated with LPS for 4 hours, with or without OI pretreatment (0.25mM, 2 hours). (**C**) qPCR of pro-inflammatory cytokine genes (*Tnfa*, *Il6* and *Il1b*) in control or *Nrf2* siRNA (#1) transfected BMDMs treated with LPS for 4 hours, with or without OI pretreatment (0.25mM, for 2 hours). Significance of qPCR was analyzed with

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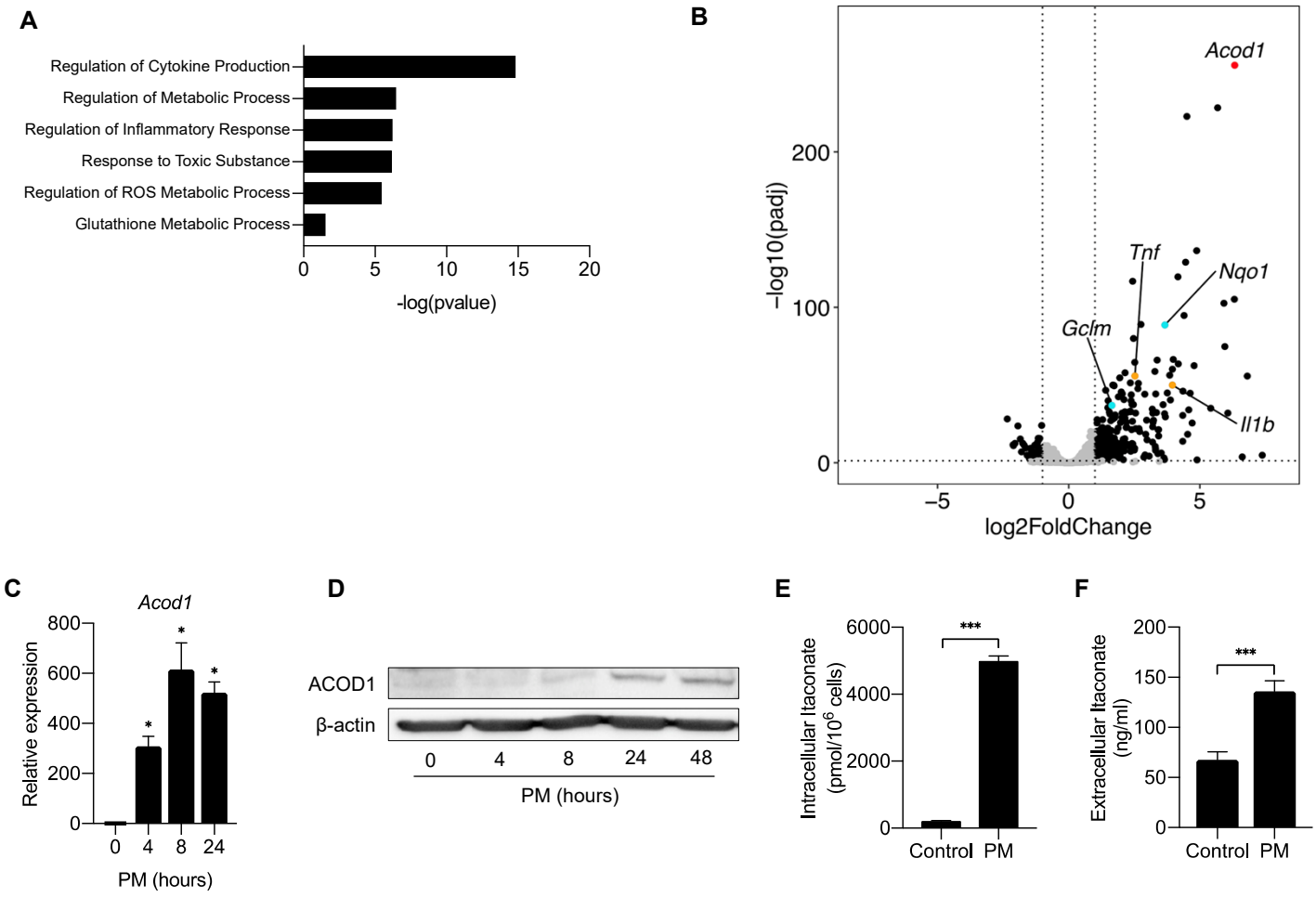
Figure 1



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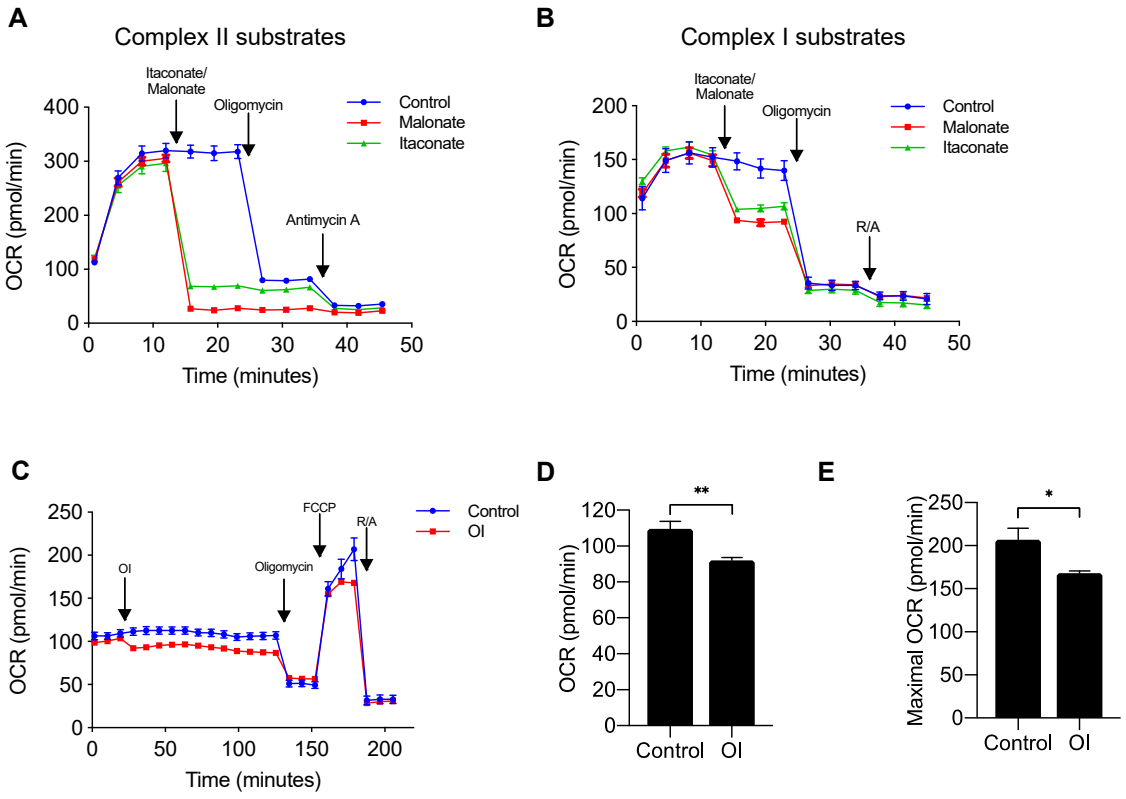

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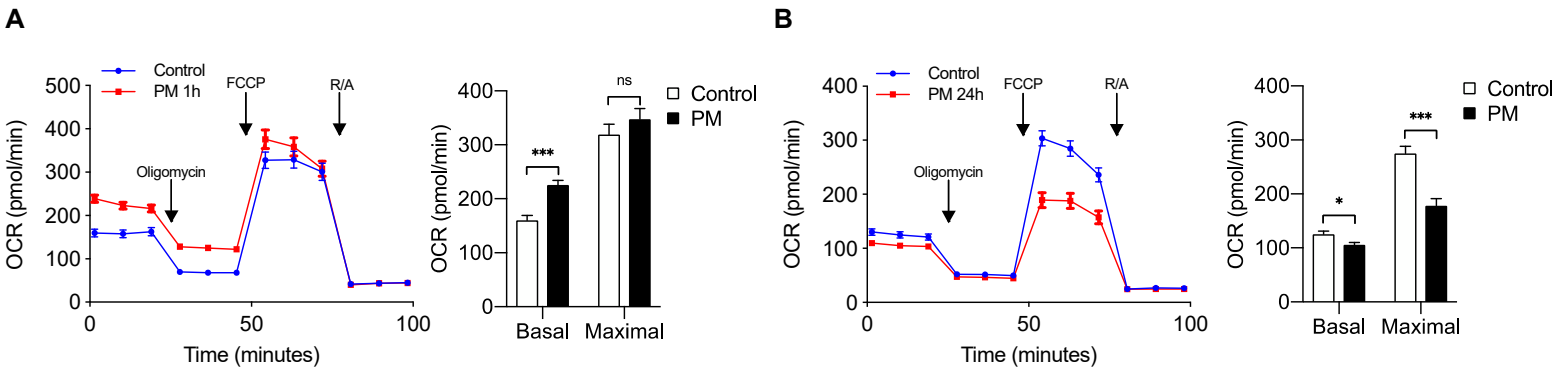

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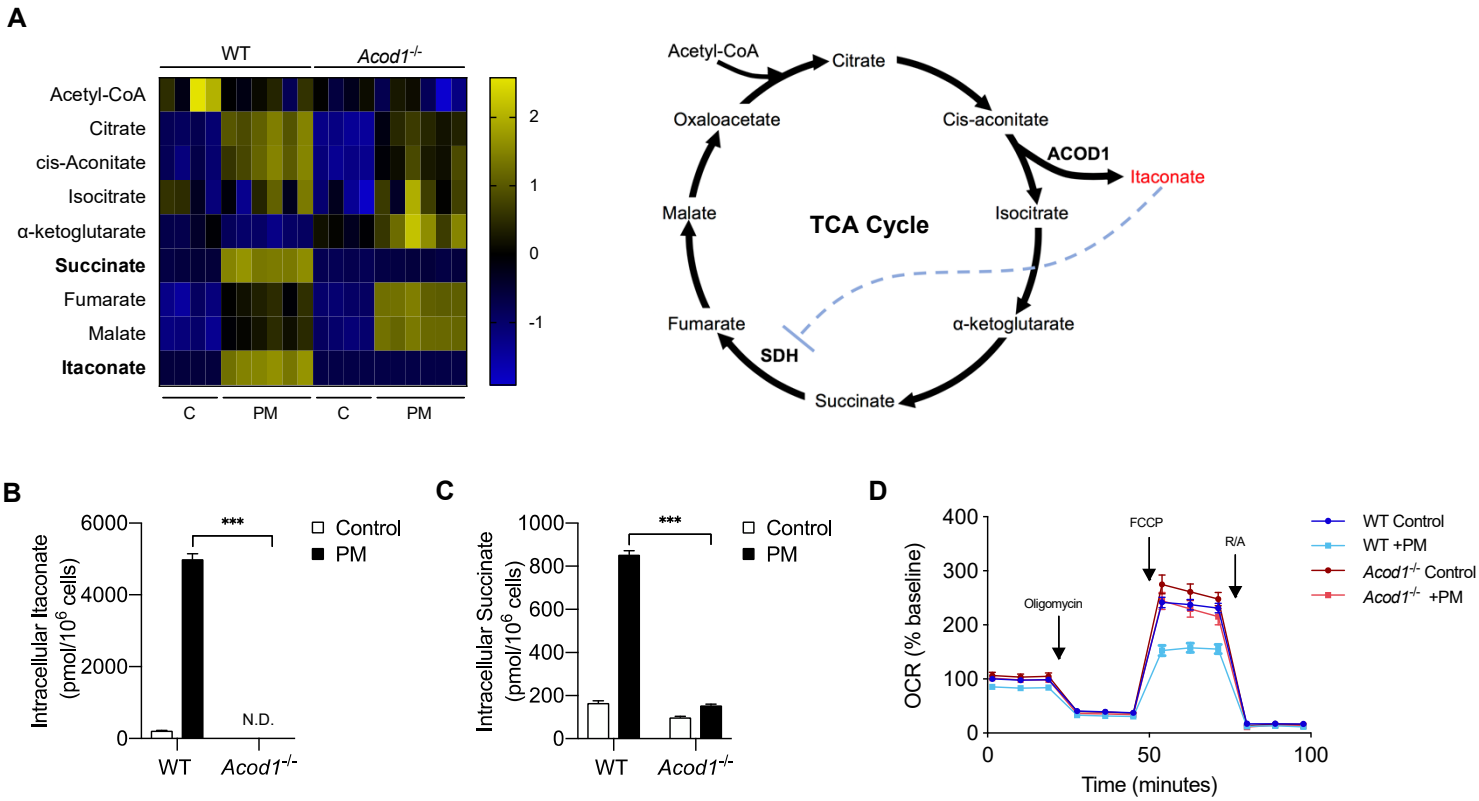

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Figure 5

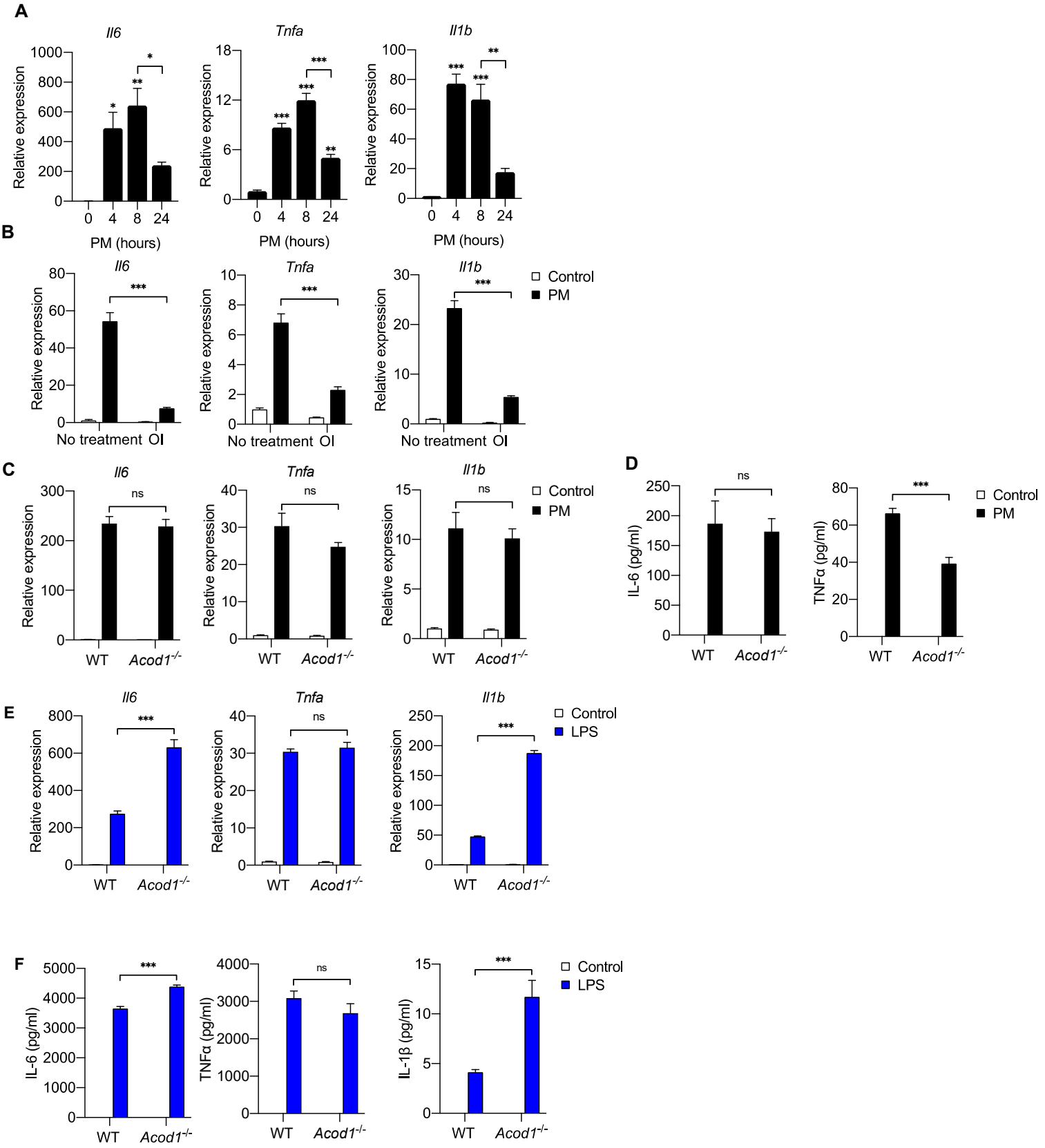


Figure 5. Exogenous but not endogenous itaconate decreases PM-induced inflammation. (**A**) We treated WT BMDMs with PM for 4, 8 or 24 hours and measured mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). Significance was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons. * $p < 0.05$, $n = 3$. (**B**) We pretreated WT BMDMs with OI or control vehicle (DMSO) for 2 hours before we treated them with PM or control (PBS) for 4 hours. We then measured mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). (**C, D**) We treated WT and *Acod1*$^{-/-}$ BMDMs with PM or PBS (control) and measured (**C**) mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). and (**D**) protein levels of IL-6 and TNFα in media (ELISA). IL-1β protein was not detectable. (**E, F**) We treated WT and *Acod1*$^{-/-}$ BMDMs with LPS or PBS (control) and measured (**E**) mRNA expression of *Tnfa, Il6* and *Il1b* (qPCR). and (**F**) protein levels of IL-6, TNFα, and IL-1β in media (ELISA). $n = 4$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6

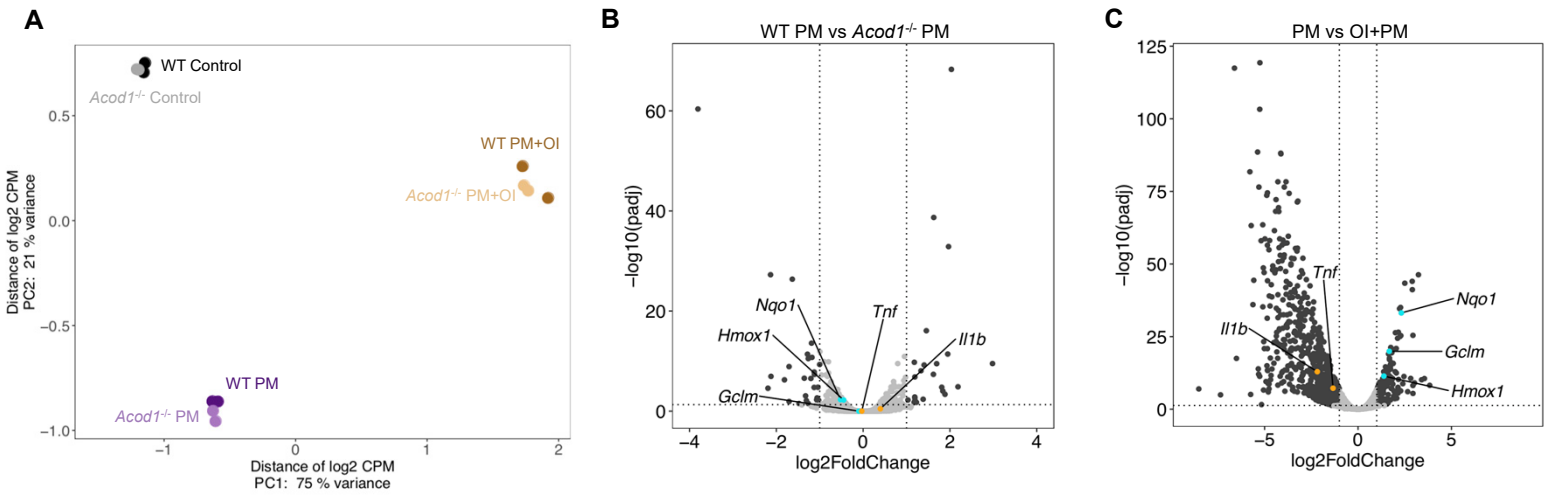


Figure 6. Endogenous and exogenous Itaconate have different effects on transcriptomic changes in response to PM. (**A**) PCA plot showing top 500 of 10,250 low expression removed gene features in WT or *Acod1*$^{-/-}$ (KO) BMDMs treated with PM and/or OI for 24 hours. (**B-C**) Volcano plots showing differentially expressed genes (DEGs) in (**B**) PM-treated *Acod1*$^{-/-}$ BMDMs compared with PM-treated WT BMDMs (51 DEGs), and (**C**) OI and PM treated BMDMs compared with only PM-treated WT BMDMs (1,030 DEGs). DEGs were identified using DESeq2 at FC > 2 and FDR adjusted p-value of $p < 0.05$. Dark gray points represent significantly different genes; light gray points represent not significantly different genes. Inflammatory genes (orange) and NRF2 target genes (turquoise) were not significantly different between WT and *Acod1*$^{-/-}$ BMDMs, while OI-pretreated BMDMs significantly expressed less inflammatory genes and more NRF2 target genes compared to BMDMs without OI pretreatment.

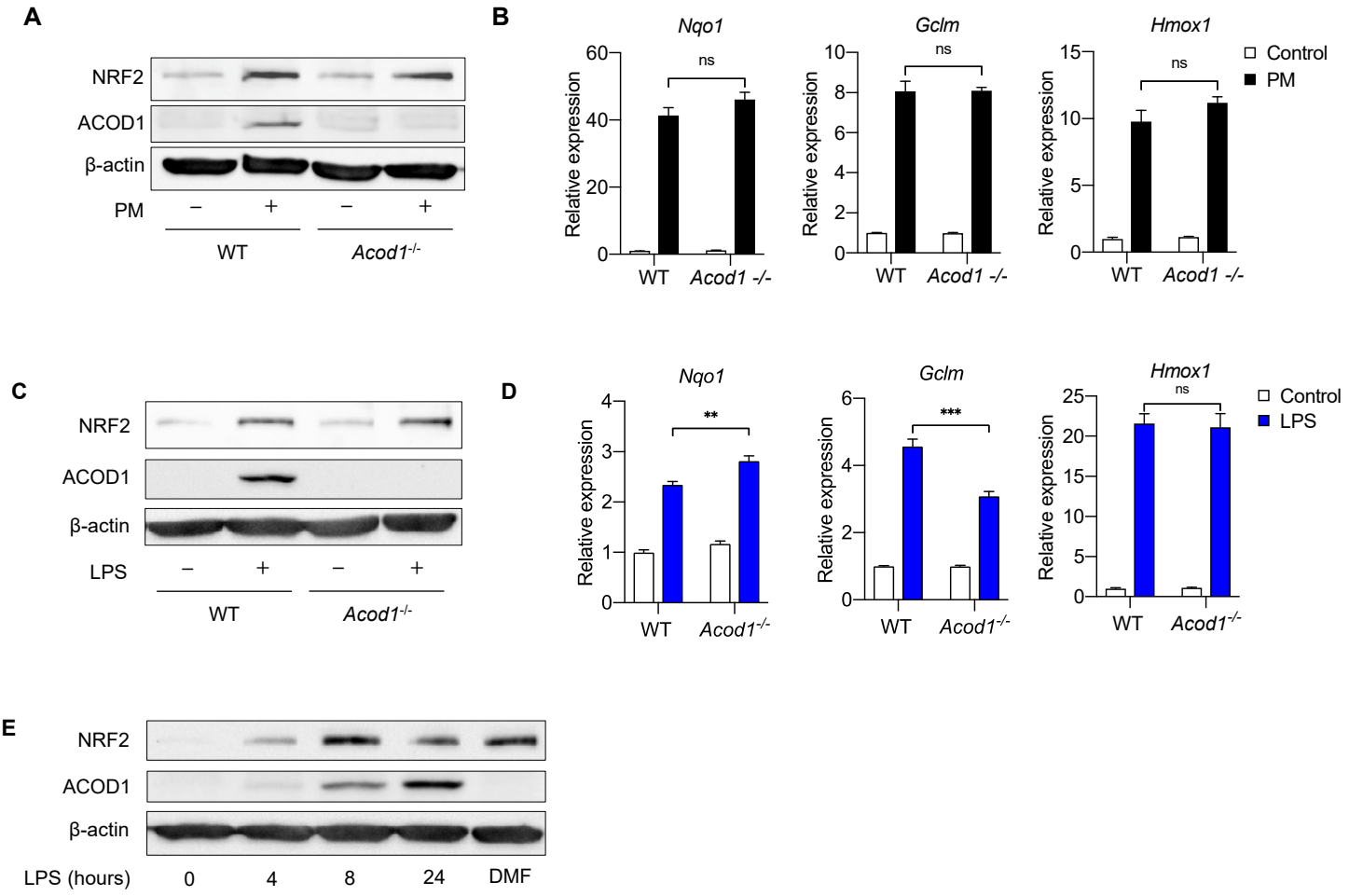


Figure 7. Acod1 and endogenous Itaconate production is not required for PM and LPS-induced activation of NRF2 pathway. (**A, B**) We treated WT and *Acod1*$^{-/-}$ BMDMs with PM for 24 hours and measured (**A**) protein expression of NRF2 (Western blot) and (**B**) mRNA expression of NRF2 target genes *Nqo1, Hmox1* and *Gclm* (qPCR). NRF2 protein and target genes are unchanged between WT and *Acod1*$^{-/-}$ (**C**, **D**) We treated WT and *Acod1*$^{-/-}$ BMDMs with LPS (100ng/ml) for 24 hours and measured (**C**) protein expression of NRF2 (Western blot) and (**D**) mRNA expression of NRF2 target genes *Nqo1, Hmox1* and *Gclm* (qPCR). NRF2 protein is not different between WT and *Acod1*$^{-/-}$ cells. (**E**) We treated WT BMDMs with PM for 4, 8, and 24 hours and measured protein expression of NRF2 and ACOD1 (Western blot) over time. Dimethyl fumarate (DMF, 0.1mM) was used as a positive control for NRF2 expression. The expression of NRF2 precedes ACOD1 and the peak expression of NRF2 occurs before the peak expression of ACOD1. Significance of qPCR data was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

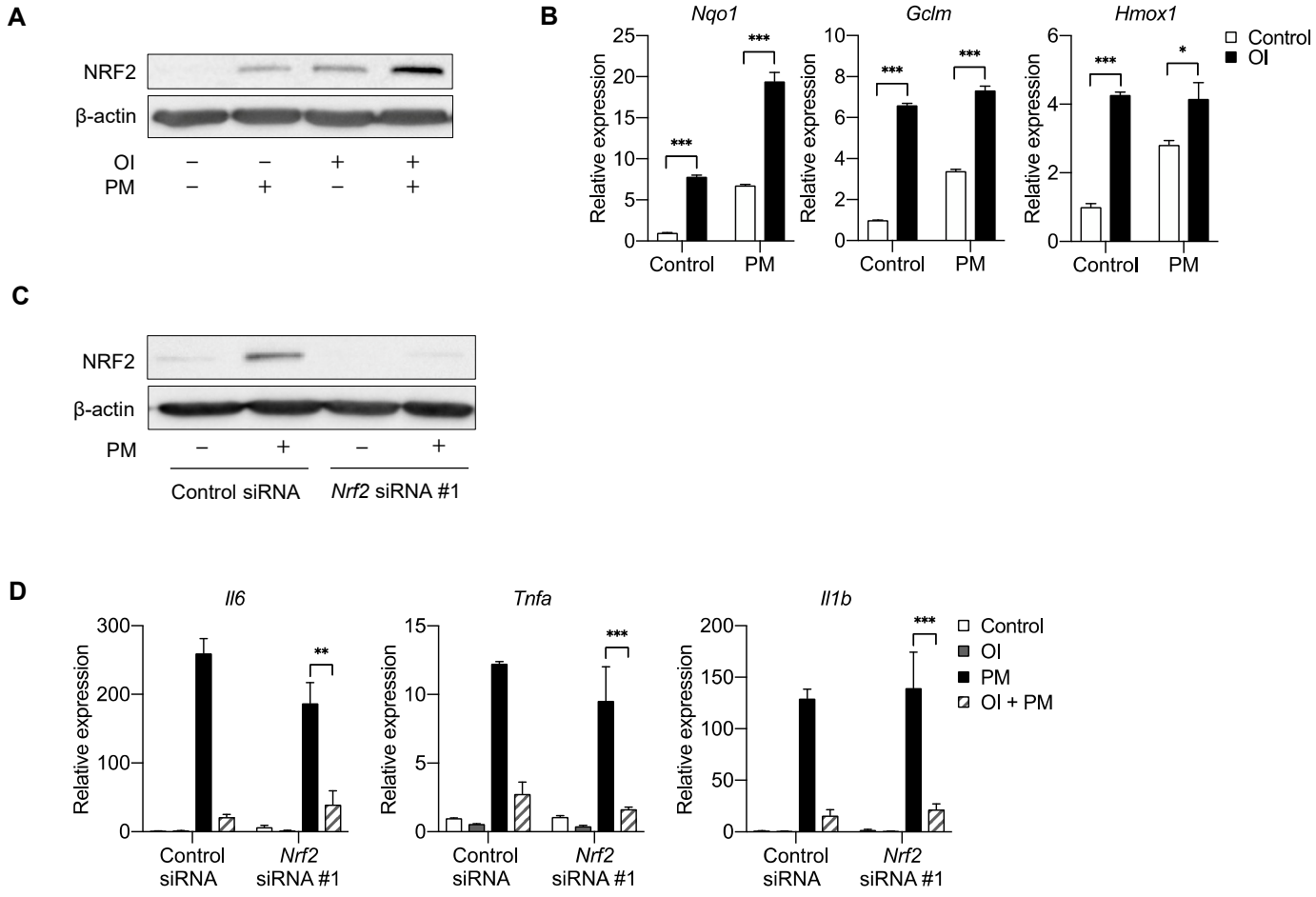


Figure 8. NRF2 is not required for the anti-inflammatory effects of exogenous itaconate (OI) on PM-induced inflammatory response. (**A**) Western blot showing upregulation of NRF2 protein following 4 hours of OI or PM treatment. The combination of OI and PM further increased NRF2 activation. (**B**) qPCR of NRF2 target genes (*Nqo1*, *Gclm*, and *Hmox1)* in WT BMDMs treated with PM for 4 hours, with or without OI pretreatment (0.25mM, for 2 hours). (**C**) BMDMs transfected with scramble control siRNA or *Nrf2* siRNA (#1), Western blot analysis of control siRNA and *Nrf2* siRNA (#1) transfected BMDMs following 4h PM treatment to induce NRF2 protein. (**D**) qPCR of pro-inflammatory cytokine genes (*Tnfa*, *Il6* and *Il1b*) in *Nrf2* siRNA transfected BMDMs treated with OI (2 hours pretreatment) and PM (4 hours). Significance of qPCR was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

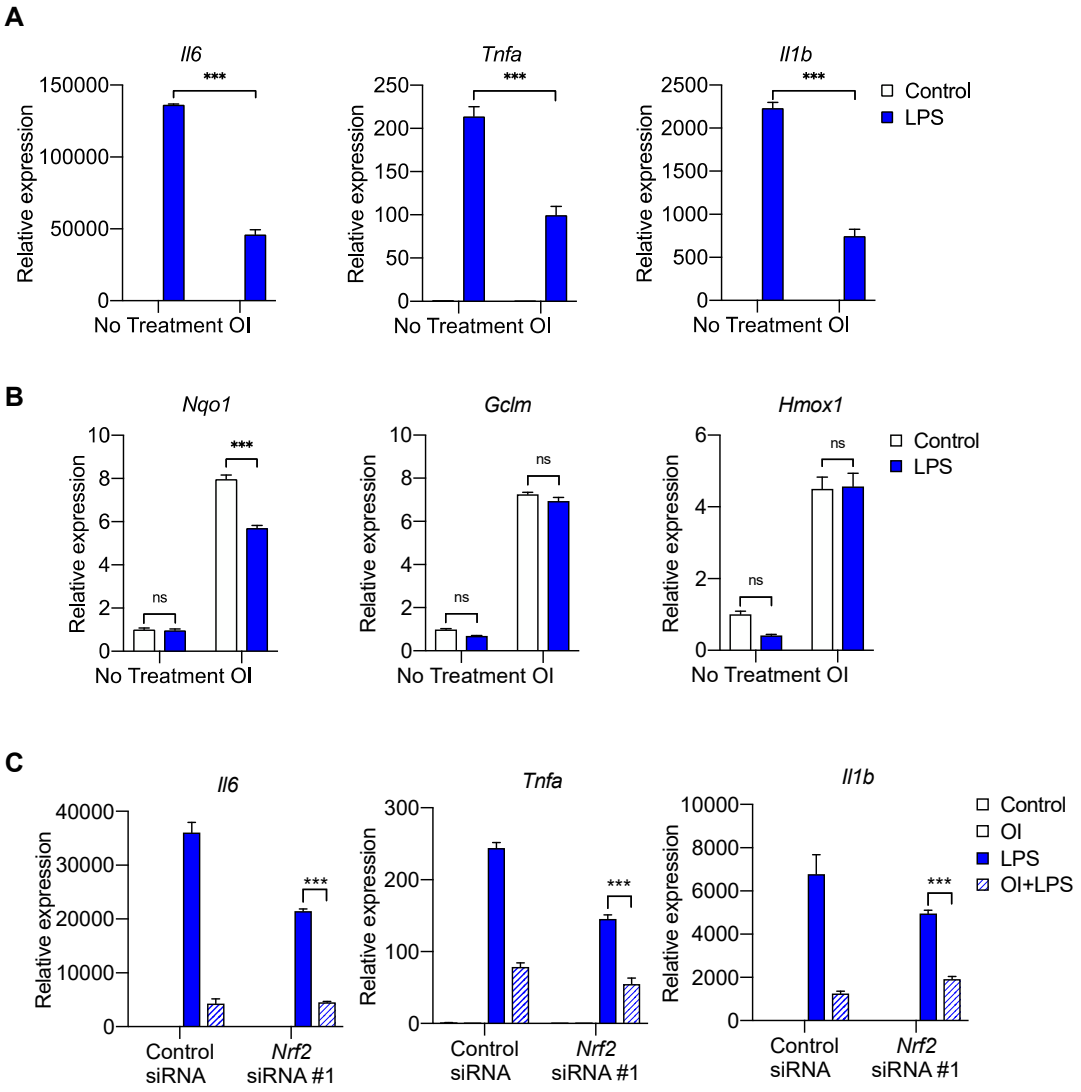
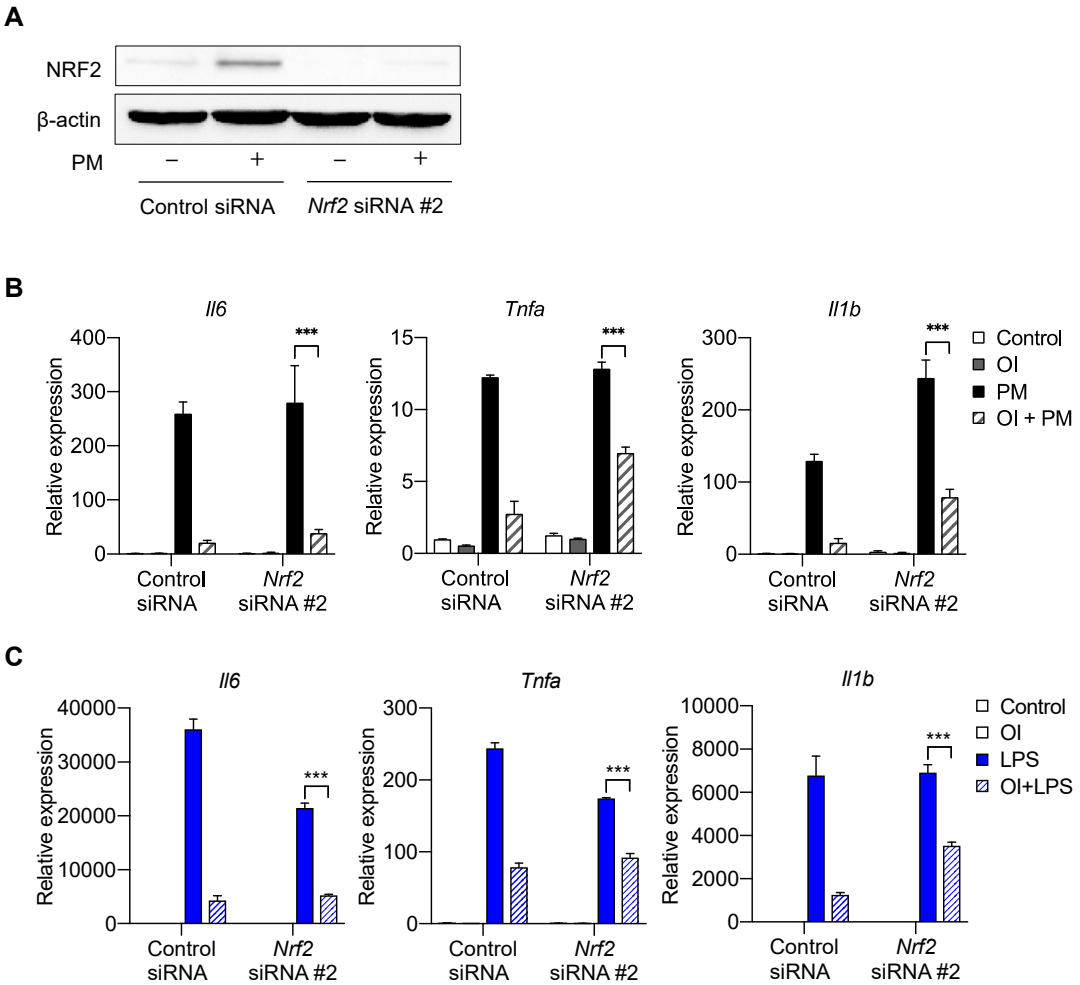


Figure 9. NRF2 is not required for the anti-inflammatory effects of exogenous itaconate (OI) on LPS-induced inflammatory response. (**A-B**) qPCR of (**A**) proinflammatory cytokine (*Tnfa*, *Il6* and *Il1b*) genes and (**B**) NRF2 target genes (*Nqo1*, *Gclm*, and *Hmox1)* in WT BMDMs treated with LPS for 4 hours, with or without OI pretreatment (0.25mM, 2 hours). (**C**) qPCR of pro-inflammatory cytokine genes (*Tnfa*, *Il6* and *Il1b*) in control or *Nrf2* siRNA (#1) transfected BMDMs treated with LPS for 4 hours, with or without OI pretreatment (0.25mM, for 2 hours). Significance of qPCR was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons, $n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Supplemental Figure 1



Supplemental Figure 1. NRF2 is not required for the anti-inflammatory effects of exogenous itaconate (OI) on LPS-induced inflammatory response. (**B-C**) qPCR of pro-inflammatory cytokine genes (*Tnfa*, *Il6* and *Il1b*) in control or *Nrf2* siRNA (#2) transfected BMDMs treated with PM (**B**) or LPS (**C**) for 4 hours, with or without OI pretreatment (0.25mM, for 2 hours). Significance of qPCR was analyzed with one-way ANOVA corrected with Bonferroni's for multiple comparisons, n = 3; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$