# Supporting Information

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### SI Materials and Methods

**Cell Transfections.** The ON-TARGETplus SMARTpool containing four different siRNA sequences, all specific to murine *Irg1* (siRNA *Irg1*), murine iNOS (siRNA iNOS), murine aconitase 2 (siRNA *Aco2*), and the corresponding nontargeting control (siRNA Ctr), were designed and synthesized by Thermo Scientific Dharmacon.

RAW264.7 macrophages were transfected with Amaxa 4D-Nucleofector Device (Lonza) using the Amaxa SG cell line 4D Nucleofector Kit for THP-1 cells according to the manufacturer's instructions.

Briefly, transfection with siRNA complexes was carried out from pelleted and resuspended cells ($1 \times 10^6$ cells per condition). Transfection reagent and siRNA were prepared according to manufacturer's instructions (Amaxa). siRNAs were added at a final concentration of 100 nM. After the nucleofection processing using "RAW264.7 (ATCC) program" on the Nucleofection device, the cells were seeded at a density of $1 \times 10^6$ cells per well in 12-well plates in DMEM supplemented with 10% FBS and incubated for 24 h.

pCMV6-Irg1 overexpressing plasmid (4 μg, *Mus musculus* immune responsive gene 1 transfection-ready DNA, OriGene), in parallel with the empty plasmid (4 μg), was transfected into $1.5 \times 10^6$ A549 cells using Lipofectamine 2000 (Invitrogen) and further incubated for 24 h. pCMV6-Entry-Irg1 plasmid was transfected into HEK293T cells by the jetPEI procedure as described previously (46) and further incubated for 48 h before extraction.

**Macrophages Bacterial Phagocytosis and Killing Assay.** Untransfected or transfected RAW264.7 macrophages (with unspecific siRNA, *Irg1*-specific siRNA or mitochondrial aconitase-specific siRNA) were seeded at a density of $25 \times 10^4$ per well in 48-well plates in 250 μL DMEM complemented with 10% FBS at 37 °C with 5% $CO_2$. After 24 h, the cells were infected with *S. enterica* serovar typhimurium at a multiplicity of infection of 1:10 (one bacteria per 10 macrophages) or 1:1 (one bacteria per one macrophage) and incubated for 1 h at 37 °C with 5% $CO_2$. Macrophages were then washed with sterile PBS and resuspend in DMEM complemented with 10% FBS and 100 μg/mL gentamicin to kill noningested bacteria and further incubated for 1 h (this was considered as time point 0 h) or 4 h (time point 4 h) at 37 °C with 5% $CO_2$. After washing with sterile PBS, macrophages were disrupted for 15 min with 250 μL $dH_2O$ to release intracellular bacteria. The amount of viable intracellular bacteria was determined by plating on Luria-Bertani agar plates using four dilutions from 1:10 up to 1:10,000 and incubation overnight at 37 °C. For metabolite extraction, macrophages were seeded at a density of $75 \times 10^4$ per well in 12-well plates. Intracellular metabolites were extracted and mRNA isolated at time point 0 h and time point 4 h for GC/MS measurements and RT-PCR, respectively. All conditions were performed in technical triplicates.

**Protein Purification and CAD Activity Assay.** HEK293T cells were extracted 48 h after transfection by scraping them into a lysis buffer containing 25 mM Hepes, pH 7.1, and 1× protease inhibitor mixture (Roche). After two freeze/thaw cycles, cell extracts were incubated for 30 min on ice in the presence of DNase I (200 U/mL extract; Roche Applied Science) and 10 mM $MgSO_4$. The crude cell extracts were centrifuged for 5 min at 16,000 × *g* (4 °C) and pellets were resuspended in lysis buffer for SDS/PAGE analysis. Flag-Irg1 was purified from the supernatant using the FlagM purification kit, according to the manufacturer's instructions (Sigma Aldrich). About 3 mg protein were loaded onto 250 μL anti-Flag affinity resin and retained proteins were eluted with a solution containing 200 μg/mL Flag peptide (3 × 400 μL fractions). Protein purity was checked by SDS/PAGE analysis. Protein concentration was measured by the Bradford assay using Bradford reagent (Bio-Rad).

*Cis*-aconitate decarboxylase activity was measured by incubating cell extracts or purified protein fractions (10 μL) at 30 °C and for 40 min in a reaction mixture containing 25 mM Hepes, pH 7.1, and 1 mM *cis*-aconitate in a total volume of 100 μL. Reactions were stopped by addition of 900 μL methanol/water (8:1) mix. After 10 min centrifugation at 16,100 × *g* and 4 °C, 100 μL of the supernatant were collected and evaporated under vacuum at −4 °C using a refrigerated CentriVapConcentrator (Labconco).

**RNA Isolation and RT-PCR.** Total RNA was purified from cultured cells using the Qiagen RNeasy Mini Kit (Qiagen) per manufacturer's instructions. First-strand cDNA was synthesized from 0.5 to 2 μg of total RNA using SuperScript III (Invitrogen) with 1 μL (50μM)/reaction oligo$(dT)_{20}$ as primer. Individual 20 μL SYBR Green real-time PCR reactions consisted of 2 μL of diluted cDNA, 10 μL of 2× iQ SYBR Green Supermix (Bio-Rad), and 0.5 μL of each 10 μM optimized forward and reverse primers in 7 μL RNase-free water. Primer sequences designed using Beacon Designer software (Bio-Rad), provided by Eurogentec, or directly designed by Thermo Scientific, are available on request. For the human Irg1 primers, the NCBI/Primer-BLAST tool available at http://www.ncbi.nlm.nih.gov/tools/primer-blast/ was used. The PCR was carried out on a Light Cycler 480 (Roche Diagnostics), using a three-stage program provided by the manufacturer: 10 min at 95 °C and 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C followed by 10-s 70–95° melting curves. All experiments included three no-template controls and were performed on three biological replicates with three technical replicates for each sample. For standardization of quantification, L27 was amplified simultaneously.

**SDS/PAGE and Western Blotting Analysis.** Heat-denatured protein samples were separated on 10% SDS-polyacrylamide gels electrophoresis followed by transfer to nitrocellulose membranes 0.2 μm (Sigma). After blocking with 5% (wt/vol) dry milk in PBS, the membrane was incubated overnight at 4 °C in primary anti-Irg1 antibody from rabbit (Sigma) diluted 1:500 in 1% BSA/PBS with constant shaking. After three washing steps with PBS containing 0.1% Tween-20, the membrane was incubated with anti-rabbit antibody coupled to horseradish peroxidase and revealed by chemiluminescence using the Amersham ECL detection reagents (GE Healthcare).

**GC/MS Sample Preparation and Procedure.** Cells grown in six-well plates were washed with 1 mL saline solution and quenched with 0.4 mL −20 °C methanol. After adding an equal volume of 4 °C cold water, cells were collected with a cell scraper and transferred in tubes containing 0.4 mL −20 °C chloroform. The extracts were vortexed at 1,400 rpm for 20 min at 4 °C and centrifuged at 16,000 × *g* for 5 min at 4 °C. 0.3 mL of the upper aqueous phase was collected in specific GC glass vials and evaporated under vacuum at −4 °C using a refrigerated CentriVap Concentrator (Labconco). The metabolite extractions of cells grown on 12-well plates were performed using half of the volumes.

The interphase was centrifuged with 1 mL −20 °C methanol at 16,000 × *g* for 5 min at 4 °C. The pellet was used for RNA isolation.

Metabolite derivatization was performed using an Agilent Autosampler. Dried polar metabolites were dissolved in 15 μL of 2% methoxyamine hydrochloride in pyridine at 45 °C. After 30 min, an equal volume of 2,2,2-trifluoro-*N*-methyl-*N*-trimethylsilyl-acetamide + 1% chloro-trimethyl-silane were added and held for 30 min at 45 °C. Metabolites extracted out of 12-well plates were derivatized using half of the reagent volumes. GC/MS analysis is described in *SI Materials and Methods*, *GC/MS Analysis*.

**Glucose Labeling Assay.** RAW264.7 macrophages were seeded at a density of $1 \times 10^6$ per well in 12-well plates in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% $CO_2$. After 24 h, the medium was changed to DMEM containing uniformly labeled 25 mM [U-$^{13}$C] glucose (Cambridge Isotope). Simultaneously, the cells were activated with 10 ng/mL LPS. After 6 h of incubation, the metabolites were extracted.

***S. enterica* Growth Analysis.** *S. enterica* serovar typhimurium bacteria were grown in liquid medium as detailed in the text in the presence different concentrations of itaconic acid or *cis*-aconitate (5, 10, 50, 100 mM). Growth was measured as OD at indicated time points.

**Mice.** All animal procedures have been performed according to the European Guidelines for the use of animals in research (86/609/CEE). All efforts were made to minimize suffering. All animals have been raised and crossed in an indoor animal house in a 12-h light/dark cycle and have been provided with water and food ad libitum.

**Cell Culture.** Mixed glial cell cultures were prepared from the brains of newborn C57BL/6 mice. After carefully removing meninges and large blood vessels, the brains were pooled and then minced in cold PBS solution. The tissue was mechanically dissociated with Pasteur pipettes and the resultant cell suspension was passed through a 21G hypodermic needle. After washes and centrifugations, the mixed glial cells were plated into poly-D-lysine (PDL, Sigma) coated six-well plates (two brains per six-well plate) in DMEM (Invitrogen) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin (Sigma), and 10% heat-inactivated FBS (Invitrogen) in a water-saturated atmosphere containing 5% $CO_2$ at 37 °C. The medium was replaced every 3–4 d. After 7–10 d, when the cultures reached confluence, microglia were detached by a 30-min shaking on a rotary shaker (180 rpm). Detached cells, mainly microglia (>95%), were then plated in multiwell plates in conditioned medium and further incubated for 3 d.

BV2 retroviral-immortalized microglia (BV-2), human embryonic kidney 293T (HEK293T), and RAW264.7 cell lines were maintained in DMEM with or without sodium pyruvate, supplemented with 10% heat-inactivated FBS (South American, Invitrogen). No antibiotics were used for BV-2; 1% penicillin/streptomycin were used for RAW264.7 and HEK293T cells.

A549 cells were cultivated in DMEM without sodium pyruvate and supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were grown and maintained according to standard cell culture protocols and kept at 37 °C with 5% $CO_2$.

For experiments, BV-2, RAW264.7, and A549 cells were seeded into multiwell plates at a density of $0.5 \times 10^5$ (BV-2) and $1.0 \times 10^5$ (RAW264.7 and A549) cells/well (six-well plates). After 3 d of culture, the cells were activated adding specific stimuli to the culture medium.

LPS 055:B5 from *Escherichia coli* (Sigma) was added at specified time points and at different doses in mouse primary microglia (1 ng/mL), BV-2, and RAW264.7 (10 ng/mL) or peripheral blood mononuclear cell (PBMC)-derived macrophages (10 μg/mL) to obtain similar activation states because of the differences in sensitivity between murine primary cultures and cell lines as well as between mouse and human cells.

**GC/MS Analysis.** GC/MS analysis was performed using an Agilent 6890 GC equipped with a 30 m DB-35MS capillary column. The GC was connected to an Agilent 5975C MS operating under electron impact ionization at 70 eV. The MS source was held at 230 °C and the quadrupole at 150 °C. The detector was operated in scan mode and 1 μL of derivatized sample was injected in splitless mode. Helium was used as carrier gas at a flow rate of 1 mL/min. The GC oven temperature was held at 80 °C for 6 min and increased to 300 °C at 6 °C/min. After 10 min, the temperature was increased to 325 °C at 10 °C/min for 4 min. The run time of one sample was 59 min.

**NO Donor Treatments.** Human PBMCs were seeded and differentiated into macrophages as described previously. Diethylamine NONOate (DEA NONOate, Sigma), an intracellular NO donor, was added at different concentrations (1, 10, 100 μM) alone or together with LPS (100 μg/mL). After 12 h of incubation, the metabolites were extracted.

**Griess Nitrite Assay.** After 12 h, 180 μL of medium was harvested and combined with 20 μL of 1 mM NaOH on ice to stop the dissociation reaction. Levels of nitrite formed from the reaction with $H_2O$ were determined using the Griess assay. In brief, 50 μL of medium sample or nitrite ion chromatographic (IC) standard (Sigma) was pipetted in triplicate in a 96-well plate. To each well, equal volumes of 1× Griess Reagent (Sigma) were added. Absorbance was read at 540 nm and nitrite concentrations were calculated.

**Sequence Alignment.** Multiple sequence alignment of *Cis*-aconitic acid decarboxylase (*Aspergillus terreus*), immune-responsive gene 1 protein homolog (human), immune-responsive gene 1 protein (mouse), and iminodisuccinate epimerase (*Agrobacterium tumefaciens*) was performed using MAFFT version 6 (1, 2) and visualized with ESPript (3). Sequences were obtained from UniProt Knowledgebase (UniProtKB) with the following accession numbers: B3IUN8 (CAD1), A6NK06 (IRG1 human), P54987 (IRG1 mouse), and Q1L4E3 (iminodisuccinate epimerase).


1. Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* 9(4):286–298.
2. Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* 30(14): 3059–3066.
3. Gouet P, Courcelle E, Stuart DI, Métoz F (1999) ESPript: Analysis of multiple sequence alignments in PostScript. *Bioinformatics* 15(4):305–308.

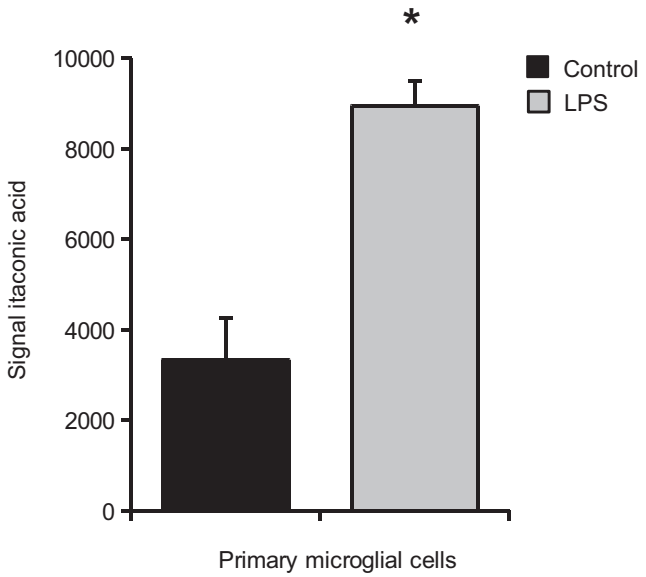


**Fig. S1.** Itaconic acid in mouse primary microglial cells. Primary microglial cells were treated for 6 h with LPS (1 ng/mL) (gray bars) or left untreated (black bars). Bars represent the mean of itaconic acid levels (± SD). *$P < 0.05$.

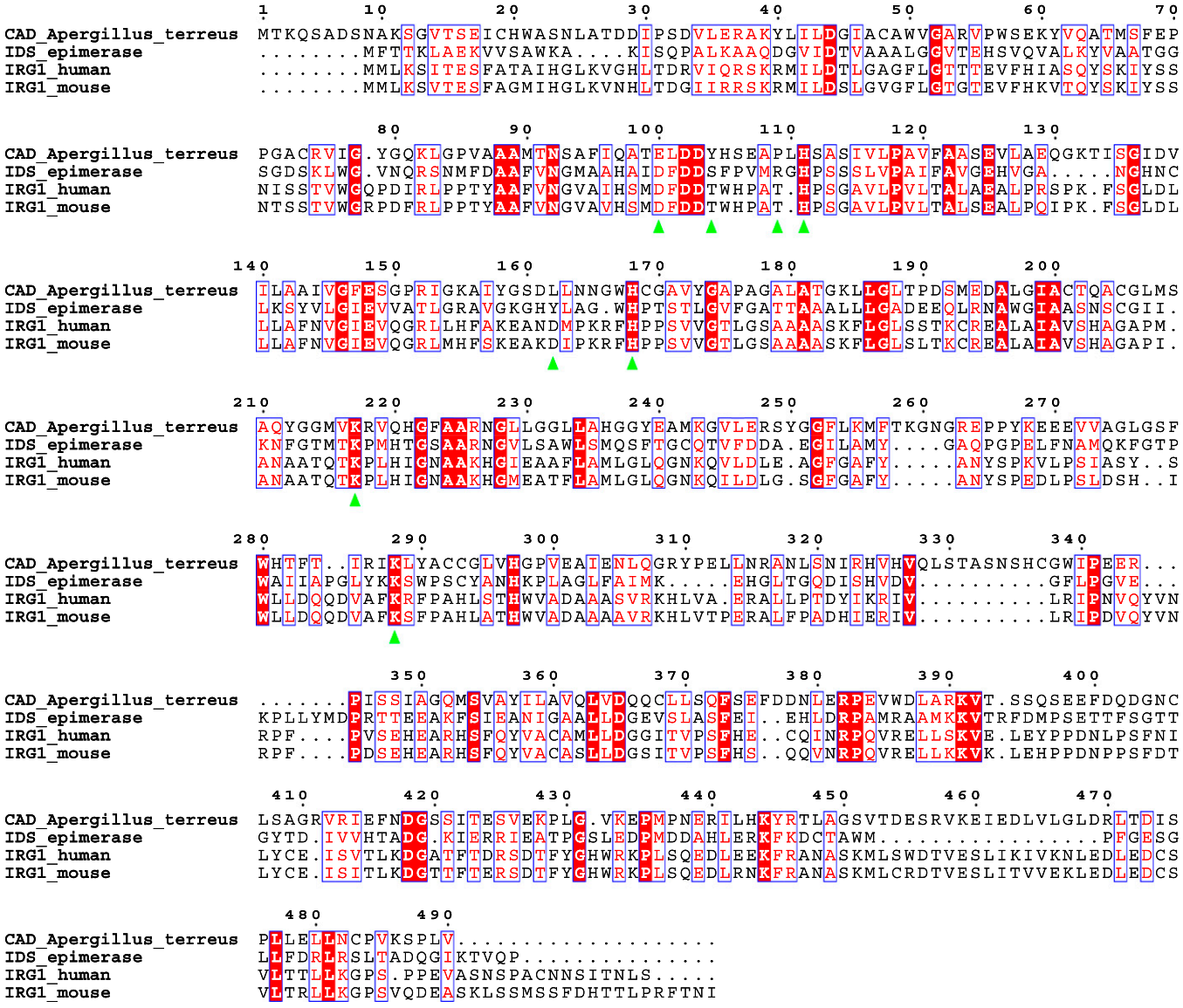


**Fig. S2.** Multiple sequence alignment of *cis*-aconitic acid decarboxylase (CAD) (*Aspergillus terreus*), immune-responsive gene 1 protein homolog (IRG1) (Homo sapiens), immune-responsive gene 1 protein (IRG1) (Mus musculus), and iminodisuccinate (IDS) epimerase (*Agrobacterium tumefaciens*). Between CAD1 and IRG1, five of eight active site residues are conserved. Conserved residues are shown in red; residues assumed to build active site are indicated with green triangles below the alignment. Figure was drawn with ESPript. Sequences were obtained from UniProt Knowledgebase (UniProtKB) with the following accession numbers: B3IUN8 (CAD1), A6NK06 (IRG1 human), P54987 (IRG1 mouse), and Q1L4E3 (IDS epimerase).

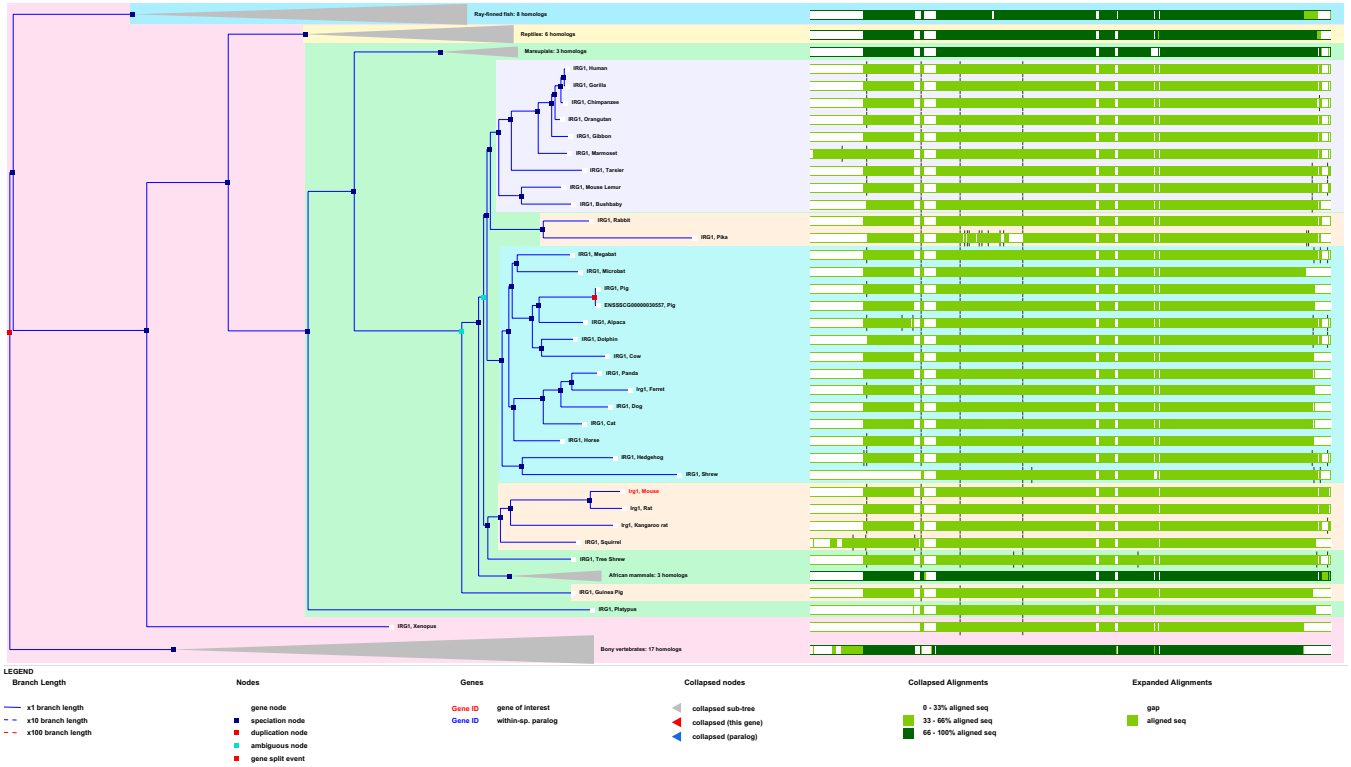


**Fig. S3.** Gene tree of mouse *Irg1*. Gene Tree was generated using the Ensembl gene orthology/paralogy prediction method pipeline (1). (*Left*) The evolutionary history of *Irg1* across species. (*Right*) A multiple sequence alignment of the associated proteins. Green bars show areas of amino acid alignment; white areas are gaps in the alignment.

1. Vilella AJ, et al. (2009) EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. *Genome Res* 19(2):327–335.

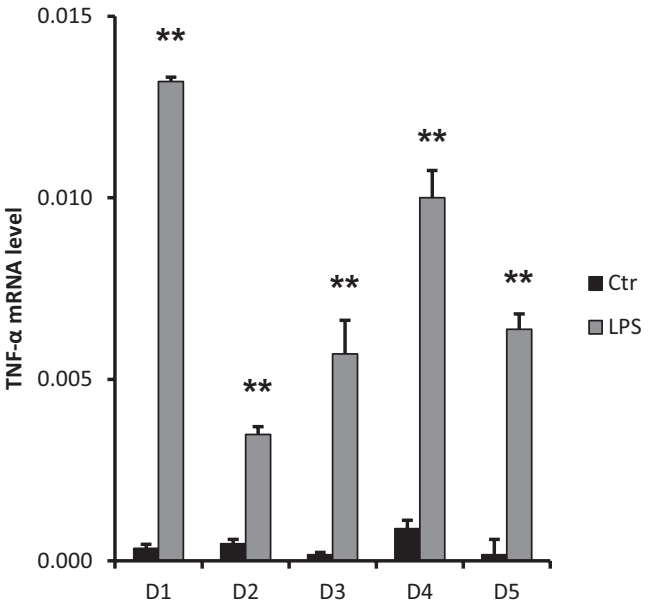


**Fig. S4.** TNF-α expression in LPS-activated human PBMC-derived macrophages. RNA extractions were performed after 6 h of LPS (10 μg/mL) stimulation of PBMC-derived macrophages from five different donors (D). The levels of TNF-α mRNA were determined by real-time RT-PCR and normalized using L27 as a housekeeping gene. Each bar represents the average expression fold change of three technical replicates (± SEM). $^{**}P < 0.01$.

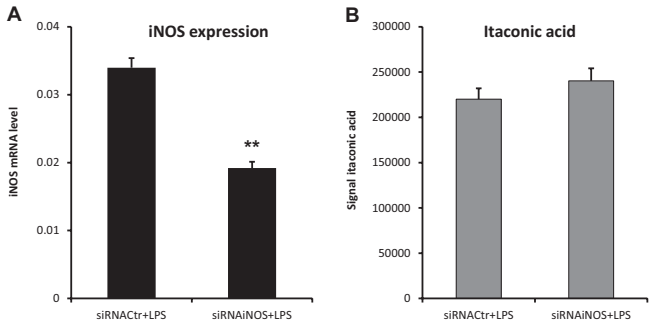


**Fig. S5.** (*A*) Levels of mRNA or (*B*) itaconic acid in LPS-activated RAW264.7 macrophages transfected with either siRNA specific for iNOS or with siRNA nontargeting control (Ctr). Metabolites and RNA extractions were performed after 6 h of stimulation. The levels of iNOS mRNA were determined by real-time RT-PCR and normalized using L27 as a housekeeping gene. Each bar represents the average expression fold change (± SEM) from three independent experiments. The levels of itaconic acid were determined by GC/MS measurements. Each bar represents itaconic acid levels (± SEM). $^{**}P < 0.01$.

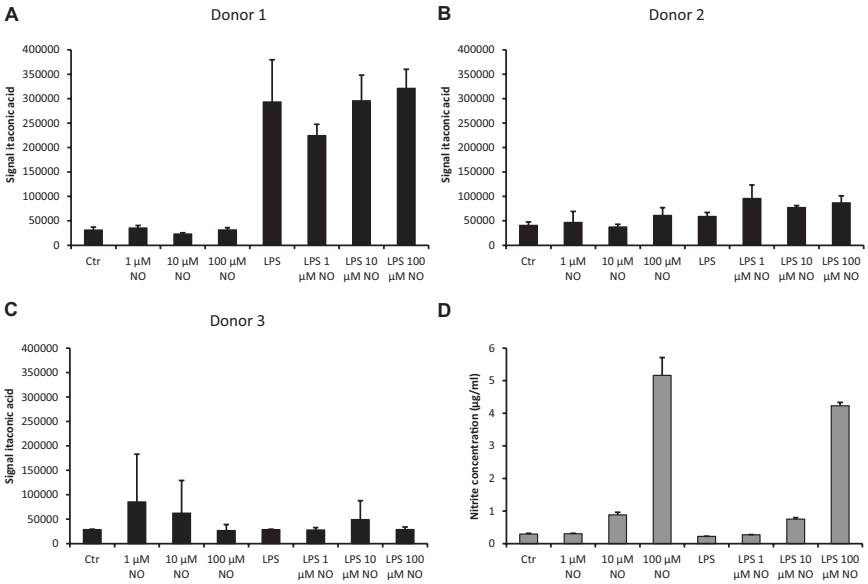


**Fig. S6.** (*A*–*C*) Itaconic acid levels in Ctr and LPS-activated (10 μg/mL) PBMC-derived macrophages from three donors treated with DEA NONOate at different concentrations (1, 10, 100 μM). Metabolites were harvested after 12 h of stimulation and the levels of itaconic acid were determined by GC/MS measurements. Each bar represents the mean of itaconic acid levels from three technical replicates (± SEM). (*D*) After 12 h, 180 μL of medium was harvested and combined with 20 μL 1 mM NaOH on ice to stop the dissociation reaction. Levels of nitrite were determined using the Griess assay and the concentrations were determined against a nitrite standard curve. Bars represent the mean of nitrite concentration (μg/mL) from the three donors (± SEM).

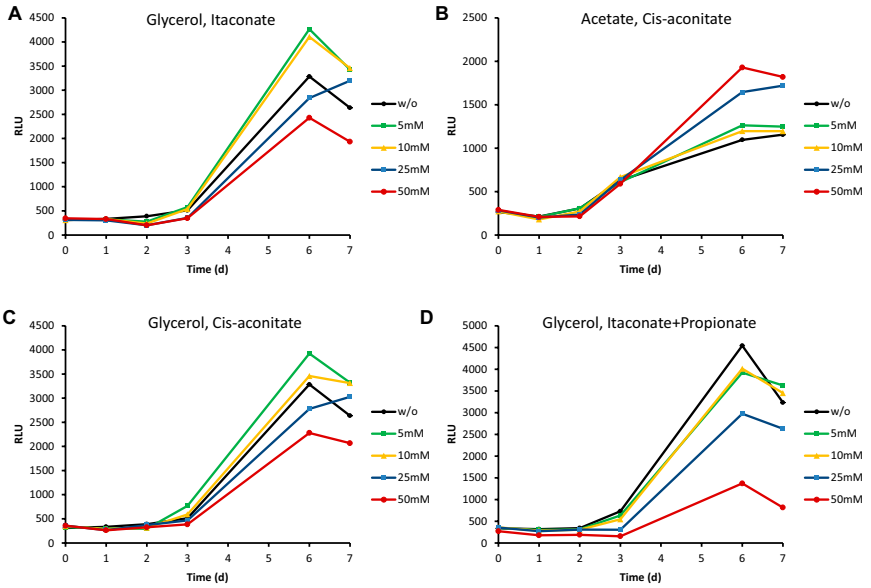


**Fig. S7.** GFP-expressing *Mycobacterium tuberculosis* bacteria were cultured in 7H9 medium supplemented with different carbon sources, without (w/o) and with various concentrations of itaconate (5, 10, 25, 50 mM) or *cis*-aconitate as indicated: (*A*) glycerol and itaconate, (*B*) acetate and *cis*-aconitate, (*C*) glycerol and *cis*-aconitate, and (*D*) glycerol, propionate, and itaconate. Growth was measured as relative light units (RLU) at indicated time points. Curves represent the mean of three technical replicates.

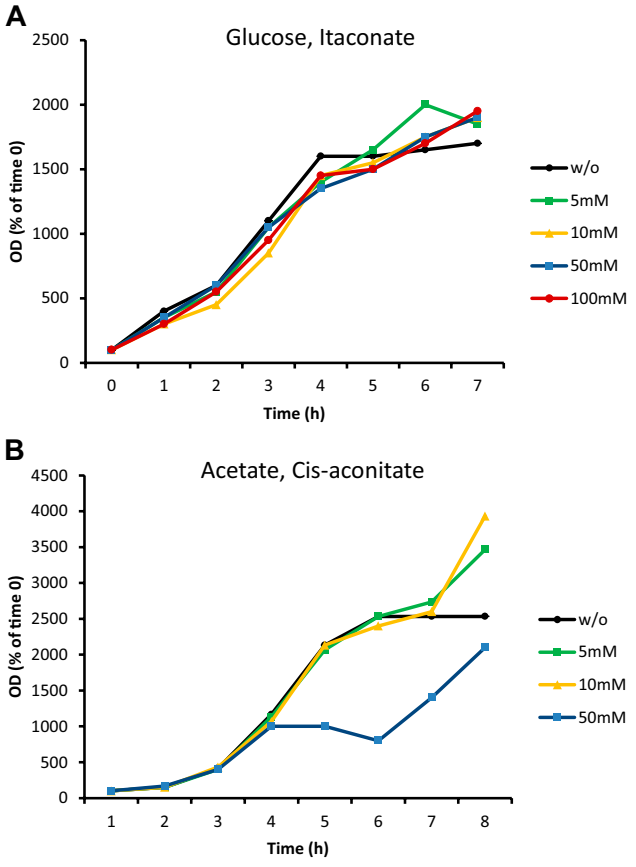


**Fig. S8.** (*A*) *Salmonella enterica* was grown in liquid medium with glucose in the presence of itaconic acid, and the OD was measured every hour (h). Curves represent the mean of two independent experiments. (*B*) *S. enterica* was grown in liquid medium with acetate as a unique carbon source without (w/o) or with increasing concentrations of *cis*-aconitate (5, 10, 50 mM). The OD was measured every hour to record the bacterial growth. Curves are calculated in the percent relative to time 0 and represent the mean of two independent experiments.

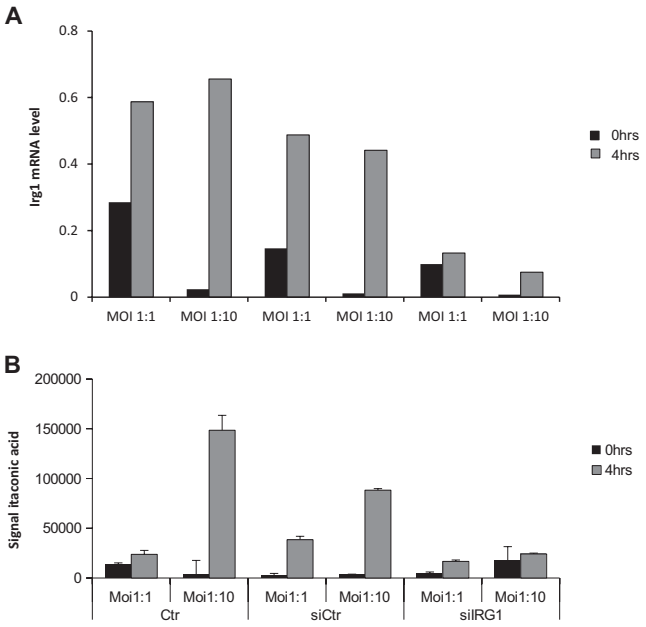


**Fig. S9.** (*A*) Levels of *Irg1* mRNA or (*B*) itaconic acid in RAW264.7 cells transfected with either siRNA specific for *Irg1* or with siRNA control under *S. enterica* infection at a multiplicity of infection (MOI) of 1:1 or 1:10 bacteria per macrophages. Infections were performed after 24 h of transfection and incubated for 0 h or 4 h after 1 h gentamycin exposure. Macrophages were then lysed to extract RNA and metabolites. Bars represent the results from one experiment.

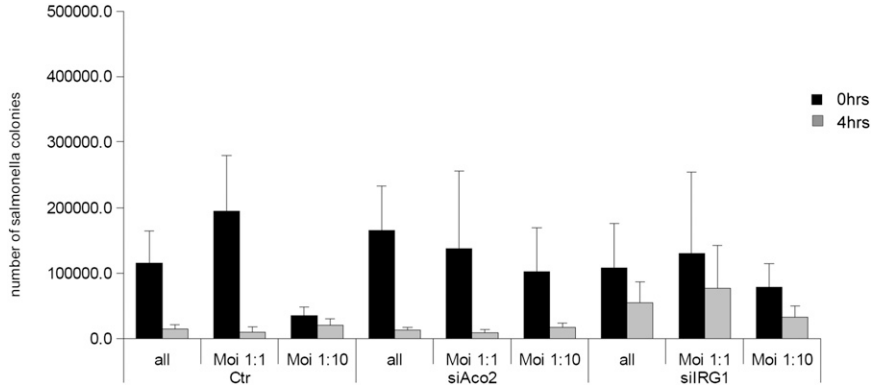


**Fig. S10.** Effect of *Irg1* silencing in macrophages on the bacterial growth. Mouse RAW264.7 cells were transfected with either siRNA specific for *Irg1* or with siRNA specific for murine aconitase 2 (*Aco2*) as control. Macrophages were infected with *S. enterica* at an MOI of 1:1 or 1:10 bacteria per macrophages. Infections were performed after 24 h of transfection and incubated for 0 h or 4 h after 1 h gentamycin exposure. Bars represent the mean of the numbers of colonies (± SEM) obtained from one experiment.