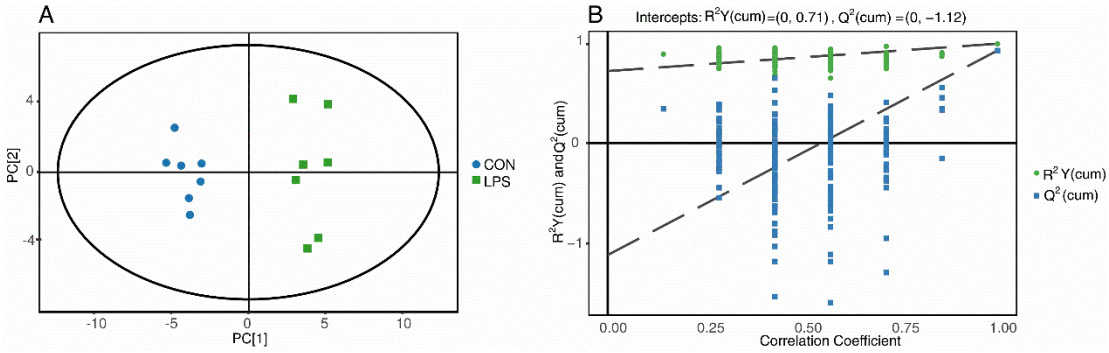
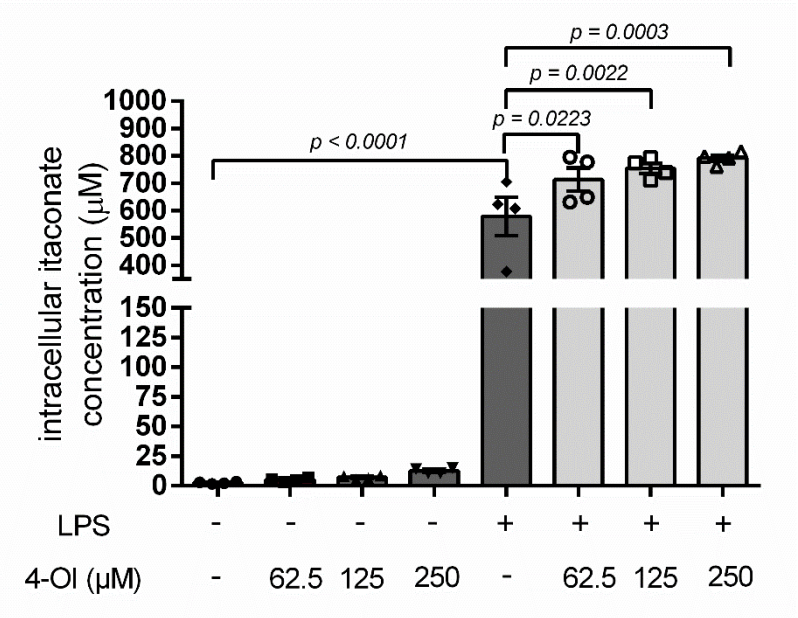
Supplementary Information

4-Octyl itaconate inhibits aerobic glycolysis by targeting GAPDH to exert anti-inflammatory effects

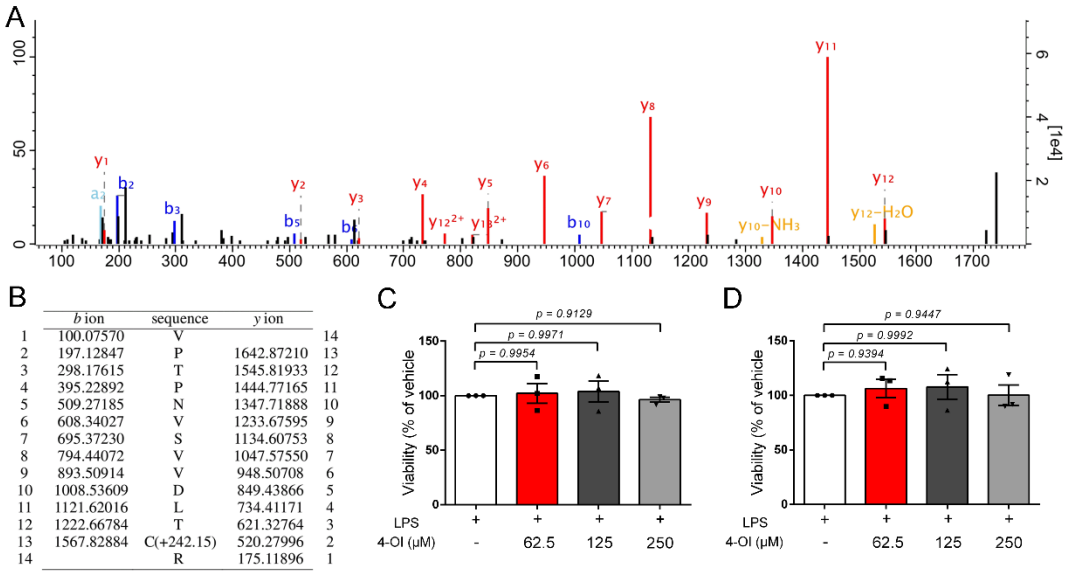
Liao et al.



Supplementary Fig. 1 LC-MS/MS metabolomics of control (CON) and LPS-stimulated (LPS) groups. (A), Score plots of the principal component analysis (PCA) model differentiated metabolites of control (CON) and LPS-stimulated (LPS) RAW264.7 macrophages. (B), Scatter plots of the statistical validations obtained by 200 permutation tests for OPLS-DA analysis in RAW264.7 macrophages. Results are from seven independent experiments.

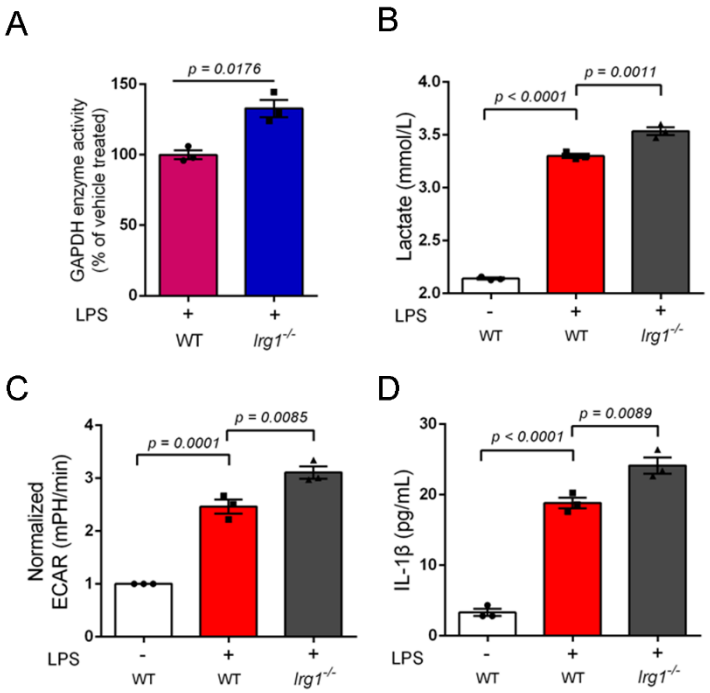


Supplementary Fig. 2 4-OI was hydrolysed to itaconate in LPS stimulated RAW264.7 macrophages. Macrophages were treated with vehicle or 4-OI at the indicated concentrations. After 3 h, cells were stimulated with or without 1 μg/mL LPS for 24 h. Itaconate levels in RAW264.7 macrophages were quantitative by LC-MS/MS. Data represent the mean ± SEM of four independent experiments. *p* values were determined by one-way ANOVA with Sidak's correction for multiple comparisons. Source data are provided as a Source Data file.

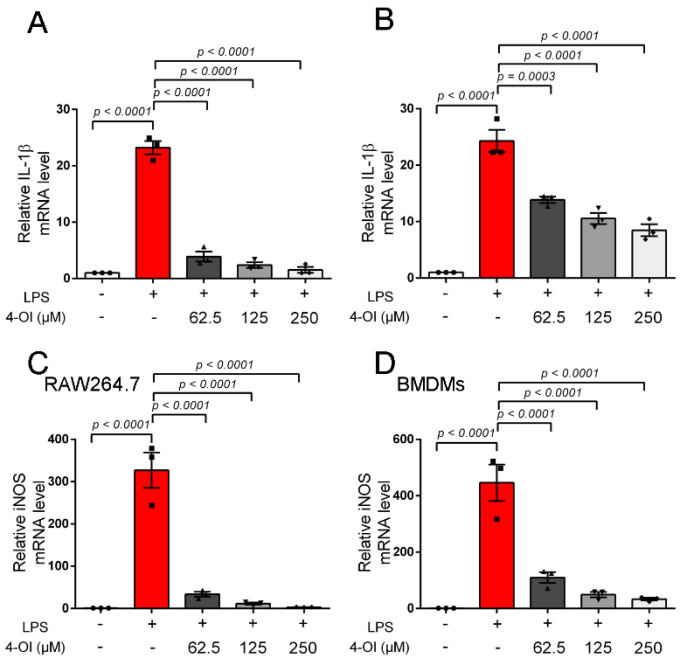


	b ion	sequence	*y* ion	
1	100.07570	V		14
2	197.12847	P	1642.87210	13
3	298.17615	T	1545.81933	12
4	395.22892	P	1444.77165	11
5	509.27185	N	1347.71888	10
6	608.34027	V	1233.67595	9
7	695.37230	S	1134.60753	8
8	794.44072	V	1047.57550	7
9	893.50914	V	948.50708	6
10	1008.53609	D	849.43866	5
11	1121.62016	L	734.41171	4
12	1222.66784	T	621.32764	3
13	1567.82884	C(+242.15)	520.27996	2
14		R	175.11896	1

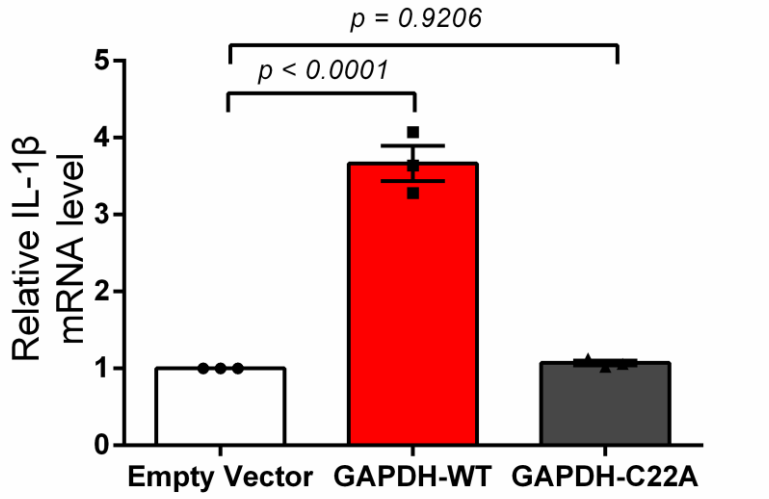
Supplementary Fig. 3 4-OI modified recombinant mouse GAPDH. (A) and (B), Representative LC-MS/MS spectra demonstrated modification of GAPDH cysteine 245 (+242.15 Da) in recombinant mouse GAPDH incubated with 4-OI (500 μM) for 4 h at 37 °C. Da = daltons. (C) and (D), RAW264.7 macrophages (C) or BMDMs (D) were treated with 100 ng/ml or 1 μg/ml LPS plus the indicated doses of 4-OI for 24 h. Viability was assessed by cell counting kit-8. Data represent the mean ± SEM of three experiments performed in duplicate. The results are non-significant by one-way ANOVA with Sidak's correction for multiple comparisons. Source data are provided as a Source Data file.



Supplementary Fig. 4 *Irg1* deficiency leads to enhanced GAPDH activity and glycolysis. WT BMDMs and *Irg1*$^{-/-}$ BMDMs were detected for GAPDH enzyme activity (A) after 100 ng/mL LPS stimulation for 24 h. Levels of lactate (B), ECAR (C) and IL-1β (D) were determined in WT and *Irg1*$^{-/-}$ BMDMs with or without LPS stimulation for 24 h. All data shown are summarized from three independent experiments. Values represent the mean ± SEM at each time point. *p* values were calculated using two-tailed Student's t-test or one-way ANOVA with Sidak's correction for multiple comparisons. Source data are provided as a Source Data file.



Supplementary Fig. 5 4-OI inhibited IL-1β and iNOS mRNA expressions. Macrophages were treated with vehicle or 4-OI at the indicated concentrations. After 3 h, cells were stimulated with LPS (1 μg/mL or 100 ng/mL) for 24 h. (A) and (B), The mRNA levels of IL-1β in RAW264.7 macrophages (A) and BMDMs (B) were measured by qRT-PCR. (C) and (D), The mRNA expression of IL-1β in RAW264.7 macrophages (C) and BMDMs (D) was identified by qRT-PCR. Data represent the mean ± SEM of three independent experiments. *p* values were determined by one-way ANOVA with Sidak's correction for multiple comparisons. Source data are provided as a Source Data file.



Supplementary Fig. 6 Wild-type GAPDH (GAPDH-WT) abolished the inhibitory effect of 4-OI. GAPDH-WT or the Cys-22 mutant (GAPDH-C22A) was overexpressed in RAW264.7 macrophages for 24 h, and the cells were then treated with LPS and 4-OI for 24 h, followed by measurement of IL-1β mRNA levels by qRT-PCR. Data represent the mean ± SEM of three experiments. *p* values were calculated by one-way ANOVA with Sidak's correction for multiple comparisons. Source data are provided as a Source Data file.

Supplementary Table 1. The sequence of mouse GAPDH peptide

	b ion	sequence	*y* ion	
1	72.04440	A		7
2	143.08152	A	820.44850	6
3	256.16559	I	749.41138	5
4	601.32659	C+(242.15)	636.32731	4
5	688.35862	S	291.16631	3
6	745.38009	G	204.13428	2
7		K	147.11281	1

4-OI modified Cys 22 of GAPDH in RAW264.7 macrophages.

Supplementary Table 2. The sequence of mouse GAPDH peptide

	b ion	sequence	*y* ion	
1	72.04440	A		7
2	143.08152	A	708.32330	6
3	256.16559	I	637.28618	5
4	489.20139	C+(130.02)	524.20211	4
5	576.23342	S	291.16631	3
6	633.25489	G	204.13428	2
7		K	147.11281	1

Itaconate covalently modified Cys 22-containing GAPDH peptide in LPS-induced RAW264.7 macrophages.

Supplementary Table 3. The sequence of recombinant mouse GAPDH peptide

	b ion	sequence	*y* ion	
1	72.04440	A		7
2	143.08152	A	820.44850	6
3	256.16559	I	749.41138	5
4	601.32659	C+(242.15)	636.32731	4
5	688.35862	S	291.16631	3
6	745.38009	G	204.13428	2
7		K	147.11281	1

4-OI covalently modified Cys 22 of recombinant mouse GAPDH.