
Figures and figure supplements

Statin-mediated reduction in mitochondrial cholesterol primes an anti-inflammatory response in macrophages by upregulating Jmjd3

Zeina Salloum et al.

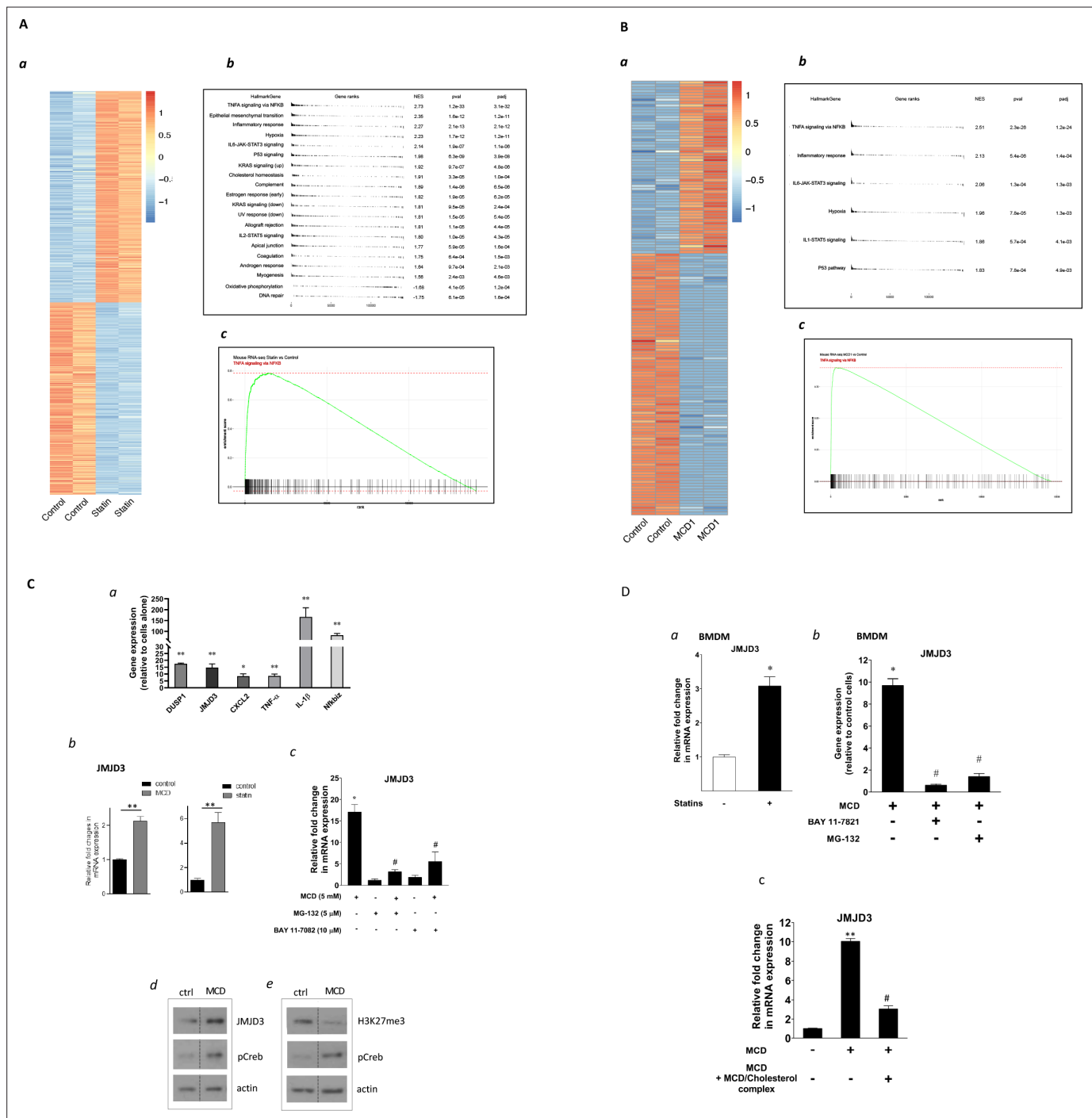


Figure 1. Statins upregulates the expression of *Jmjd3* in macrophages through NF- κ B. (A) Statins activate NF- κ B pathways in RAW 264.7 cells. (a) Heatmap of differentially expressed genes with or without statin (lovastatin, 7 + 200 μ M mevalonate; 2 days). (b) Pathways identified by gene set enrichment analysis (GSEA) of differentially expressed genes in (a). (c) The details of most highly represented pathway, TNFA signaling vis NF- κ B. (B) Methyl- β -cyclodextrin (MCD) activate NF- κ B pathways in RAW 264.7 cells. (a) Heatmap of differentially expressed genes with or without MCD (5 mM, 1 hr). (b) Pathways identified by GSEA of differentially expressed genes in (a). (c) The details of most highly represented pathway, TNFA signaling vis NF- κ B. (C) Statins and MCD upregulate *Jmjd3* in RAW 264.7 cells. (a) Reverse transcriptase quantitative PCR (RT-qPCR) of genes with or without MCD (5 mM; 1 hr). (b) *Jmjd3* gene expression in MCD- or statin-treated RAW 264.7 macrophages. (c) Effect of NF- κ B inhibitors, MG-132 (5 μ M) and BAY11-7082 (10 μ M) on *Jmjd3* expression in MCD-treated RAW macrophages. (d) Western blotting of JMJD3 protein expression and (e) levels of H3K27me3 in macrophages treated with 5 mM MCD (1 hr). The pCREB was used as internal control for cholesterol depletion and actin a loading control. Original blots are in source data. (D) Statin/MCD upregulates *Jmjd3* in bone marrow-derived macrophages (BMDMs). (a) *Jmjd3* gene expression in statin-treated BMDMs (10 μ M pravastatin + 200 μ M mevalonate; 2 days). (b) Effect of NF- κ B inhibitors, MG-132 [5 μ M] and BAY11-7082 [10 μ M], on

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Jmjd3 expression in MCD-treated BMDMs. (c) *Jmjd3* expression in cholesterol repletes MCD-treated macrophages. Graphs are representative of 3 independent experiments with 3 replicates per condition and are presented as means \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's *t*-test. An asterisk (*) or double asterisks (**) indicate a significant difference with $p < 0.05$ and $p < 0.001$, respectively. A hashtag (#) indicates a significant difference between MCD without or with inhibitors, $p < 0.05$.

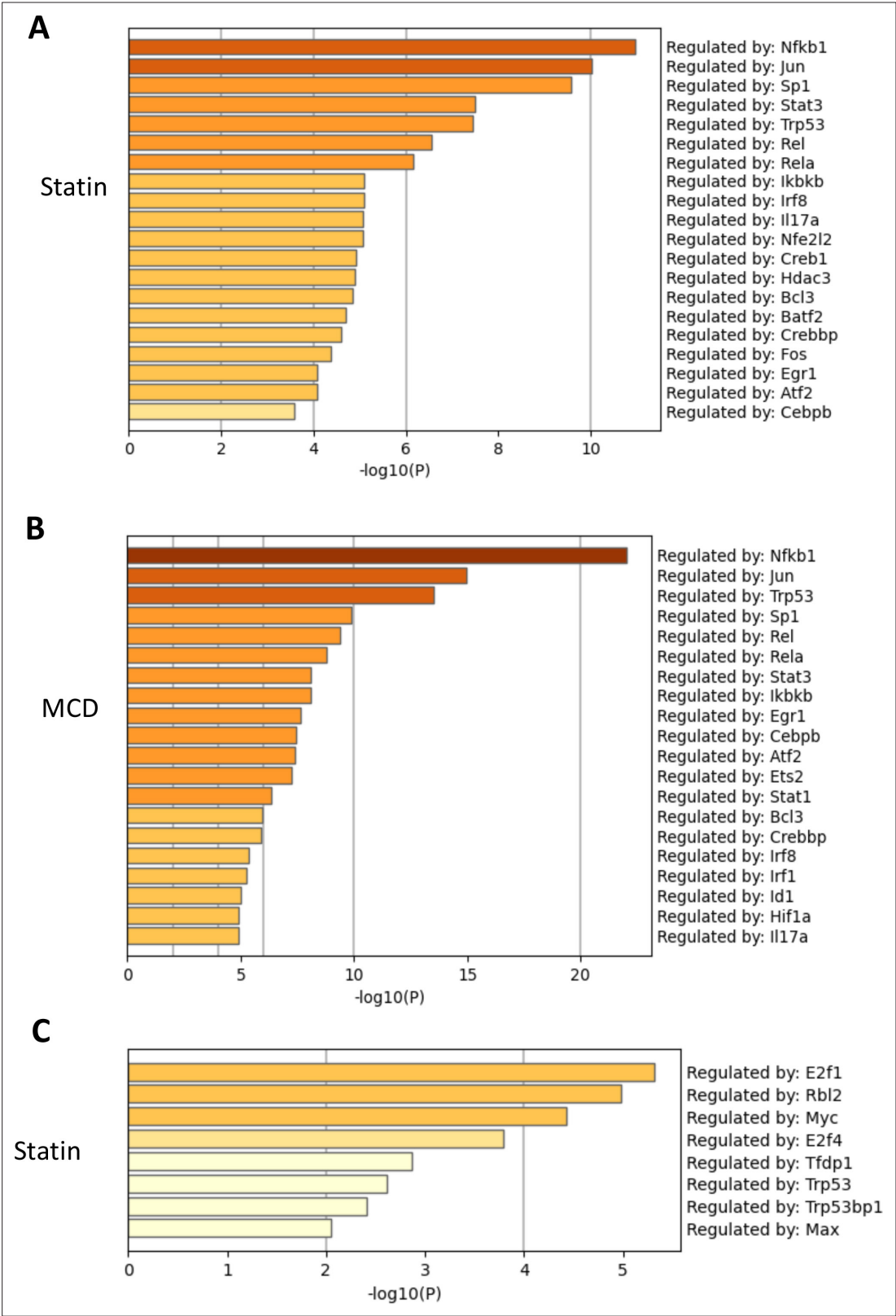


Figure 1—figure supplement 1. Cellular cholesterol contents regulate NF- κ B pathway. RAW macrophages were treated with methyl- β -cyclodextrin (MCD) or statins, and RNA-seq was performed as in **Figure 1**. TF regulatory pathways of activated genes were analyzed using Metascape; top TFs employed by either lovastatin (**A**) (7 + 200 μ M mevalonate) or MCD (**B**) (5 mM, 1 hr). The genes downregulated by statin is analyzed in (**C**). No TF is identified in MCD downregulated genes.

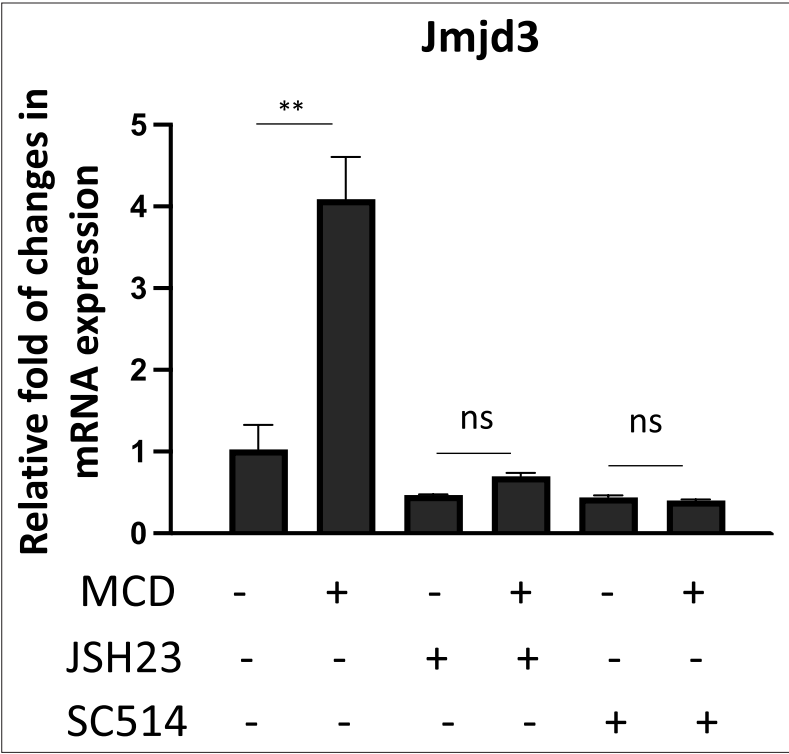


Figure 1—figure supplement 2. The expression of *Jmjd3* activated by methyl- β -cyclodextrin (MCD) requires NF- κ B activity. Effect of NF- κ B inhibitors, JSH23 (10 μ M) and SC514 (10 μ M), on *Jmjd3* expression in MCD-treated RAW macrophages. Graphs are representative of 3 independent experiments with 3 replicates per condition and are presented as means \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's t-test. Double asterisks (**) indicates a significant difference $p < 0.001$.

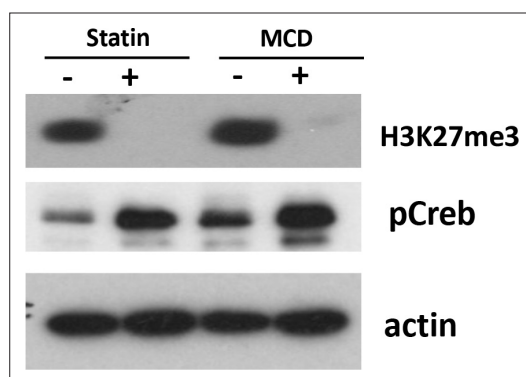


Figure 1—figure supplement 3. Levels of H3K27Me3 are decreased in macrophages treated with statin or methyl- β -cyclodextrin (MCD) (lovastatin, 7 + 200 μ M mevalonate; 2 days) or MCD (5 mM, 1 hr). The pCREB was used as internal control for cholesterol depletion and actin a loading control. Original blots are in source data.

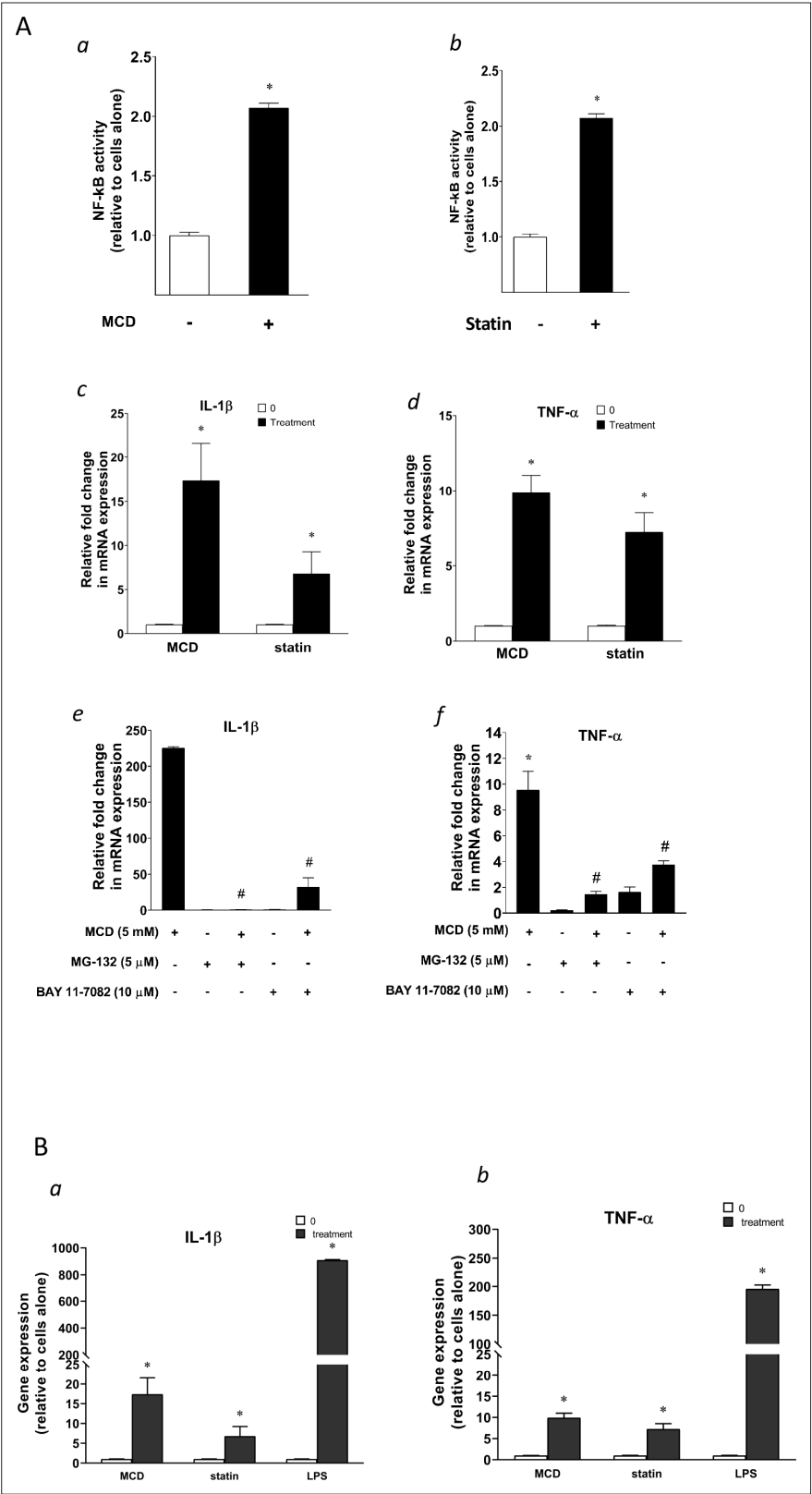


Figure 2. Cellular cholesterol contents regulate NF- κ B pathway. **(A)** NF- κ B activation by methyl- β -cyclodextrin (MCD) **(a)** and statins **(b)** using RAW blue macrophages. Reverse transcriptase quantitative PCR (RT-qPCR) of *Il1b* **(c)** and *Tnfa* **(d)** in RAW 264.7 cells with or without MCD (5 mM; 1 hr), or with or without (10 μ M compactin + 200 μ M mevalonate; 2 days). Effect of NF- κ B inhibitors (MG-132 [5 μ M] and BAY11-7082 [10 μ M]) on *Il1b* **(e)** and

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Tnfa (f) expression in MCD-treated RAW 264.7 macrophages. (B) The gene expression activated by MCD, statin, or lipopolysaccharide (LPS). RT-qPCR of *Il1b* (a) and *Tnfa* (b) in RAW 264.7 cells with or without MCD (5 mM; 1 hr), simvastatin (10 + 200 μ M mevalonate; 2 days) or LPS (100 ng/ml; 3 hr). Graphs are representative of 3 independent experiments with 3 replicates per condition and are presented as means \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's t-test. An asterisk (*) indicates a significant difference with $p < 0.05$. A hashtag (#) indicates a significant difference between MCD without or with inhibitors with $p < 0.05$.

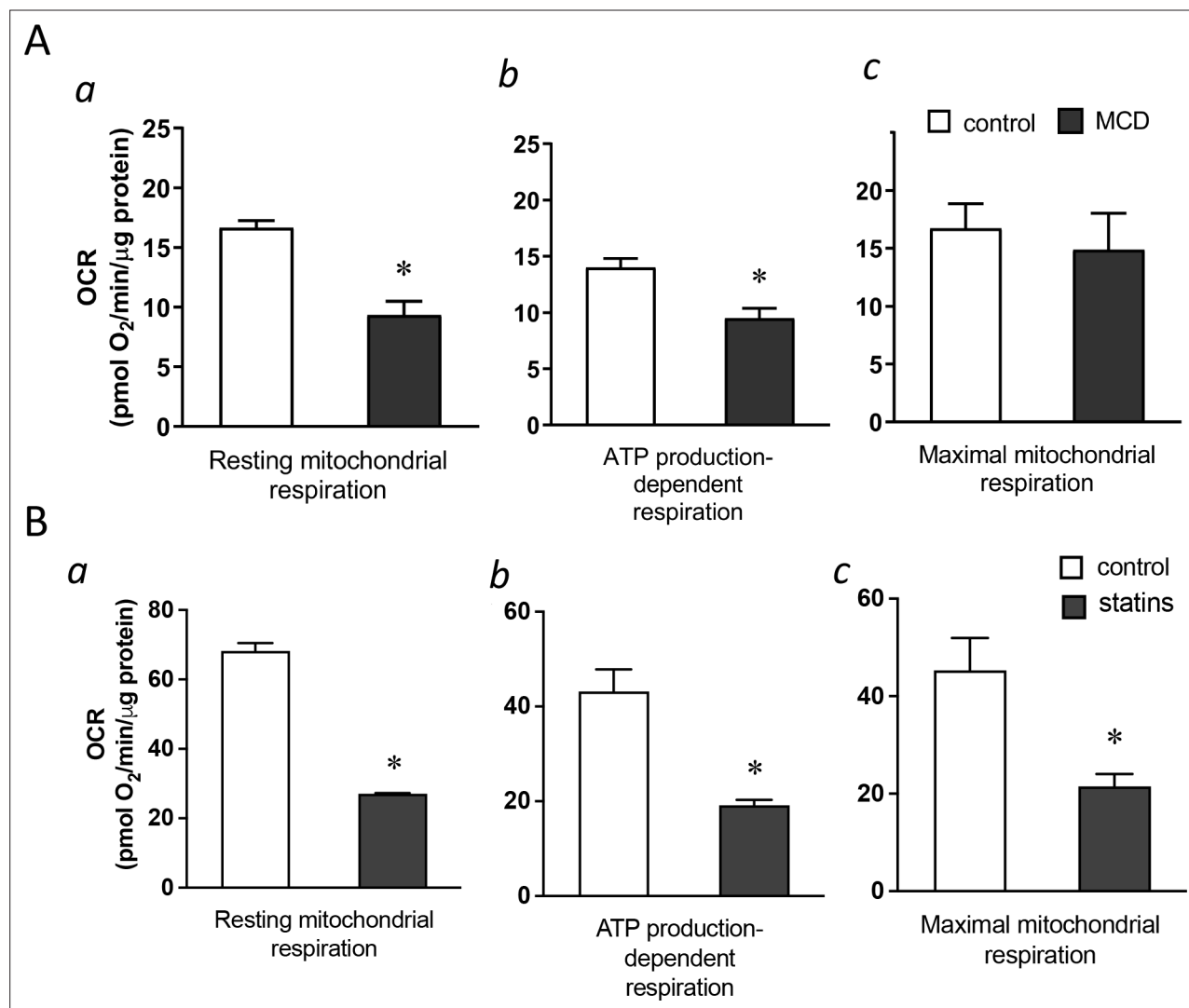


Figure 3. Cholesterol levels modulate mitochondrial respiration. **(A)** Mitochondrial oxygen consumption rates (OCRs) of bone marrow-derived macrophages (BMDMs) treated with 1 hr methyl- β -cyclodextrin (MCD) (5 mM) or without. **(a)** OCR for mitochondrial resting respiration. **(b)** OCR representing mitochondrial ATP production-linked respiration. **(c)** OCR representing maximal respiration. **(B)** Mitochondrial OCRs in BMDMs treated compactin or without compactin (10 + 200 μ M mevalonate; 2 days). **(a)** OCR for mitochondrial resting respiration. **(b)** OCR representing mitochondrial ATP production-linked respiration. **(c)** OCR representing maximal respiration. Data are representative of 3 independent experiments with 3 samples per group and data are presented as mean \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's *t*-test. An asterisk (*) indicates a significant difference, $p < 0.05$.

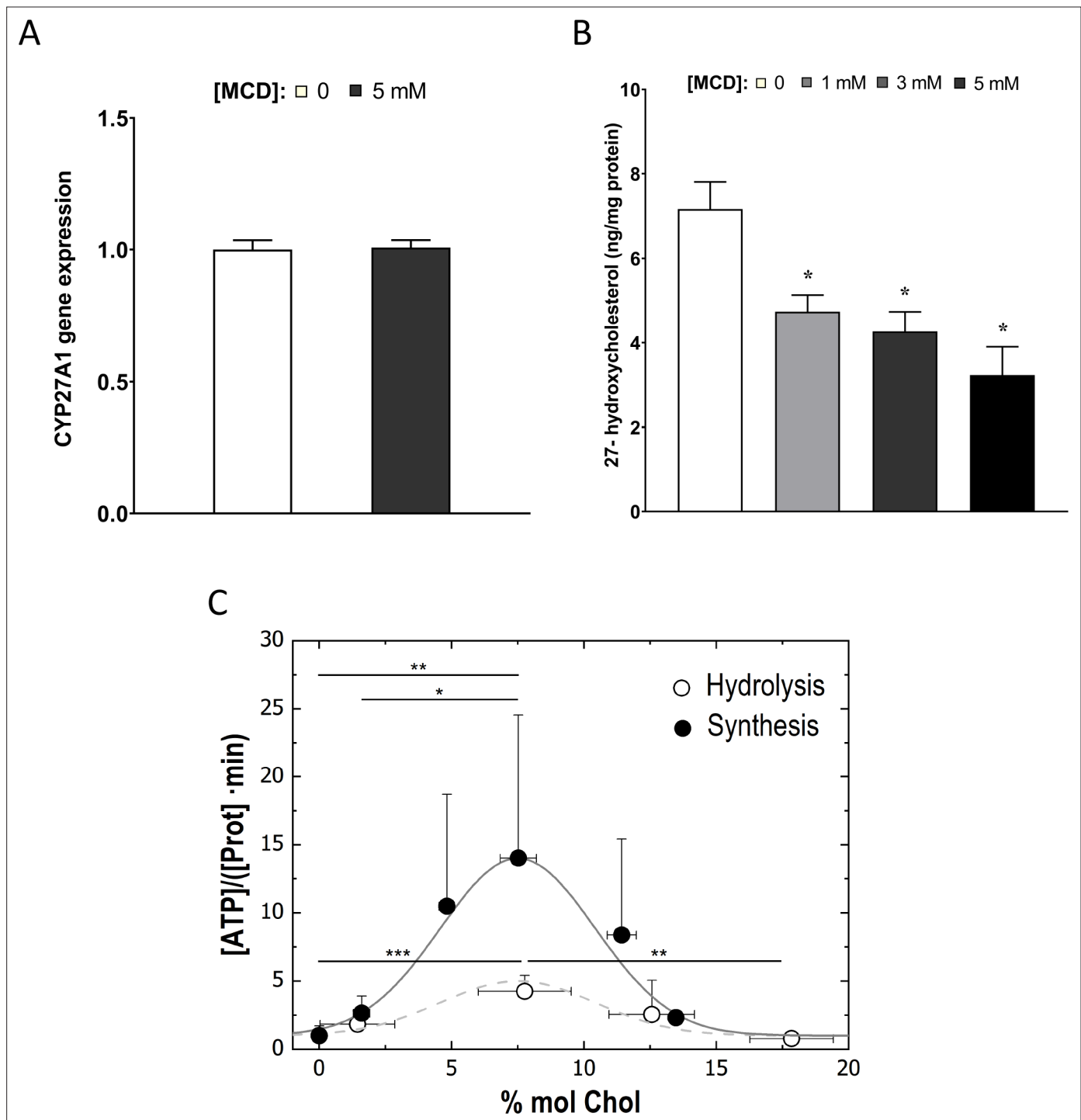


Figure 4. Cholesterol levels modulate the mitochondrial ATP synthase activity. **(A)** Sterol 27-hydroxylase, *Cyp27a1*, expression in 1 hr methyl- β -cyclodextrin (MCD)-treated and control RAW macrophages. **(B)** 27-Hydroxycholesterol (27-HC) analysis by ultraperformance liquid chromatography/electrospray ionization/tandem mass spectrometry. 27-HC levels were normalized to the protein content in the whole cell pellet. **(C)** ATP hydrolysis and synthesis in cholesterol-doped inner membrane vesicles (IMVs); ATP hydrolysis was performed by adding a total concentration of 2 mM ATP to 200 mM IMVs (lipid concentration) and incubated for 30 min. Concentration of phosphates from ATP hydrolysis was measured using the malaquite green assay. ATP concentration after synthesis was measured using ATP detection assay kit (Molecular Probes) with a luminometer GloMax-Multi Detection. Data in B are from 3 samples per group and data are presented as mean \pm standard deviation (SD). An asterisk (*) indicates $p < 0.05$. Data in (C) are representative of at least two independent experiments with three replicates and presented as means \pm SD. Statistical analysis was performed using the Tukey analysis of variance (ANOVA) test. (*), (**), and (***) indicate a significant difference with $p < 0.05$, 0.01, and 0.001, respectively.

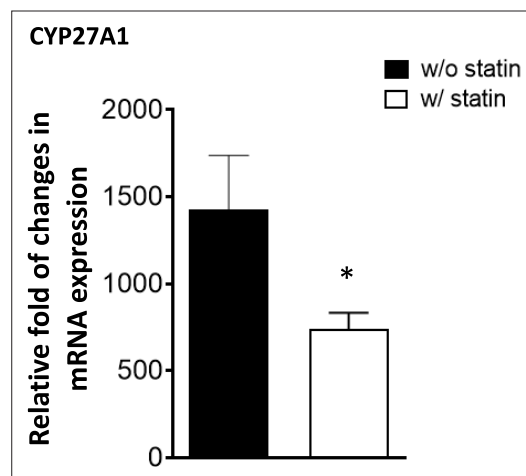


Figure 4—figure supplement 1. Sterol 27-hydroxylase, Cyp27a1, expression. RAW 264.7 macrophages were treated with compactin (7 + 200 μ M mevalonate) for 2 days and the expression of Cyp27a1 analyzed. (*) indicates a significant difference with $p < 0.01$.

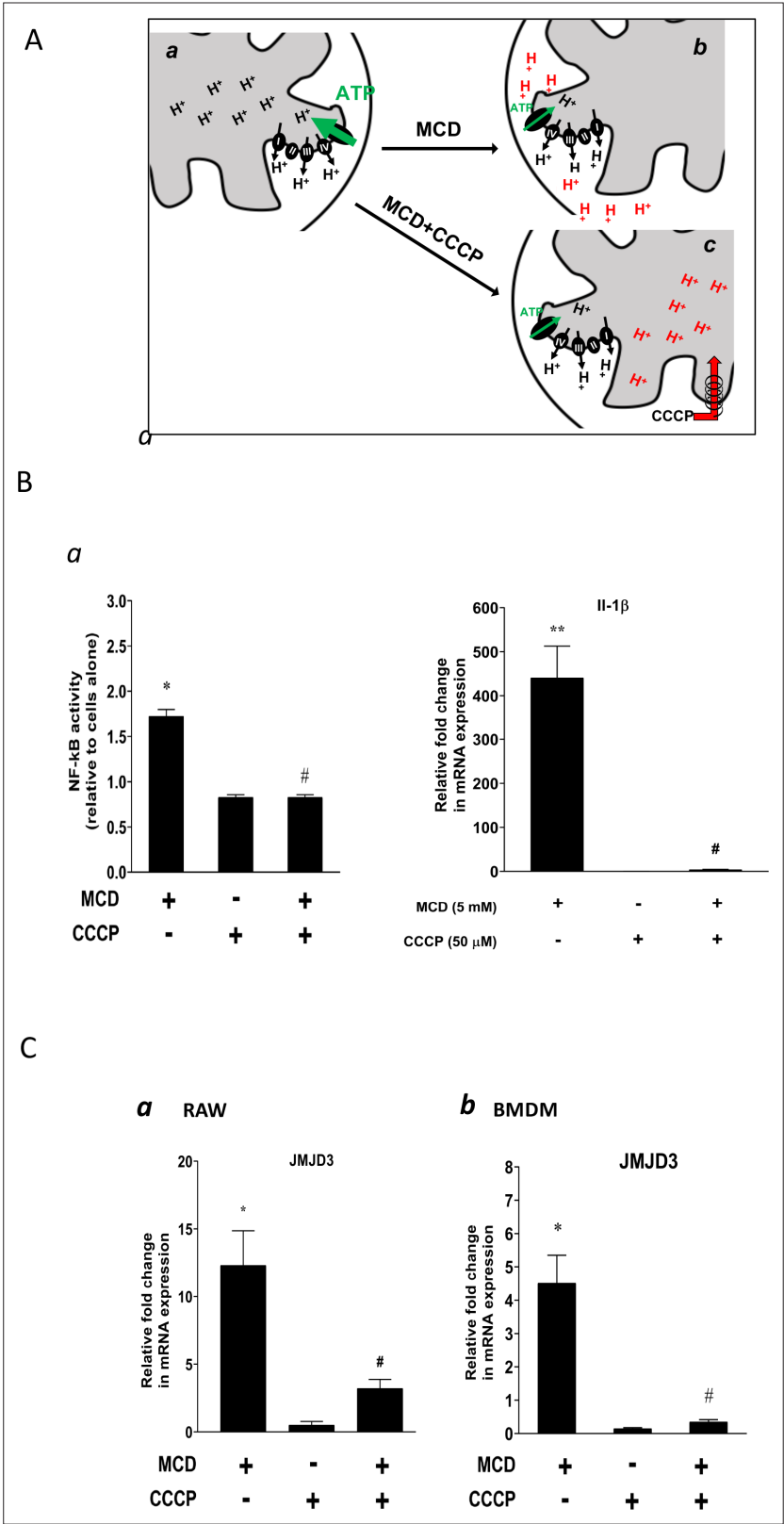


Figure 5. Effect of proton flux on NF- κ B activation and *Jmjd3* expression in methyl- β -cyclodextrin (MCD)-treated cells. **(A)** Schematic of potential mechanism induced by MCD, and MCD/carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) on mitochondrial proton flux. **(B)** Effect of CCCP on NF- κ B activity and *Jmjd3* expression: **(a)** on NF- κ B activity in RAW blue macrophages (CCCP = 50 μ M); **(b)** effect of CCCP on *Il1b* expression in RAW 264.7

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macrophages (CCCP = 50 μ M). **(C)** Effect of CCCP **(a)** on *Jmjd3* expression in RAW 264.7 macrophages (CCCP = 50 μ M) and **(b)** on bone marrow-derived macrophages (BMDMs) (CCCP = 200 μ M). Data are representative of 3 independent experiments with 3 samples per group and data are presented as mean \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's *t*-test. Asterisks (*) and (**) indicate a significant difference with $p < 0.05$ and $p < 0.001$. A hashtag (#) indicates a significant difference between MCD without or with inhibitors with $p < 0.05$.

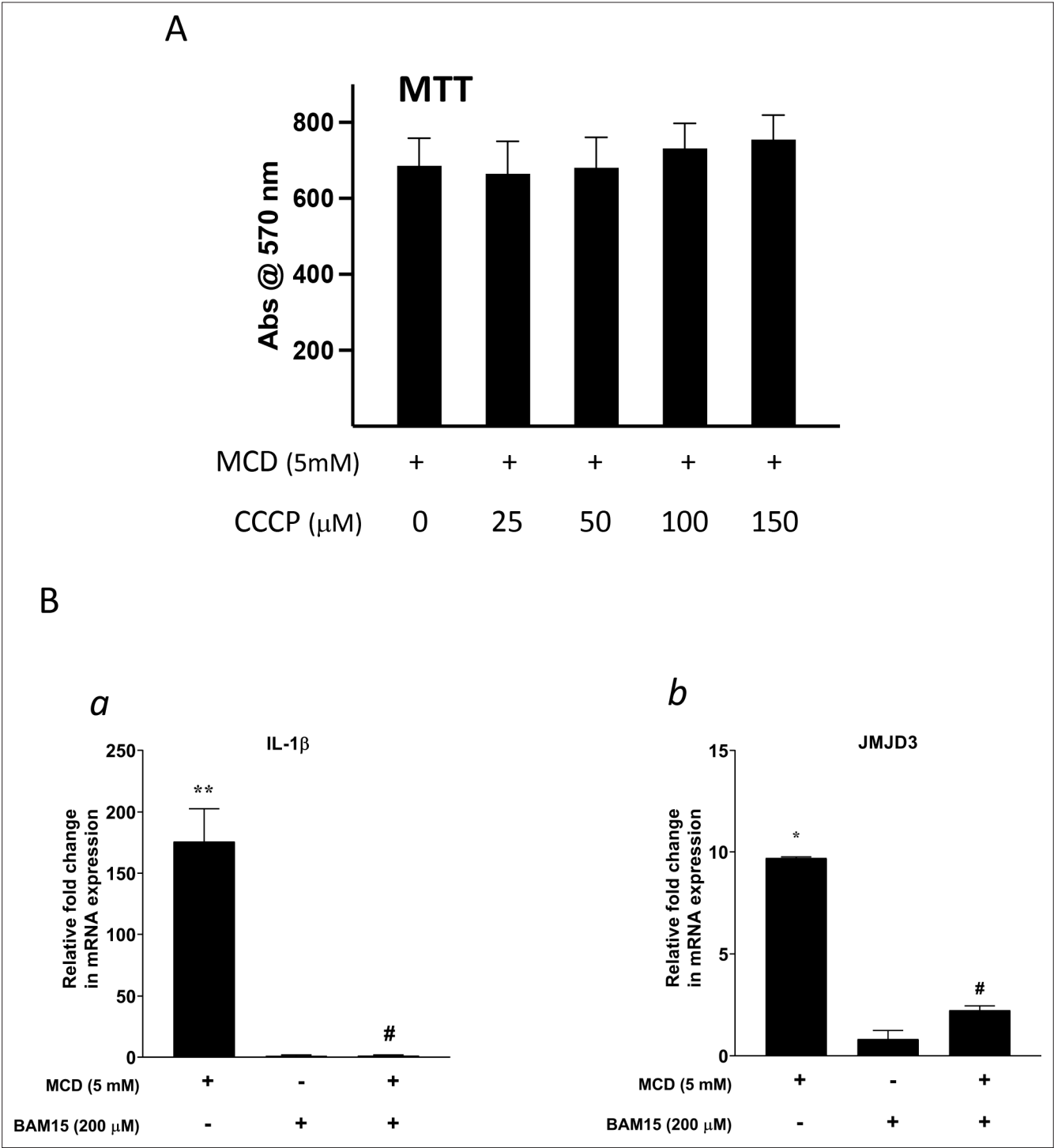


Figure 5—figure supplement 1. The effect of proton flux Inhibitors. **(A)** The toxicity of carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **(B)** Effect of BAM15 (200 μM) on Il1b (a) and Jmjd3 (b) gene expression in methyl-β-cyclodextrin (MCD)-treated RAW264.7 macrophages. Data are representative of 3 independent samples per condition and are presented as mean ± standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's *t*-test. Asterisk (*) and hashtag (#) indicate a significant difference between MCD without or with inhibitors with *p* < 0.05; (**) indicates a significant difference with *p* < 0.01.

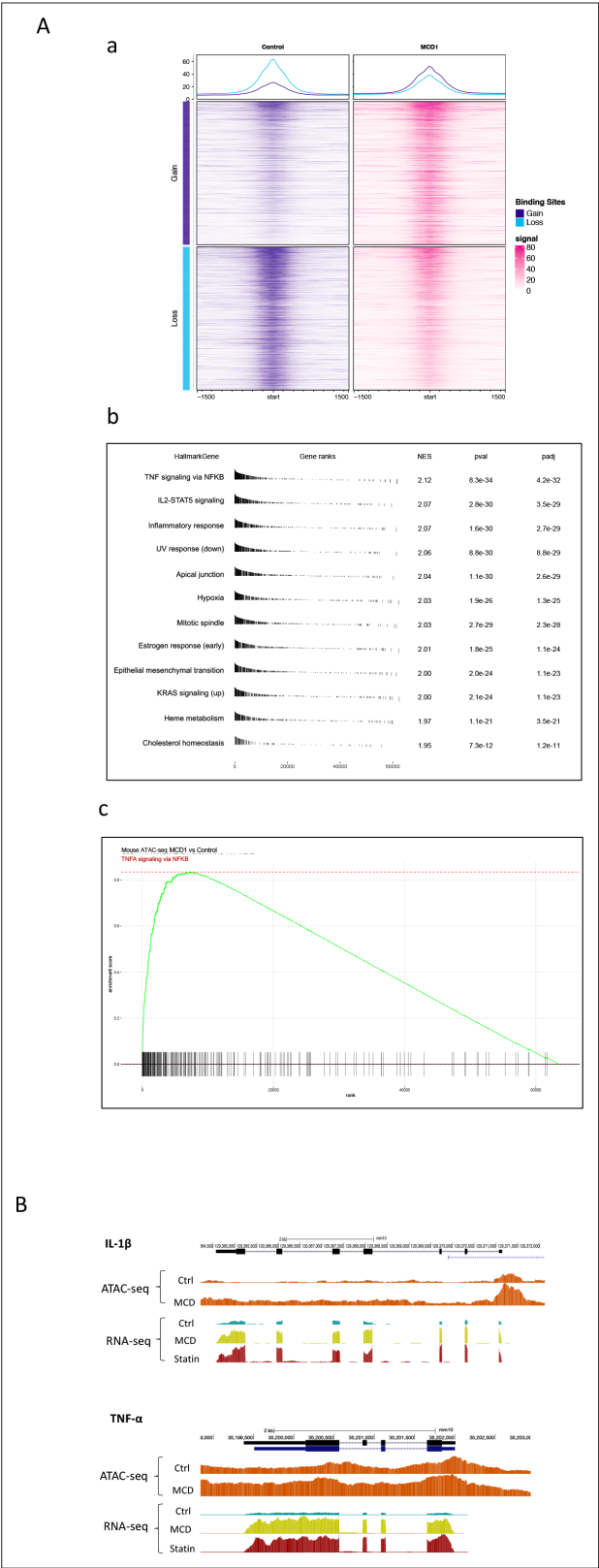


Figure 6. Cholesterol modulates macrophage epigenetic modifications. **(A)** ATAC-seq in control and methyl-β-cyclodextrin (MCD) (5 mM, 1 hr) treated RAW 264.7 macrophages. **(a)** Summit-centered heatmap of differentially accessible ATAC-seq signals. **(b)** Pathways identified by gene set enrichment analysis (GSEA) of differentially assessable genes in **(a)**. **(c)** The details of most highly represented pathway, TNFA signaling vis NF-κB. **(B)** ATAC- and RNA-seq profiles alignment from ATAC- and RNA-seq for the genomic loci of *Il1b* (top) and *Tnfa* (bottom).

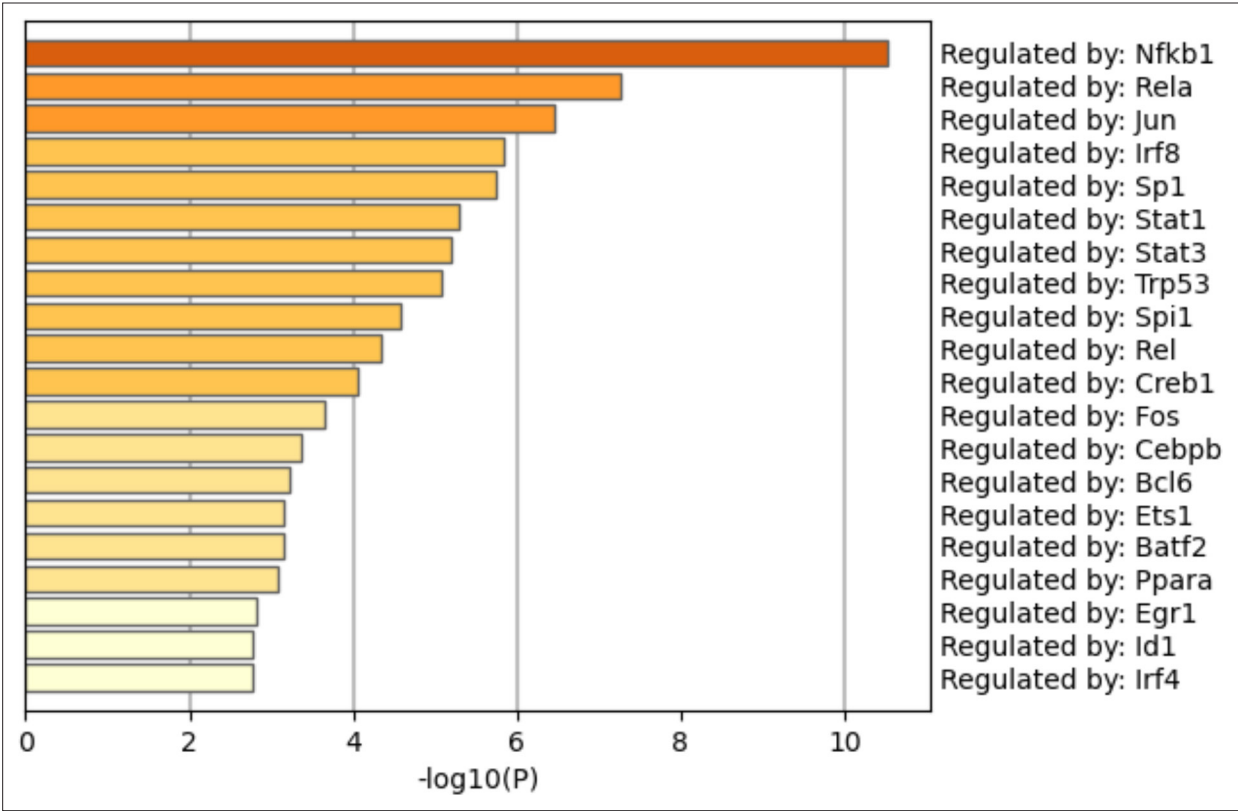


Figure 6—figure supplement 1. Pathway analysis among the genes opened by methyl- β -cyclodextrin (MCD) in ATAC-seq. Regulatory pathways of genes opened by MCD were analyzed using Metascape.

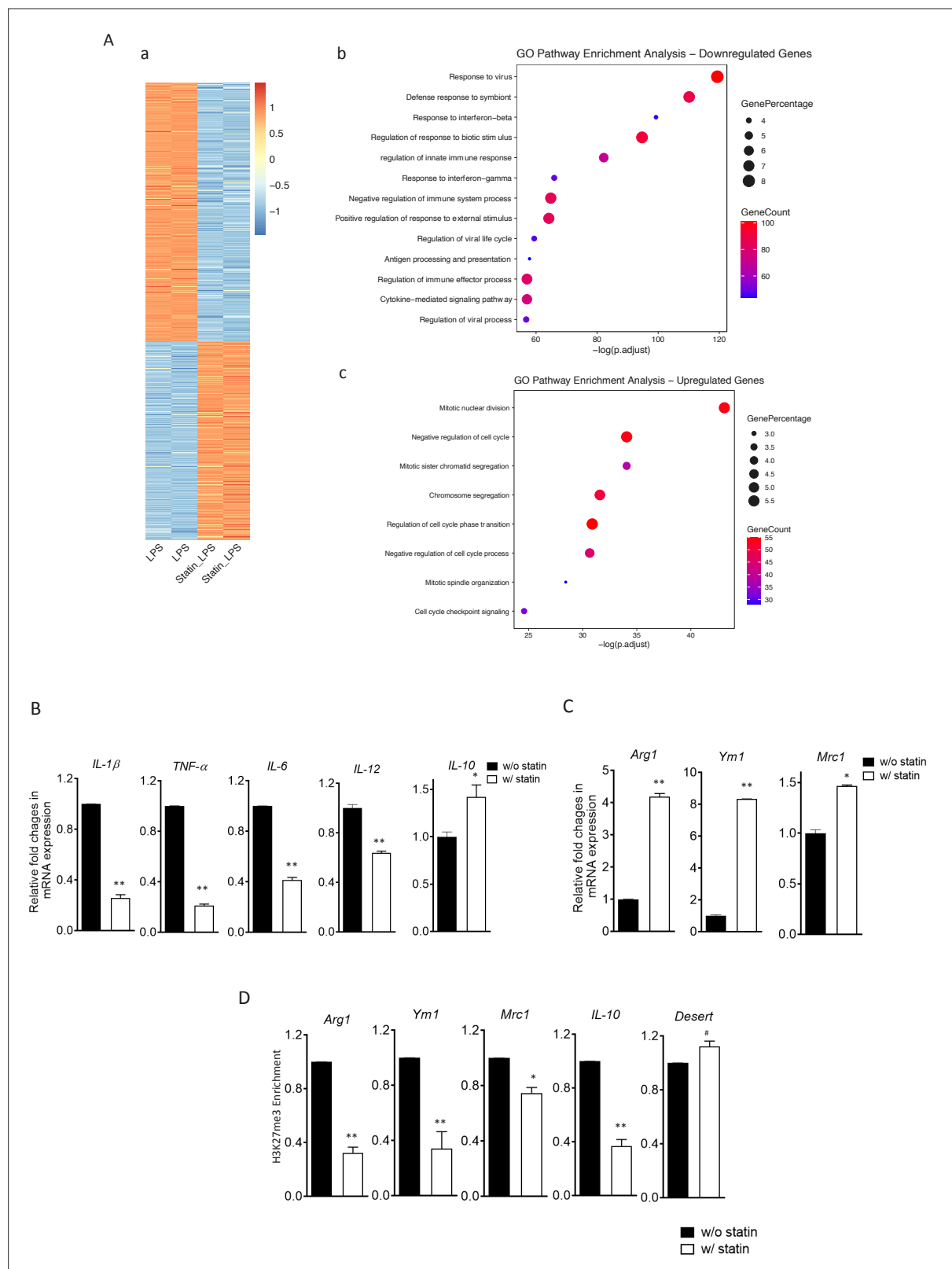


Figure 7. Statins suppress proinflammatory cytokines and enhance anti-inflammatory factors in lipopolysaccharide (LPS)- or IL-4-activated macrophages. **(A)** RAW 264.7 macrophages treated with statins (lovastatin, 7 + 200 μ M mevalonate; 2 days) or without were stimulated with LPS (100 ng/ml) for 3 hr. **(a)** Heatmap of differentially expressed genes by statins. **(b)** Gene Ontology (GO) analysis of downregulated genes by statins. **(c)** GO analysis of upregulated genes by statin. **(B)** Bone marrow-derived macrophages (BMDMs) treated with or without compactin (10 + 200 μ M mevalonate; 2 days)

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are stimulated by LPS (50 ng/ml, 3 hr). Gene expressions are analyzed by qPCR. **(C)** BMDMs treated with or without compactin are stimulated by IL-4 (20 ng/ml, 6 hr) and gene expressions are analyzed by qPCR. **(D)** Chromatin-immunoprecipitation (ChIP) analysis of H3K27me3 in BMDMs with or without compactin treatment. The inactive gene *desert* is used as input control. Data are representative of at least 2 independent experiments with 3 samples per group and data are presented as mean \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's *t*-test. Asterisks (*) and (**) indicate a significant difference with $p < 0.05$ and $p < 0.001$. A hashtag (#) indicates not significant.

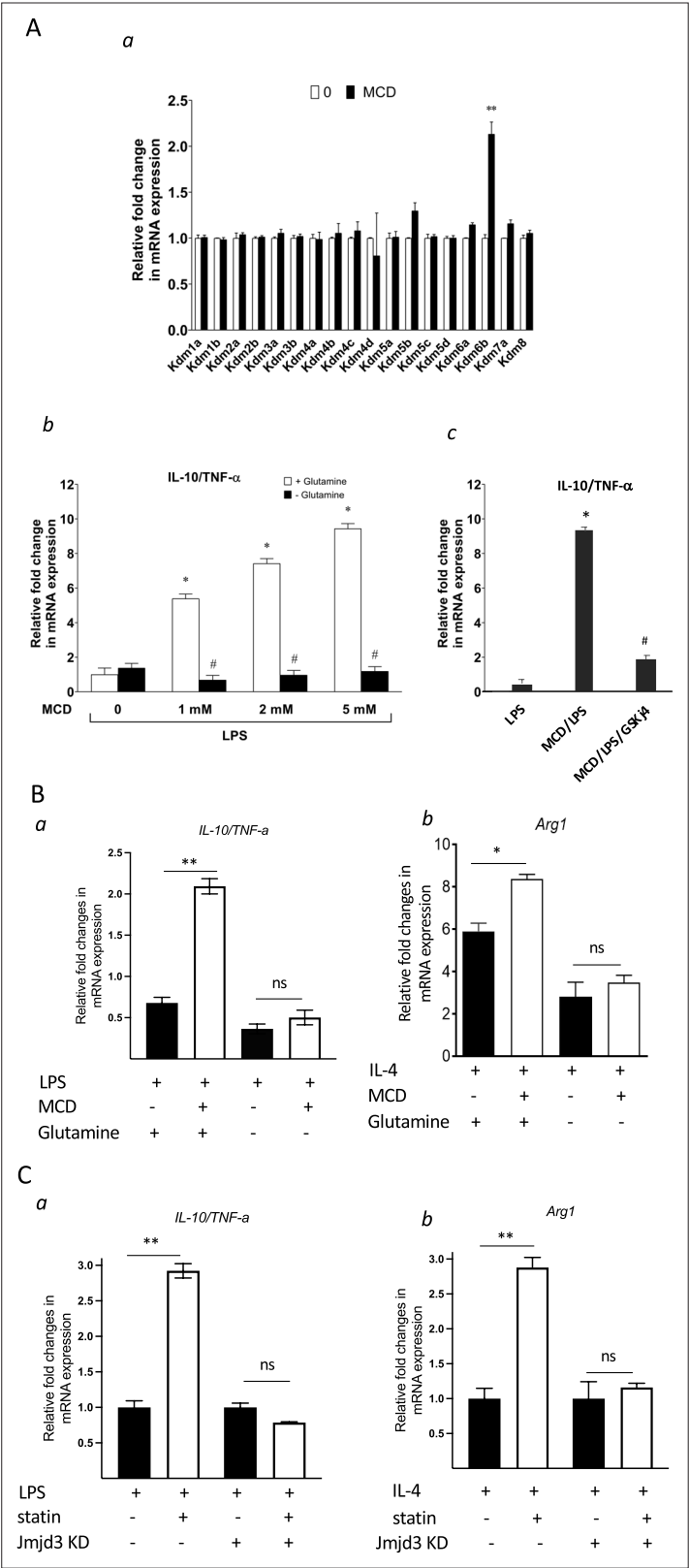


Figure 8. Cholesterol reduction suppresses proinflammatory phenotypes and enhances the expression of anti-inflammatory factors, depending on *Jmjd3* and its enzymatic activity. **(A)** RAW macrophages were treated with methyl- β -cyclodextrin (MCD) (5 mM, 1 hr). **(a)** The expression of JmJC demethylase family with or without MCD treatment. **(b)** After lipopolysaccharide (LPS) stimulation (100 ng/ml, 3 hr), in the presence of glutamine

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or without, the expression of *Il10* and *Tnfa* was analyzed by qPCR to generate the ratio of *Il10/Tnfa*. **(c)** Effect of GSJK4, a JMJD3 inhibitor, on *Il10/Tnfa*. **(B)** **(a)** Bone marrow-derived macrophages (BMDMs) treated with or without MCD are stimulated by LPS (50 ng/ml, 3 hr) in the presence of glutamine or without. Expression of *Il10* and *Tnfa* was analyzed by qPCR to generate the ratio of ratio of *Il10/Tnfa*. **(b)** BMDMs treated with or without MCD are stimulated by IL-4 (20 ng/ml, 6 hr) and expression of *Arg1* is analyzed by qPCR. **(C)** wt and *Jmjd3* KD RAW macrophages were treated with compactin (10 + 200 μ M mevalonate; 2 days) and then stimulated with LPS (100 ng/ml, 3 hr) or IL-4 (20 ng/ml, 6 hr). Expression of *Il10* and *Tnfa* **(a)** or *Arg1* **(b)** was analyzed by qPCR. Data are representative of 3 independent experiments with 3 samples per group and data are presented as mean \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's t-test. Asterisks (*) and (**) indicate a significant difference with $p < 0.05$ and $p < 0.001$. A hashtag (#) indicates a significant difference between MCD without or with glutamine with $p < 0.05$.

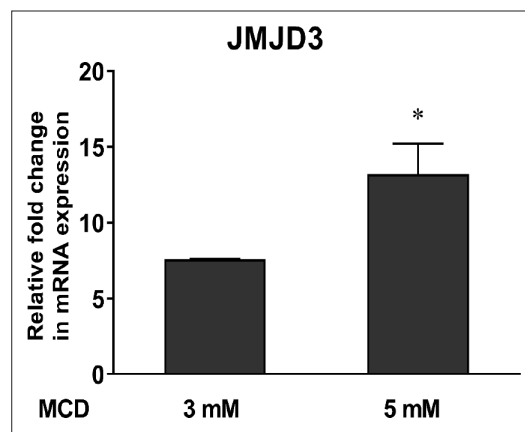


Figure 8—figure supplement 1. Expression of the *Jmjd3*. In 264.7 macrophages with increasing doses of methyl- β -cyclodextrin (MCD) (mean \pm standard deviation [SD]). An asterisk (*) indicates a significant difference with $p < 0.01$.

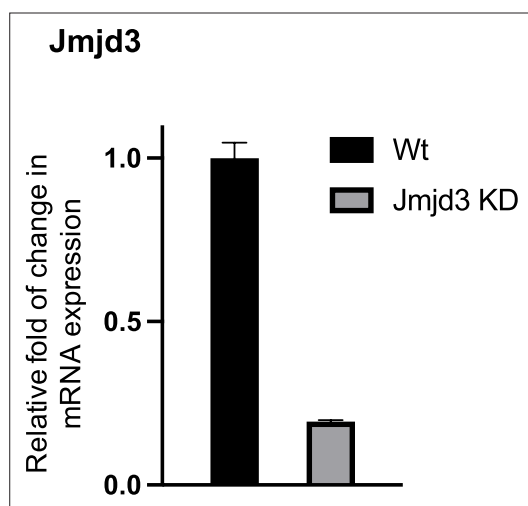


Figure 8—figure supplement 2. Expression of JMJD3 in wt and Jmjd3 KD macrophages (mean \pm standard deviation [SD]), $p < 0.001$.

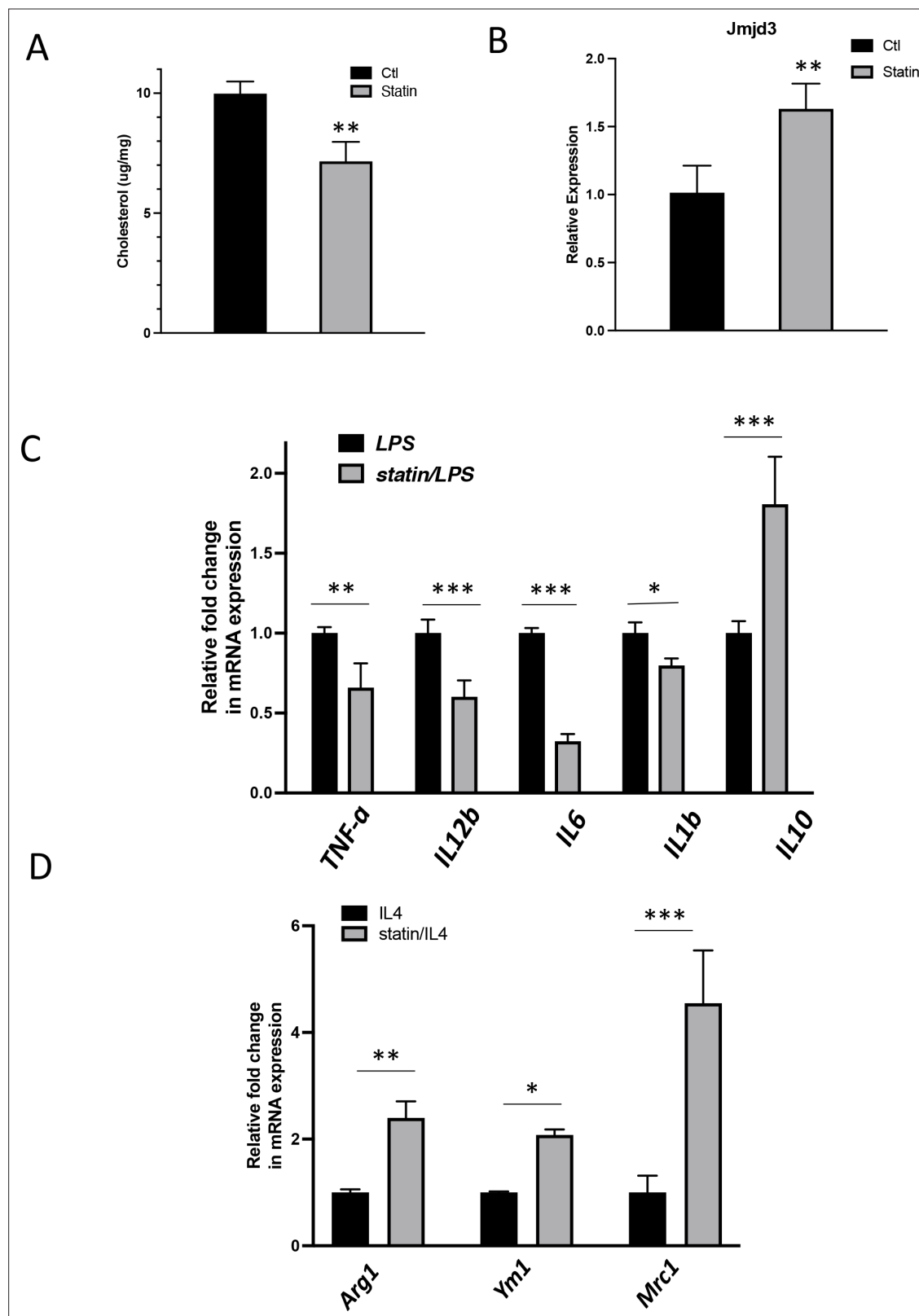


Figure 9. In vivo statin feeding in mice reduces cholesterol and upregulates *Jmjd3* in the peritoneal macrophages, which conveys anti-inflammatory phenotype. **(A)** Total cholesterol contents (free and esterified) in freshly isolated mouse peritoneal macrophages from simvastatin-fed (100 μ g/kg/day, 14 days) and control mice. **(B)** *Jmjd3* gene expression in peritoneal macrophages from simvastatin-fed (statin) and control mice. **(C)** Freshly isolated mouse peritoneal macrophages from simvastatin-fed and control mice are stimulated by lipopolysaccharide (LPS) (100 ng/ml, 6 hr). Gene expressions

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are analyzed by qPCR. **(D)** Freshly isolated mouse peritoneal macrophages from simvastatin-fed and control mice are stimulated by IL-4 (20 ng/ml, 6 hr) and gene expressions are analyzed by qPCR. Data are representative of 3 independent experiments with 3–4 samples per group and presented as mean \pm standard deviation (SD). Statistical analysis was performed using unpaired, two-tailed Student's *t*-test. Asterisks (*), (**), and (***) indicate a significant difference with $p < 0.05$, $p < 0.005$, and $p < 0.001$, respectively.