Supplemental Material

METHODS:

Materials: Stearic acid was purchased from Nu-chek Prep, Inc (Elysian, MN). Triacsin C (TC) was obtained from Enzo Life Sciences International, Inc (Plymouth Meeting, PA). Thioglycollate was purchased from Sigma-Aldrich (St. Louis, MO). An endpoint chromogenic LAL assay from Lonza (Basel, Switzerland) was used to confirm the absence of endotoxin contamination in fatty acid preparations (data not shown). Antibodies to detect IRE-1α, phosphorylated PERK, BiP, phosphorylated JNK, cleaved caspase-3, and β-actin were purchased from Cell Signaling (Danvers, MA). Forward and reverse primers to amplify the XBP-1 gene were purchased from Integrated DNA Technologies (Coralville, IA). Primer-probe sets for real-time detection of *Il6*, *Tnf*, *Ccl2*, *Il1b* and *Chop* gene expression were obtained from Applied Biosciences (Foster City, CA). PBA was purchased from Millipore (Billerica, MA). SS and LPS were purchased from Sigma-Aldrich (St. Louis, MO). IL-13 was obtained from R&D Systems (Minneapolis, MN).

Macrophage Collection: Three mL of 3% thioglycollate medium was injected into the peritoneal cavity of WT, TLR4-KO, or TLR2-KO mice on a C57BL/6 background. Three days later, macrophages were collected in serum-free Dulbecco's Modified Eagle's Medium (DMEM) and washed. Cells were then plated in DMEM containing 10% FBS at a density of 1.5×10^6 cells per well of a 12-well plate or 3.0×10^6 cells per well of a 6-well plate and incubated at 37 °C for 4 h. At this point, non-adherent cells were removed and the remaining cells were incubated for an additional 48 h at 37 °C before treatment.

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Stearic Acid Treatment: Peritoneal macrophages were treated with stearic acid at a final concentration of 90 μM. Stearic acid was dissolved in ethanol and then added to DMEM supplemented with 5% FBS to achieve a fatty acid to albumin ratio of 3:1. Peritoneal macrophages were treated with 90 μM stearic acid in the presence or absence of 2.5 μM TC. Cells treated with TC were pre-treated for 30 min prior to stearic acid exposure. For time-course treatments, cells were exposed to stearic acid and TC for 2, 4, 8, 16, or 24 h. All controls [vehicle-treated control, TC alone, tunicamycin (1 μM), and stearic acid alone] were treated for 24 h in media containing 5% FBS. For all other studies, macrophages were treated with stearic acid and TC for 16 h. An endpoint chromogenic LAL assay was used to confirm that there was no endotoxin contamination in media used for cell treatments. For co-treatments with PBA or SS: macrophages were treated with stearic acid and TC for 16 h, as describe above, in the presence of PBA (6 mM) or SS (1 mM or 5 mM). For WT and TLR4-KO experiments: As a control, macrophages were stimulated with LPS (10 μM), the known ligand for TLR4, and inflammatory cytokine expression was assessed. For all genes analyzed, WT macrophages responded to LPS treatment while TLR4-KO cells failed to respond (data not shown).

Macrophage Polarization: MPMs were plated in DMEM containing 10% FBS, as described above. Cells were then washed in PBS and serum-free media was added to the cells 4 h prior to polarization. All macrophages were grown in serum free media. MØ macrophages were untreated. M1 macrophages were treated with 10 ng/ml of LPS and M2 macrophages were treated with 4 ng/ml of IL-13. All polarization treatments were for 24 h. After polarization, cells were washed with PBS before stearic acid and TC

treatments for 16 h in the presence of respective polarization agents. Thus, cells were treated with LPS or IL-13 for a total of 40 h by the end of the study. Additionally, control cells were treated with polarization agents for the duration of the studies. These cells were washed with PBS after 24 h of polarization (as treated cells were) and then re-exposed to the polarization agent in the absence of stearic acid and TC for the remaining 16 h.

Western Blots: After treatment, cells were collected in lysis buffer containing 20 mM Tris-HCL (pH 8.0), 150 nM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 0.5 mM PMSF. A modified Lowry protocol was used to quantify protein concentration, and 10-15 μg of protein was electrophoresed through a 4-12% Bis-Tris gel (Invitrogen), transferred to a nitrocellulose membrane, and immunoblotted with antibodies generated against proteins involved in ER stress, inflammatory, and apoptotic pathways. Band intensity was quantified using ImageJ64 software.

Real-time RT-PCR: Cells were treated as described above and collected in TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit from Bio-Rad. Real-time RT-PCR analysis was performed using an iQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). Primer-probe sets (Assays-on-Demand) were purchased from Applied Biosystems (Foster City, CA). The expression of the following genes was assessed: *18S* (4352930E), *Ccl2*

(Mm00441242_m1), *Tnf* (Mm00443258_m1), *Il6* (Mm00446190_m1), *Chop/Ddit3* (Mm00492097_m1), *Il1b* (Mm01336189_m1). The data was analyzed using the Pfaffl method[1] and presented as relative expression.

Analysis of XBP-1 Splicing: cDNA was generated from treated cells, as described above. The *Xbp1* gene was amplified using the following primers: forward: 5'-TGA GAA CCA GGA GTT AAG AAC C-3'; reverse: 5'-TTC TGG GTA GAC CTC TGG GAG TTC C-3'. The resulting PCR product was electrophoresed on a 1.8% agarose gel for 1 h. Expression of the spliced form of *Xbp-1* was normalized to an internal β-actin standard (forward primer: 5'-TGA CCC AGA TCA TGT TTG AGA CC-3'; reverse primer: 5'-CCA TAG CCA AGA AGG AAG GC-3').

TUNEL Staining: Cells were plated at 250,000 cells/well in an 8-well chamber slide in DMEM containing 10% FBS. Cells were treated as previously described. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche-Applied Science) according to manufacturer's instructions. After TUNEL staining, cells were counterstained with DAPI (Vector Laboratories, Burlingame, CA) and imaged at 20X magnification using an inverted Leica Conventional fluorescent microscope (Vanderbilt Shared Imaging Resource). One or two fields of view (at least 300 cells) were counted for each well of the chamber slide using Histometrix 6 software. Experiments were repeated at least 3 separate times.

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Statistical Analysis: GraphPad Prism 5.0 software was used for all statistical analyses. Data was analyzed using one-way ANOVA when more than two treatment groups were compared or two-way ANOVA to compare measurements with two different variables. For all studies, each "n" indicates macrophages collected from a different mouse with studies performed on separate occasions. Outliers were excluded from the data for each individual parameter using the Grubbs outlier test.[2] A p of ≤ 0.05 was considered significant.

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Supplemental Data:

Supplementary Figure I. Intracellular stearic acid accumulation increases inflammatory cytokine gene expression in MPMs.

MPMs were co-treated with stearic acid (18:0, 90 μM) and TC (2.5 μM) for 2 to 24 h in DMEM containing 5% FBS. All controls [vehicle-treated control, TC alone, tunicamycin (1 μM), and 18:0 alone] were treated for 24 h. Analysis of A) *Tnf*, B) *Il6*, C) *Il1b*, D) *Ccl2*, and E) *Ccl3* gene expression by real-time RT-PCR. Data are presented as mean ± SD, n = 3/group. Abbreviations: C, vehicle-treated control; TC, triacsin C; Tunica, tunicamycin; 18:0, stearic acid. * $p<0.05$, ** $p<0.01$ compared to control.

Supplementary Figure II. Higher levels of stearic acid, in the absence of TC, are sufficient to induce MPM ER stress, inflammation, and apoptosis.

MPMs were treated with stearic acid (18:0, 250 or 500 μM) for 16 h in DMEM containing 5% FBS. A-E) Western blot analysis of: A) phospho-PERK, B) BiP, C) IRE-1α, D) phospho-JNK1/2, and E) cleaved caspase-3. Data are presented as mean ± SD, n = 3-5/group. Abbreviations: C, vehicle-treated control; 18:0, stearic acid. * $p<0.05$, ** $p<0.01$ compared to control.

Supplementary Figure III. PBA co-treatment does not attenuate inflammatory cytokine expression in response to intracellular stearic acid accumulation in MPMs.

MPMs were co-treated with stearic acid (18:0, 90 μM) and TC (2.5 μM) in the presence or absence of PBA (6 mM) for 16 h in DMEM containing 5% FBS. Analysis of A) *Il6* and B) *Il1b* gene expression by real-time RT-PCR. Data are presented as mean ± SD, n =

7/group. Abbreviations: C, vehicle-treated control; TC, triacsin C; 18:0, stearic acid; PBA, 4-phenyl butyric acid. Groups not connected by the same letter are significantly different, $p<0.05$.

Supplementary Figure IV. Confirmation of MPM Polarization State.

MPMs were treated with vehicle, LPS (10 ng/mL), or IL-13 (4 ng/mL) for 24 h to induce a Mθ, M1, or M2 polarization state, respectively. A-C) RNA was collected and real-time RT-PCR was performed in order to confirm macrophage polarization: A) *Tnf*, B) *Arg1*, and C) *Il10*. Gene expression of *Nos2* (iNOS) was not detected in Mθ or M2 macrophages, but was seen in M1 macrophages. Data are presented as mean ± SD, n = 3/group. Abbreviations: Mθ, unpolarized macrophage; M1, LPS polarized macrophage; M2, IL-13 polarized macrophage. # $p<0.05$ compared to M1.

Supplemental Figure V. Consequences of Stearic Acid Accumulation in MPMs.

Co-treatment of MPMs with stearic acid and TC increases intracellular stearic acid content, leading to TLR4/2-indepenedent inflammatory signaling. This inflammation results in ER stress-mediated apoptosis of macrophages. Polarization of MPMs to a pro-inflammatory M1 phenotype increases the susceptibility of the macrophages to inflammation and ER stress, but not apoptosis, during stearic acid accumulation. Abbreviations: TC, triacsin C; LPS, lipopolysaccharide; M1, LPS polarized macrophage.

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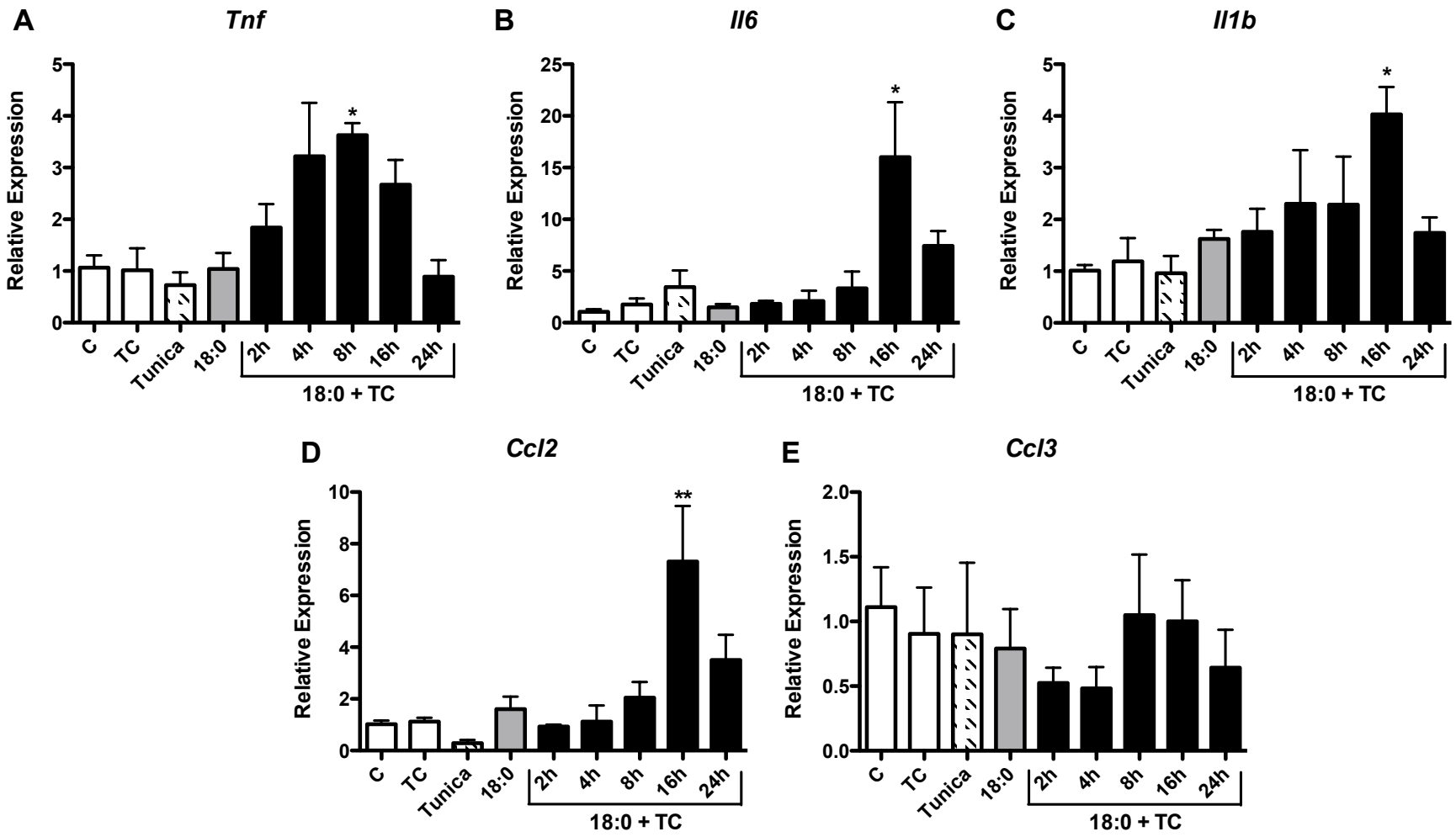
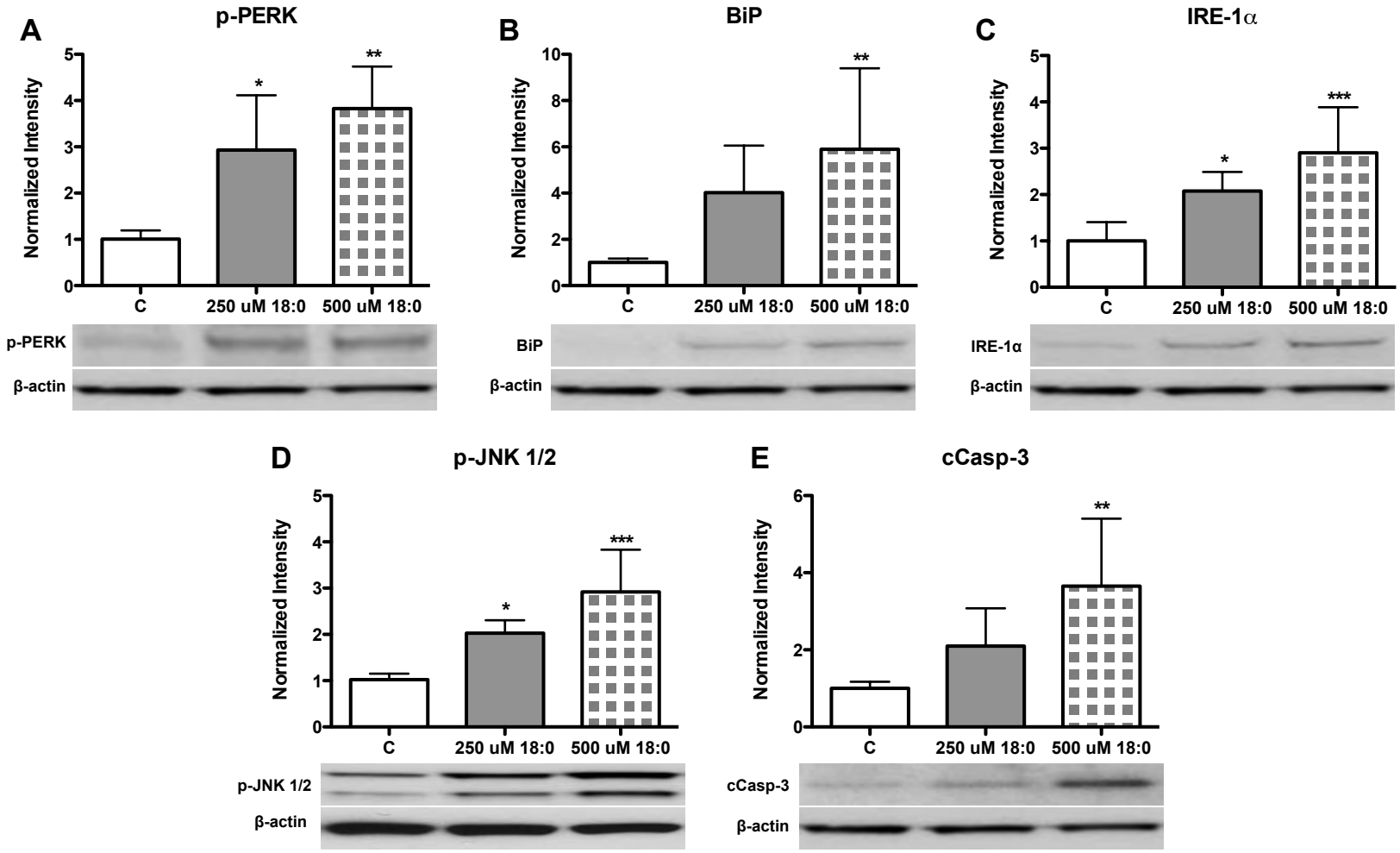
References

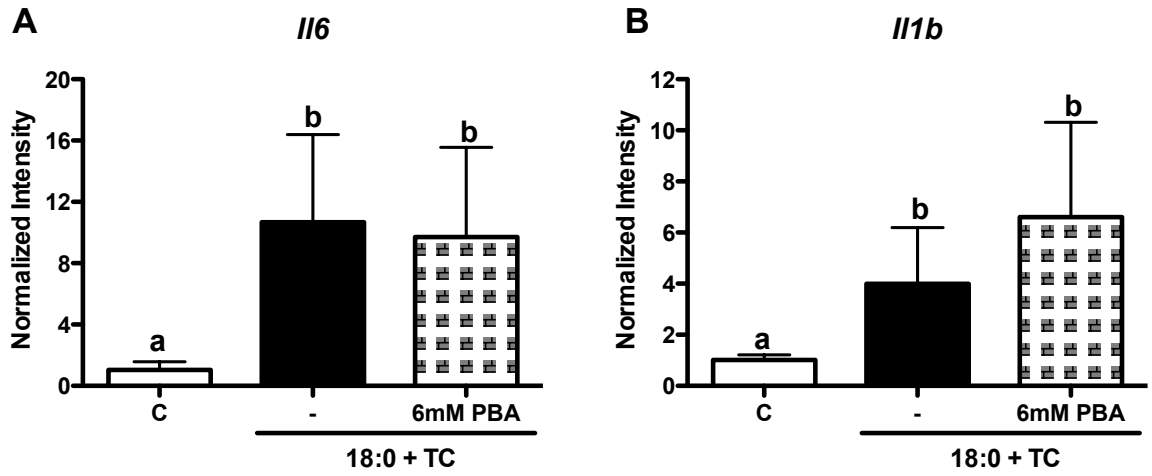
1. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001;29:2002-2007
2. Grubbs FE. Procedures for detecting outlying observations in samples. *Technometrics*. 1969;11:1-21



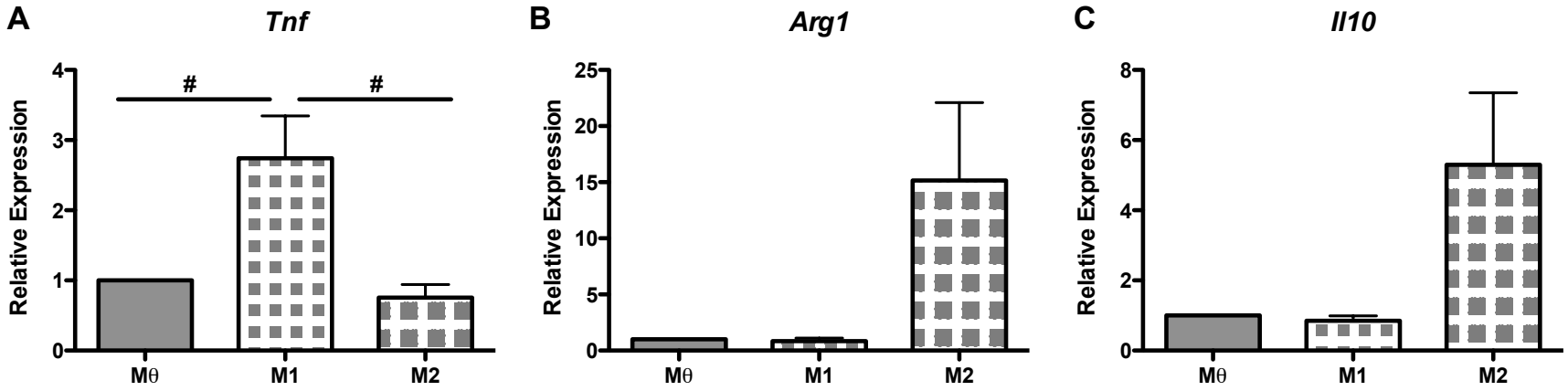
Anderson, *et al*. Supplemental Figure I



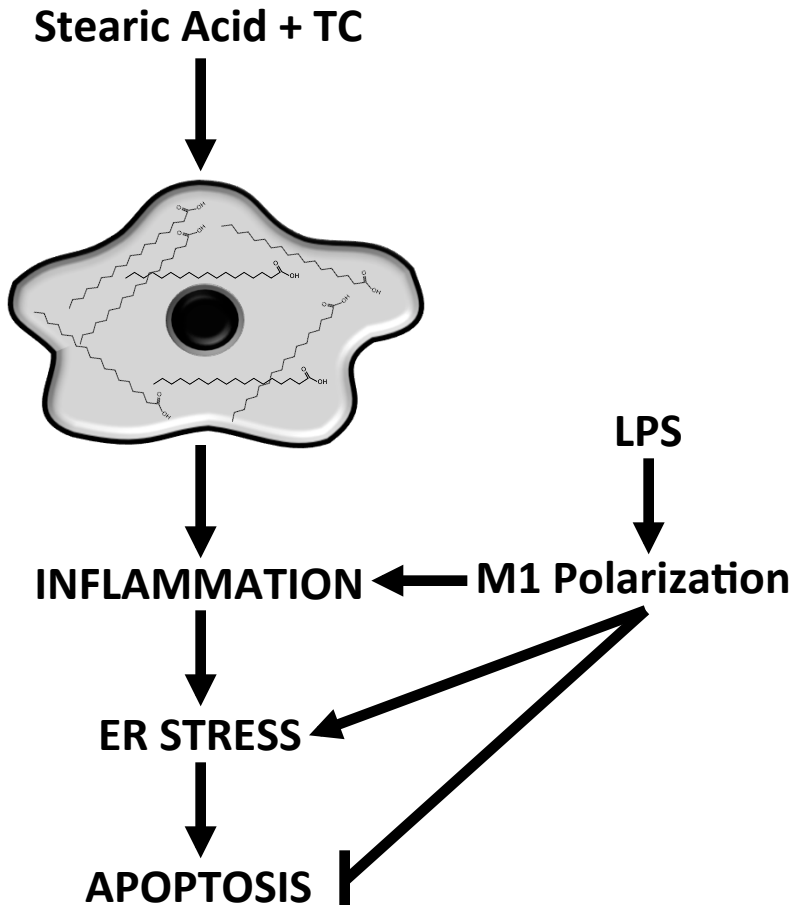
Anderson, *et al*. Supplemental Figure II



Anderson, *et al*. Supplemental Figure III



Anderson, *et al*. Supplemental Figure IV



Anderson, *et al*. Supplementary Figure V