SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Interaction of Macrophages with Apoptotic Cells Leads to an Increase in Drp1, Which Promotes Efferocytosis, Related to Figures 1 and 2.

(A) Cre-/- and Cre+/- bone marrow-derived macrophages were rendered apoptotic by treatment with 35 μg/ml 7-ketocholesterol and then incubated with WT bone marrow-derived macrophages at a ratio of 10:1 for 45 minutes. The unengulfed ACs were removed by rinsing, and whole cell lysates were immunoblotted for Drp1 and β-actin. The bar graph shows densitometric quantification of immunoblots normalized to β-actin (n=3 biological replicates).

(B) Cre-/- and Cre+/- peritoneal macrophages were assayed for *Drp1* mRNA relative to *Actb* by RT-QPCR (n=3 biological replicates).

(C) Total cells lysates of Cre-/- and Cre+/-peritoneal and bone marrow (BM)-derived macrophages were immunoblotted for Drp1 and two mitochondrial proteins, PDH1α and VDAC1.

(D) Cre-/- and Cre+/- bone marrow-derived macrophages were incubated with or without apoptotic Jurkat cells for 45 minutes, and then lysates were immunoblotted for Drp1.

(E) Cre-/- and Cre+/- peritoneal macrophages were immunostained using antibodies against Drp1 (green) and the mitochondria marker cytochrome C oxidase IV (COX IV) and viewed by confocal microscopy. Bar, 1 μm.

(F) Bone marrow-derived macrophages were treated for 30 min with 1 μg/ml actinomycin D or 10 μg/ml cycloheximide then incubated with or without apoptotic Jurkat cells for 45 minutes. Lysates were immunoblotted for Drp1 and β-actin. The bar graph shows densitometric quantification of immunoblots normalized to β-actin (n=4 biological replicates).

(G) CypHer5E-labeled apoptotic Jurkat cells were incubated with PKH67-labeled macrophages at 10:1 ratio for 45 minutes. The unengulfed ACs were removed by rinsing, and the macrophages were detached from the plate for flow cytometric analysis

of CypHer5E^{+} PKH67^{+} cells, which reflects efferocytosis. Representative contour plots and quantified data are shown (n=3 biological replicates).

(H) Macrophages were treated with 10 μM MDIVI-1 or vehicle control, and then the cells were incubated with PKH26-labeled apoptotic Jurkat cells were for 1 hour at a 10:1 AC:macrophage ratio (left) or for 2 hours at a 5:1 AC:macrophage ratio (right). Efferocytosis was measured as the total percent of macrophages positive for PKH67-labeled ACs (n=4 biological replicates).

Figure S2. Drp1-Deficient Macrophages Differentiate Normally and Show Expected Patterns of Polarization to Stimuli, and Effect of MDIVI-1 on Mitochondrial Length, Related to Figures 1 and 2.

(A) Macrophages differentiated from bone marrow cells of Cre-/- and Cre+/- mice were analyzed by flow cytometry for cell-surface F4/80, CD68, and FcγRI. Average MFI per macrophage is shown (n=3 biological replicates)

(B and C) Cre-/- and Cre+/- bone marrow-derived macrophages were treated with LPS and IFNγ or with IL-4 and then assayed for *Nos2, Tnfa, Il6, Arg1, Mrc1,* and *Retnla* mRNA by RT-qPCR, with normalization to *36B4* (n=3 biological replicates).

(D) Macrophages were treated with vehicle control or 10 μM MDIVI-1 followed by incubation with PKH67-labeled apoptotic Jurkat cells for 45 minutes at a 5:1 AC:macrophage ratio. Unengulfed cells were rinsed away, and, a after a 2-hour incubation period, the macrophages were labeled with MitoTracker Red FM and incubated for 45 minutes with CellVue Claret-labeled ACs at a 5:1 AC:macrophage ratio. Macrophages that had not engulfed an AC or engulfed 2 ACs were visualized by confocal microscopy and quantified for average mitochondrial length (n=3 biological replicates with 10 cells per treatment group quantified).

(E) Vehicle-treated Cre-/- and Cre+/- macrophages or MDIVI-1-treated Cre+/- macrophages were subjected to the two-stage efferocytosis assay described in Figure 2C (n=4 biological replicates).

For all panels, values are mean + S.E.M.; *$p < 0.05$; n.s., not significant.

Figure S3. Macrophages with Impaired Mitochondrial Fission Have a Defect in Phagosome Sealing, Delayed Phagosome Acidification, and Defects in Large Particle Internalization, Related to Figures 3 and 4.

(A) Macrophages transfected with scrambled RNA or *Mff* siRNA were incubated with PKH67-labeled apoptotic Jurkat cells and MitoTracker Red FM for 45 minutes at a 5:1 AC:macrophage ratio. Macrophages that had or had not engulfed an AC (AC^+, AC^-) were visualized by confocal microscopy. Mean mitochondrial length was measured in 0.5 μm z-sections in at least 10 cells per group across four biological replicates (n=3 with $\geq$ 10 cells quantified per group). Also shown is an immunoblot of Mff in ScrRNA- and *Mff* siRNA-treated macrophages.

(B) Macrophages treated with ScrRNA or *Mff* siRNA were subjected to the two-stage efferocytosis assay described in Figure 2C.

(C) Macrophages treated with ScrRNA or *Mfn1* siRNA were immunoblotted for Mfn1, Drp1, and β-actin (n=3 biological replicates).

(D) Macrophages treated with ScrRNA, *Drp1* siRNA, or *Mfn1* siRNA were immunostained using an antibody against the mitochondria marker, ATP5A, and viewed by confocal microscopy. Bar, 1 μm.

(E) Macrophages treated with ScrRNA, *Mfn1* siRNA, or *Drp1* siRNA were incubated for 1 hour with PKH67-labeled apoptotic Jurkat cells at a 10:1 AC:macrophage ratio and then efferocytosis was measured. The data are quantified as percent of macrophages positive for PKH67-labeled ACs (n=3 biological replicates).

(F) Macrophages treated with ScrRNA or *Mff* siRNA were subjected to the phagosome sealing assay after 45 minutes of incubation with ACs, as described in Figure 3 (n=4 biological replicates).

(G) Images from the time-lapse fluorescence microscopy experiment in Figure 3B examining time to CypHer5E positivity. Arrows in 0-min images show the AC being monitored. Bar, 5 μm.

(H) Cre-/- and Cre+/- macrophages were incubated for 16 hours with dual-labeled rhodamine and fluorescein dextran to label lysosomes. Lysosomal pH was determined

by analyzing the fluorescein/rhodamine ratio (n=3 biological replicates with 30 cells per group quantified per experiment).

(I) Cre-/- and Cre+/- macrophages were subjected to the two-stage phagocytosis assay described in Figure 2C, using biotinylated versions of the indicated particles instead of biotinylated ACs (n=3 biological replicates).

Values are mean ± S.E.M.; *p < 0.05; n.s., not significant.

Figure S4. Drp1-Deficient Macrophages Have Defects in AC Corpse Degradation and Phagosomal ROS, Quantification of ATP, and Illustrative Images of GCaMP6f Fluorescence Over Time, Related to Figures 4 and 5.

(A) Time-lapse confocal fluorescence microscopy of Cre-/- and Cre+/- macrophages incubated with apoptotic PMNs labeled with CypHer5E. Time to AC fragmentation was quantified as the time interval between the first frame in which the AC became CypHer5E + (t = 0 minutes) and the frame in which there was the first evidence of fragmentation of the CypHer5E signal (arrows). The time interval between each frame was ~3.57 minutes, some of which are displayed here. Bar, 10 μm. The bar graph shows the mean ± S.E.M., *p < 0.0001 (n = 15 cells per group).

(B) Cre-/- and Cre +/- macrophages were treated with 200 nM rapamycin (Rap) or vehicle control (Veh) for 18 hours. The cells were then fixed with 2% paraformaldehyde, permeabilized with digitonin, and immunostained for LC3. Fluorescence microscopic analysis was conducted to quantify the average number of LC3+ punctae (red spots) per cell (n=200 cells per group).

(C) Cre-/- and Cre+/- macrophages were incubated for 45 minutes with H2DCFDA-labeled apoptotic Jurkat cells that were loaded with the oxidant, TBHP (50 μM), or vehicle control. Unengulfed ACs were then removed by rinsing, and the macrophages were then assayed for H2DCFDA fluorescence as a measure of ROS (n=3 biological replicates).

For panels B-C, values are mean ± S.E.M.; *p < 0.05; n.s., not significant.

(D) Cre-/- and Cre +/- macrophages were incubated for 45 minutes under control conditions (no ACs) or with ACs, and relative ATP levels were quantified using an ATP fluorescence assay (n=3 technical replicates).

(E-G) Images from the time-lapse fluorescence microscopy experiment in Figure 5A-B examining Cyto- and Mito-GCaMP6f fluorescence intensity over time in AC- and AC+ Cre-/- and Cre +/- macrophages; and images and quantified data for a similar experiment using ER-GCaMP6f-150 (n=3 cells for AC^- macrophages and n=10 cells for AC^+ macrophages, with 2 plates of cells examined for each condition; values are mean ± S.E.M, *$p < 0.05$ for the two AC^+ groups relative to the two AC^- groups, with no significant difference between the Cre-/- and Cre+/- groups). Bars, 10 μm.

Figure S5. Control Experiments Related Calcium Dynamics and Lysosome Acidification and Summary of Pathway Related to Figures 5 and 6.

(A) Wildtype macrophages were treated with 5 μM BAPTA-AM, and membrane-bound LC3 MFI was quantified by flow cytometry as in Figure 4C (n=3 biological replicates).

(B) Cre-/- and Cre+/- macrophages were incubated with 2 μM ionomycin (Iono) or vehicle control (Veh), and membrane-bound LC3 MFI was quantified by flow cytometry as in Figure 4C (n=4 biological replicates).

(C) Immunoblot of MCU1 and β-actin from macrophages incubated for 45 minutes without or with apoptotic Jurkat cells (ACs) (n =3 biological replicates).

(D) Cre-/- and Cre+/- macrophages were transfected with scrambled RNA or *Mcu* siRNA and then incubated for 45 minutes with PKH67-labeled apoptotic Jurkat cells and MitoTracker Red FM at a 5:1 AC:macrophage ratio. Mitochondrial length was length measured as in Figure 1 (n=10 cells analyzed per group).

(E) WT macrophages were treated with 1 μM bafilomycin (Baf) or vehicle control (Veh), incubated with PKH67-labeled ACs, and then assayed for mitochondrial length in macrophages containing or not containing ACs (n=3 biological replicates).

(F) Control and bafilomycin-treated macrophages were incubated with biotinylated ACs for the indicated times and then subjected to the phagosome sealing assay described in Figure 3 (n=3 replicates for each time point).

For all panels, values are mean ± S.E.M.; *p < 0.05; n.s, not significant.

(G) Proposed model linking mitochondrial fission in macrophages engaging an AC to high-burden efferocytosis.

Figure S6. Quantification of Apoptotic Cells and Cytokine mRNAs in the Thymuses of Dexamethasone-Treated Mice and Validation of *Mcu*$^{-/-}$ and MDIVI-1-Treated Mice, Related to Figure 7.

(A) Representative images of thymuses from Cre-/- and Cre+/- mice 18 hours after i.p. injection of PBS or 250 μg dexamethasone. Bar, 1 mm.

(B) Thymocytes from Cre-/- and Cre+/- mice were treated with PBS or 100 μM dexamethasone for 2 hours then stained with Annexin V and analyzed for the percentage of annexin V^{+} cells by flow cytometry (n=4 biological replicates per group).

(C) The thymuses of dexamethasone-treated Cre-/- and Cre+/- mice were assayed for the indicated mRNAs by RT-qPCR and expressed as fold-change relative to the Cre-/- value (n=4 mice per group).

(D) The thymuses of dexamethasone-treated Cre-/- and Cre+/- mice were surface-immunostained for F4/80 and assayed for F4/80+ cells by flow cytometry (n=8 mice per group).

(E) Irradiated WT CD1 mice were transplanted with bone marrow from WT CD1 mice or *Mcu*$^{-/-}$ mice on the CD1 background. After 6 weeks, the mice were sacrificed, and spleen lysates were immunoblotted for MCU and β-actin.

(F) Cre-/- and Cre+/- mice were injected i.p. every 12 hours for 3 treatments with 20 mg/kg MDIVI-1 or vehicle control. Macrophages were isolated from the thymuses of the mice in (C) and then fixed, immunostained stained for COXIV, and viewed by confocal fluorescence microscopy. Bar, 5 μm. Average mitochondrial length was measured in ≥ 10 cells per group across three biological replicates.

(G) Thymocytes from Cre-/- mice were incubated for 2 hours with 100 μM dexamethasone or vehicle control with or without 10 μM MDIVI-1. The cells were then

stained with annexin V and analyzed for the percentage of annexin V^+ cells by flow cytometry (n=3 biological replicates).

For all panels, values are mean ± S.E.M.; *p < 0.05; n.s., not significant.

Figure S7. Systemic Parameters in WD-Fed *Ldlr*$^{-/-}$ Mice With or Without Myeloid Drp1 Deficiency, Related to Figure 7.

(A) Aortic smooth muscle cells isolated from Cre-/- and Cre+/- mice were assayed for *Drp1* mRNA relative to *Actb* by RT-QPCR (n=3 biological replicates).

(B-F) Body weight, fasting blood glucose, plasma cholesterol, triglyceride levels, and plasma lipoprotein profile from 12-week WD-fed *Drp1*$^{f/fl}$ *Lysmcre*$^{+/-}$ (Cre-/-) and *Drp1*$^{f/fl}$ *Lysmcre*$^{+/-}$ (Cre+/-) mice on the *Ldlr*$^{-/-}$ (LDLR-KO) background.

(G) Aortic root cross-sections of 12-week WD-fed mice were quantified for total lesion area and immunostained for Mac3, followed by analysis of Mac3+ cells/section (n=12-14 mice per group)..

(H) Peritoneal macrophages from Cre-/- and Cre+/- mice were treated for 24 hours with vehicle (Veh), 50 µg/ml oxidized LDL (oxLDL), or 35 µg/ml 7-ketocholesterol (7-KC). The cells were then stained with Annexin V and analyzed for the percentage of annexin V^+ cells by flow cytometry (n=3 replicates per group).

For all panels, values are mean ± S.E.M.; *p < 0.05; n.s., not significant.