Supplemental Material

Cathepsins Drive Anti-Inflammatory Activity by Regulating Autophagy and Mitochondrial Dynamics in Macrophage Foam Cells

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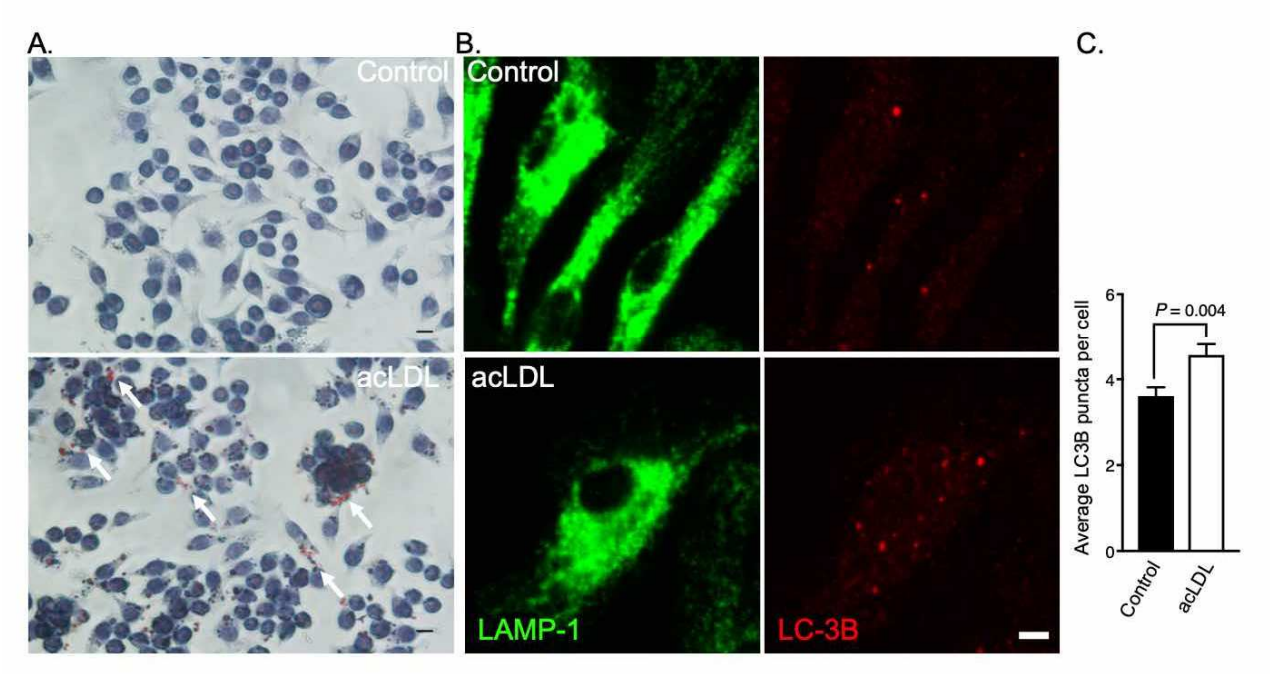
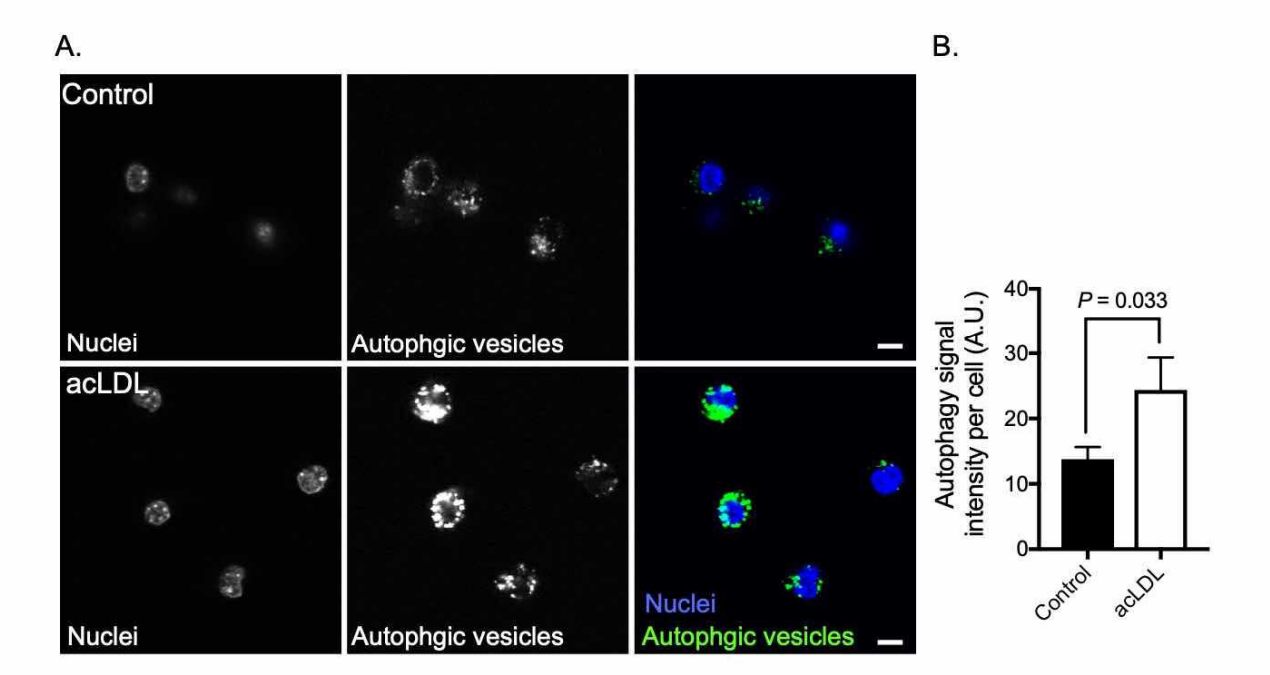
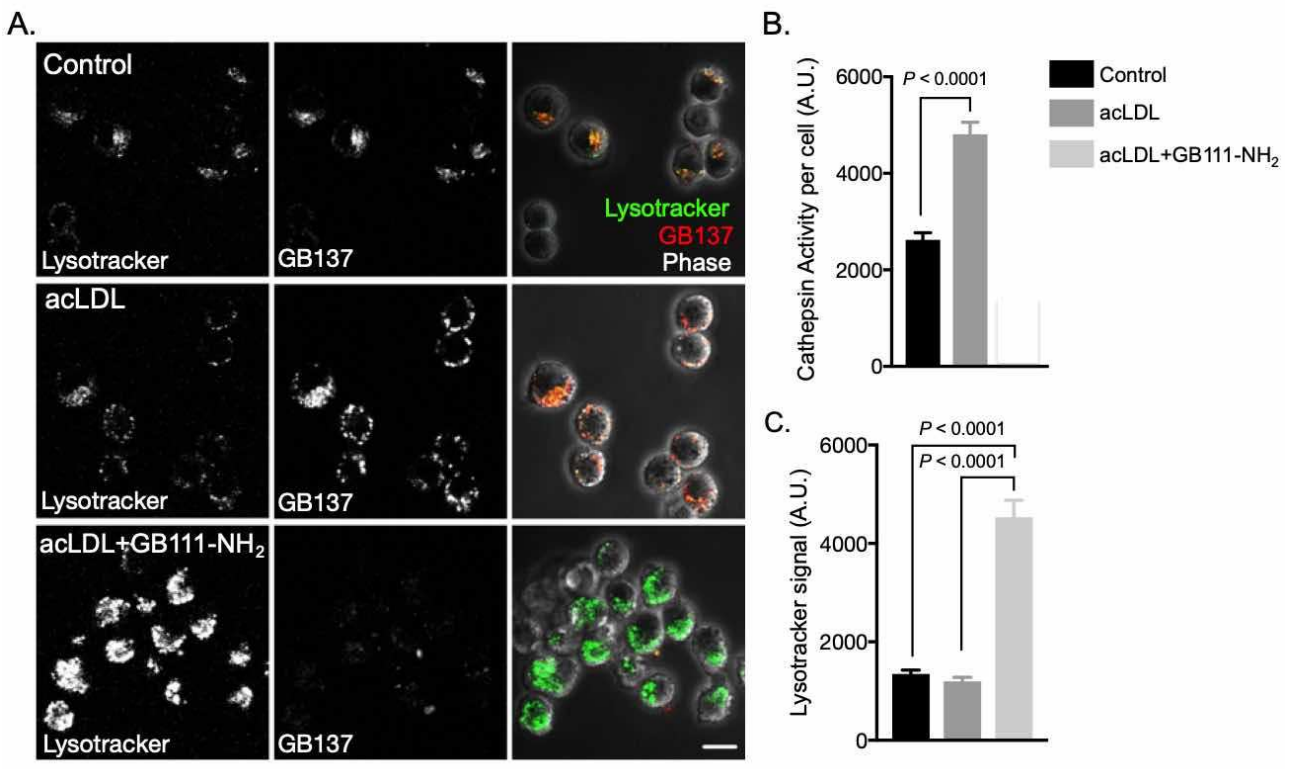


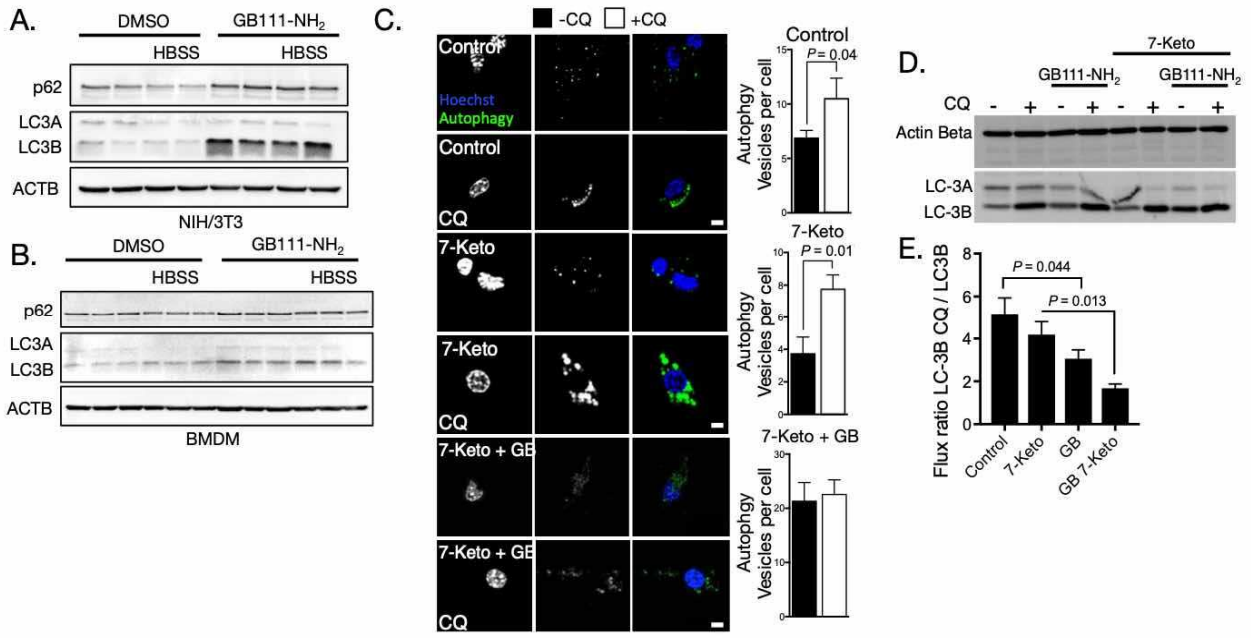
Supplementary Figure 1. Autophagy is induced in macrophage foam cells. (A). Primary macrophages were stimulated with acetylated LDL cholesterol (acLDL) (100μg/mL) or vehicle control (PBS x1 for 24 h). Cells were fixed in 4% PFA and stained with oil red O for neutral lipids; and counter stained with hematoxylin. Lipid accumulation is evident in acLDL stimulated cells (see arrows) compared to controls. The scale bar is 10μm. **(B)** Primary macrophages were stimulated as described in panel A and stained for autophagosomes (LC-3B) 24 h later. Representative confocal micrographs of methanol fixed cells, stained for LC-3B, red, (autophagosomes) or LAMP-1, green, (lysosomes). The scale bar is 5μm. **(C)** Quantification of LC-3B puncta per cell (n=498 cells for acLDL) and (n=583 cells per control).



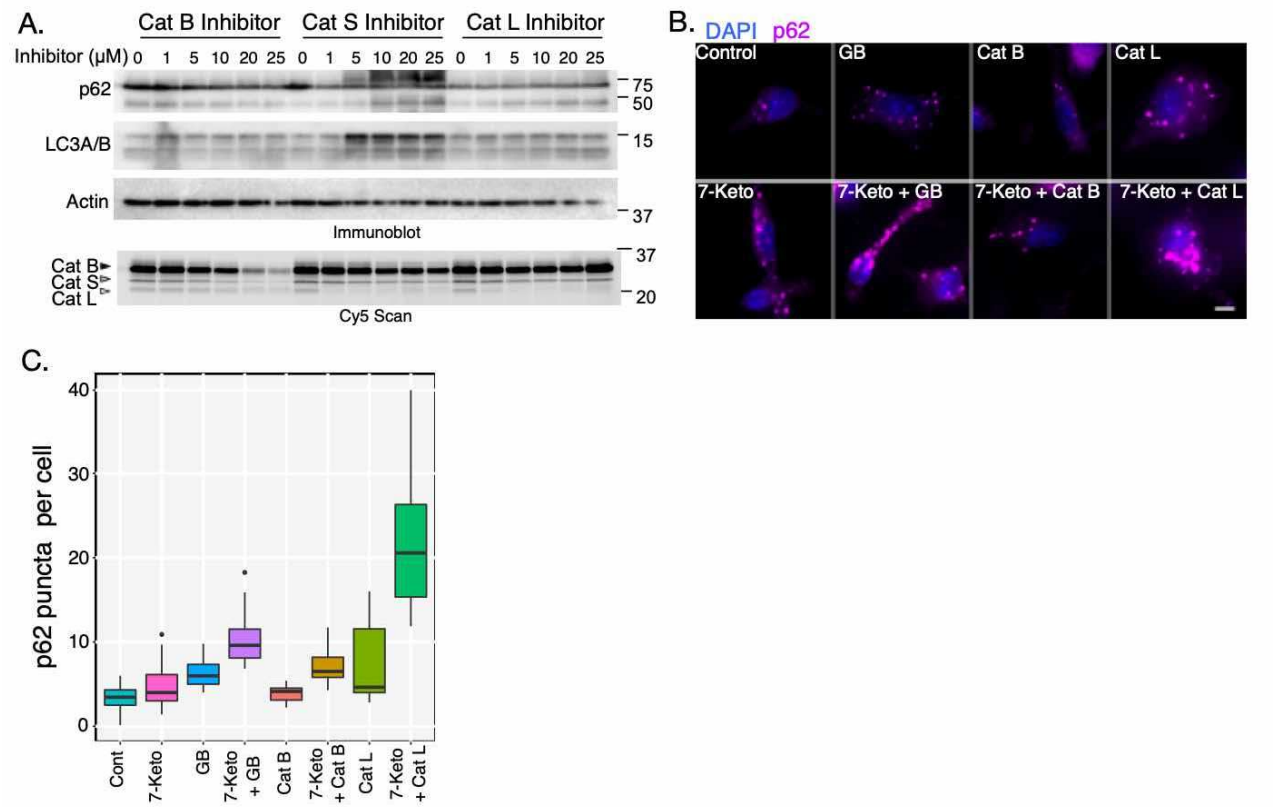
Supplementary Figure 2. Autophagy is induced in macrophage foam cells. (A). Raw 264.7 cells were incubated in the presence of acLDL (100μg/mL) for 24 h and autophagic vesicles were visualized by confocal microscopy 24 h later using a CytoID autophagy detection kit, (as described in the Methods). The scale bar is 5μm **(B)**. Quantification of the autophagy signal from acLDL-treated cells (n=76 cells) or controls (n=149 cells). Nuclear stain appears in blue and autophagy vesicles in green.



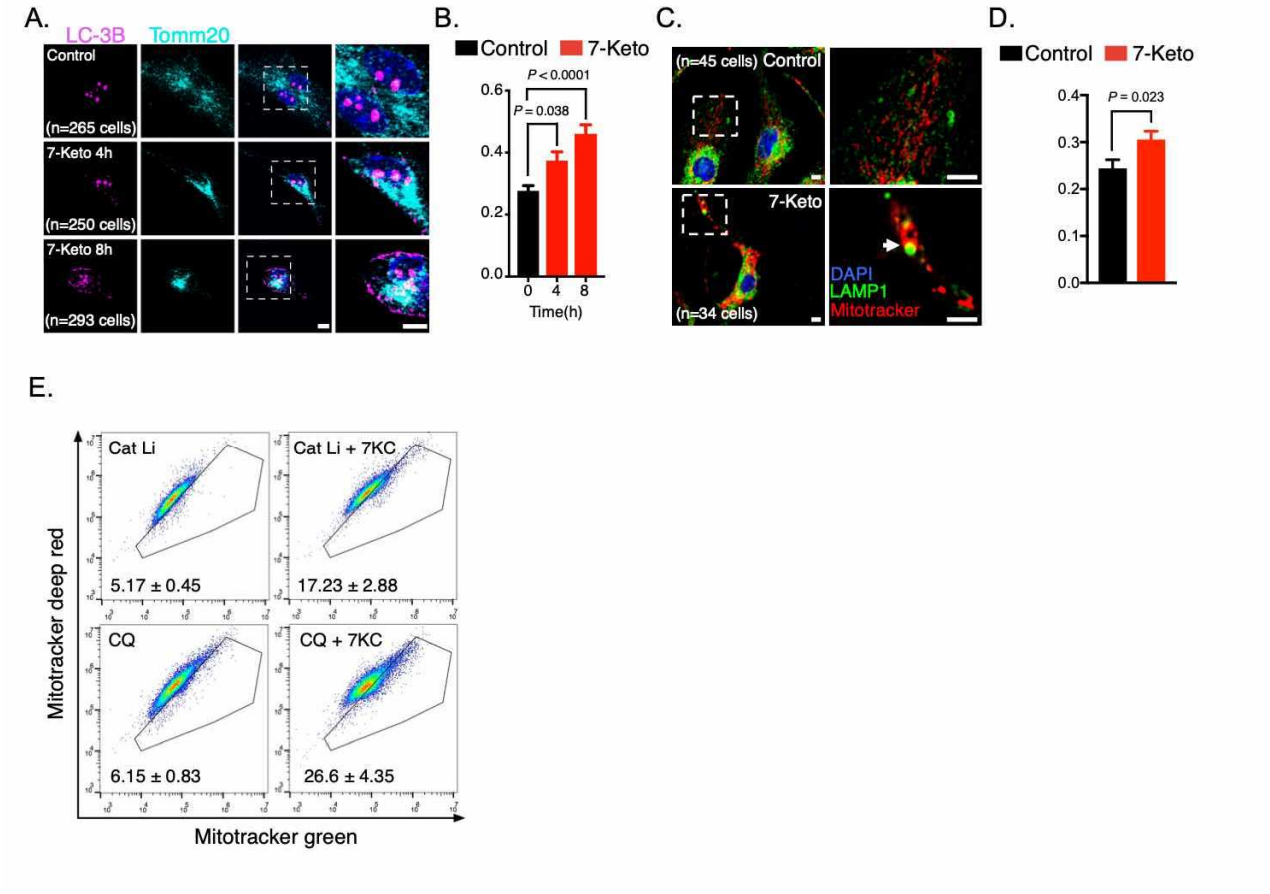
Supplementary Figure 3. Transformation of macrophages into foam cells. (A). Representative confocal images showing increased cathepsin activity in macrophage-like foam cells, Raw264.7. The cells were treated with acLDL (100μg/mL) for 24 h. Cathepsin activity was then determined by the fluorescent signal from GB137 (1μM) 24 h later, in the presence of 2% fetal bovine serum. Lysotracker (50nM) was added to the cells 30 min before microscopy examination. Cathepsin inhibitor, GB111-NH_2 (5μM) was added 1 h before GB137 and demonstrated complete blockade of cathepsin activity. The scale bar is 10μm. Lysotracker stain is in green, cathepsin activity (GB137) in red. Bar graphs present the mean ± SEM and one-way ANOVA with Tukey's correction for multiple comparisons.



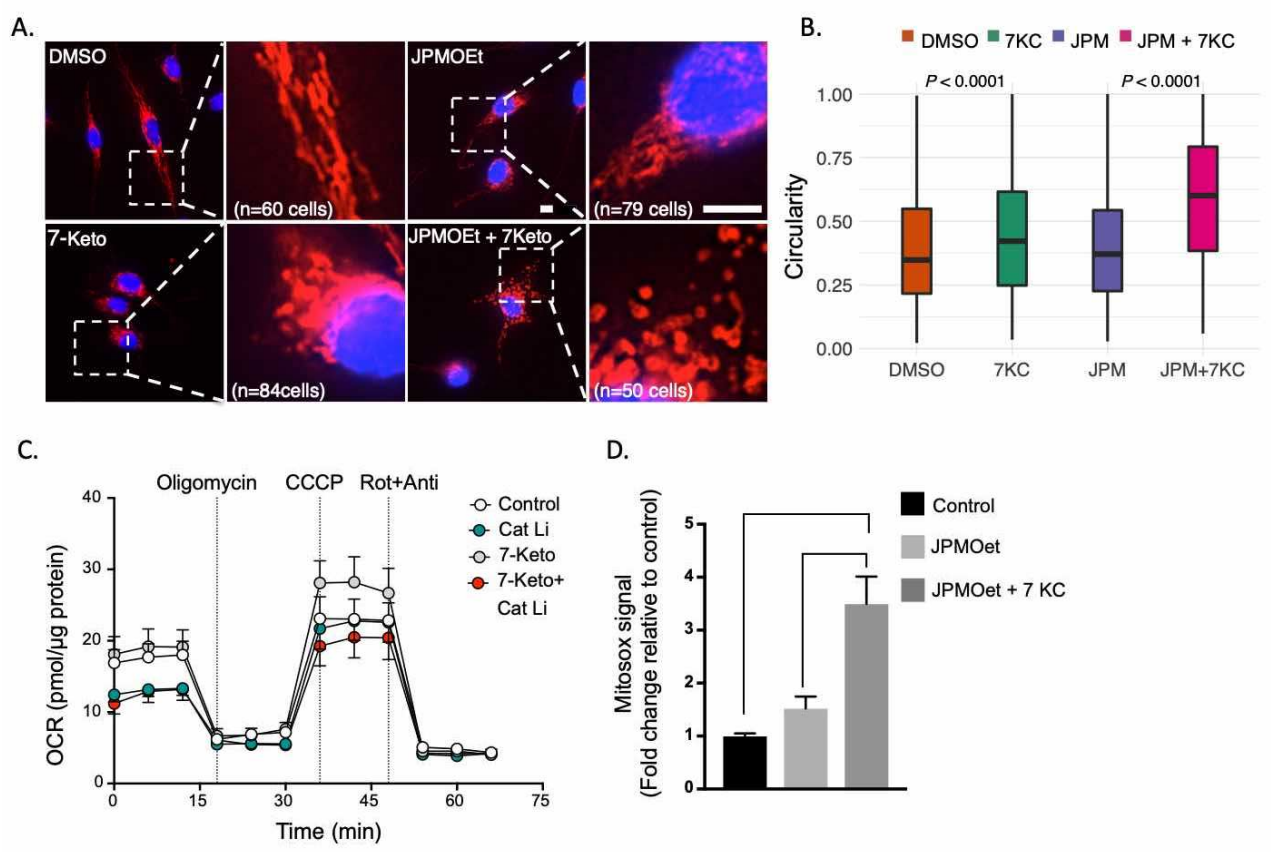
Supplementary Figure 4. Cathepsin inhibition. (A) NIH3T3 cells were treated with GB111-NH2 (5µM) for 16 h. Then, the cells were washed once with PBS x1 and incubated with HBSS or complete media for 2 h. Lysates were analysed as in Fig 2a, (n=2 biological samples). **(B).** BMDM were treated as described in **(A)** with 4 hours of incubation with HBSS (n=3 biological samples). **(C).** Raw 263.7 cells were treated with GB111-NH2 (10µM) or DMSO for 16 h before the addition of 7-Keto (50µM) for 4 hours. Chloroquine (CQ) (50µM) was added in indicated samples for the last 2 hours. Autophagic vacuoles were detected by the CytoID® Autophagy detection kit according to the manufacturer's instructions. Cells were fixed in 4% PFA and nuclei were stained with Hoechst. Representative confocal micrographs showing autophagic vacuoles (green) and nuclei in (blue). Bar graphs present the mean ± SEM and statistical difference, as computed with Student's t-test. The scale bar is 5µm. **(D,E).** Autophagy flux measurement as indicated by LC-3B turnover in primary macrophages treated with or without CQ. Macrophages were treated and analyzed as described in panel A. The ratio of normalized LC-3B expression between CQ treated and CQ untreated macrophages is expressed as the rate of lysosomal LC-3B degradation (n=6 biological samples). One-way ANOVA with Tukey's correction for multiple hypotheses was used to determine statistical significance.



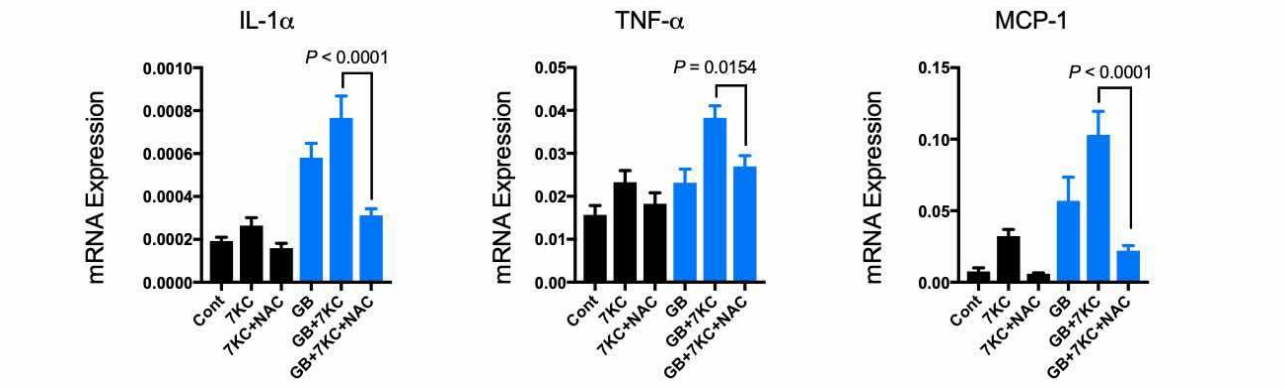
Supplementary Figure 5. Selectivity and potency of commercial cathepsin inhibitors and their impact on autophagy. (A) Primary macrophages were treated with increasing doses of inhibitors for cathepsins B, L and S for 16 h, as indicated. Cells were then treated with the activity-based probe, GB123, for another two h, after which the cells were lysed and resolved on SDS-PAGE. Selectivity of the cathepsin inhibitors was determined by fluorescent scan (Cy5, bottom) of residual cathepsin activity. Gels were then blotted on PVDF and reacted with indicated antibodies (top). **(B)** Autophagic degradation in the presence of cathepsin inhibitors was assessed by fluorescent microscopy examination of p62 levels. Primary macrophages were stimulated with inhibitors for cathepsins (CatB 25μM, CatL 25μM or GB111-NH2 10μM) after which, 7-Keto (50μM) was added for another 4 h. Cells were fixed and permeabilized with cold methanol for 10 min at -20°C and then stained for p62 (magenta) or DAPI (blue) for the nucleus. **(C)** Quantification of p62 puncta per cell is presented as box and whiskers plot. The scale bar is 5μm.



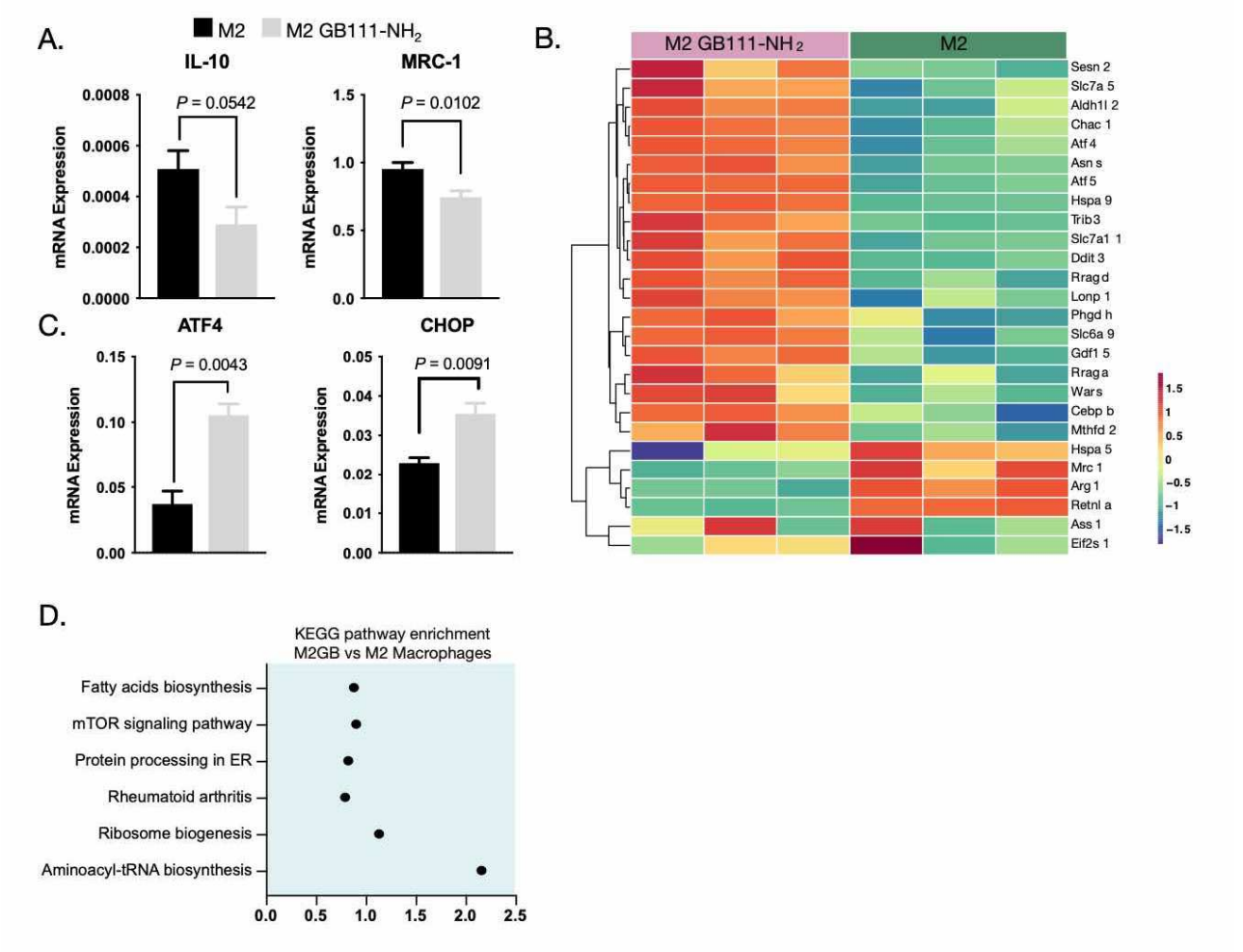
Supplementary Figure 6. Cathepsin inhibition delays mitochondrial clearance. (A) Exposure to 7-Keto cholesterol induces mitophagy in primary macrophages. Representative confocal micrographs of macrophages stimulated with 7-Keto (50μM) at different time points. Cells were then fixed and permeabilized with chilled methanol and stained with LC-3B (autophagosomes, magenta) or Tomm20 (mitochondria, cyan). **(B)** Quantification of LC-3B colocalization with Tomm20. One-way ANOVA with Dunnet's correction for multiple hypotheses. **(C)** 7-Keto induces lysosomal degradation of damaged mitochondria. Confocal images of macrophages stimulated with 7-Keto (50μM) or vehicle control for 4 h. Cells were stained with mitotracker deep red (100nM) for 30 min and then fixed and permeabilized with chilled methanol. Lysosomes were stained with LAMP-1 (green) and nuclei with DAPI (blue). **(D)** Quantification of mitochondrial overlap with lysosomes. Statistical significance was determined by Student's t-test. **(E)** Accumulation of mitochondrial damage due to lysosomal inhibition. Primary macrophages were treated with cathepsin L inhibitor (Cat Li 25μM) for 16 h, after which 7-Keto (50μM) was added for another 4 h. Chloroquine (CQ 100μM) (as a positive control) was added in parallel to 7-Keto. Cells were then stained with mitotracker deep red and mitotracker green (100nM) for 30 min and prepared for flow cytometry analysis (n=3-4 biological replicates). The gated population represents cells with damaged mitochondria. Data are presented as mean ± SEM. The scale bar is 5μm.



Supplementary Figure 7. Cathepsin inhibition alters mitochondrial shape and function. (A) Primary macrophages were treated with pan-cathepsin inhibitor JPM-OEt (50µM) or DMSO for 16 h followed by treatment with 7-Keto (50µM) for another 4 h. Mitotracker deep red (100nM) was added for 30 min and the cells were then fixed in cold methanol for 10 min. Mitochondria were visualized by fluorescent microscopy (magnification x100). Magnified rectangles are presented on the right. The scale bar is 5µm. **(B)** Quantification of mitochondrial circularity is presented in a box and whiskers plot. **(C)** Mitochondrial function was assessed by the Seahorse assay. Primary macrophages were treated with cathepsin L inhibitor (Cat Li 25µM) or DMSO for 16 h and then with 7-Keto or ethanol for another 2 h. Mitochondrial oxygen consumption was measured by Seahorse and values were normalized to protein content. Data are presented as the mean ± SEM of 4-6 biological replicates. **(D)** Primary macrophages were treated as described in panel (A) and mitochondrial ROS production was measured by flow cytometry using mitosox (1µM), according to the manufacturer's protocol. The data are expressed as fold change relative to the control, and are presented as the mean ± SEM of 7-8 biological replicates.



Supplementary Figure 8. Cathepsin inhibition in the settings of metabolic stress mediate inflammatory cytokine expression through reactive oxygen species. Primary macrophages were treated with GB111-NH2 (10μM) for 16 h or with DMSO vehicle. Before the addition of 7-Keto (50μM) for 4 h, the cells were administered N-acetyl cysteine (NAC) 10mM for 4 h or vehicle. Normalized expression to the 36B4 housekeeping gene of indicated inflammatory cytokines are presented in bar graphs and represent the mean ± SEM. One-way ANOVA with Fisher's Least Significant Distance for multiple comparisons (n=8-12 biological replicates per group).



Supplementary Figure 9. Cathepsin inhibition decreases M2 markers in parallel to elevation in the cellular stress-related gene signature. Mouse primary macrophages were treated with GB111-NH_2 (10μM) or DMSO for 16 h and then stimulated with IL-4 for another 24 h. **(A)** Normalized expression of IL-10 and MRC-1, classical M2 markers are shown (n=7-8 biological replicates per group). **(B)** Heat map presenting differentially expressed genes from RNA-seq experiments, indicating an increase in stress-related genes and a marked reduction in classical M2 markers are highlighted in a blue rectangle. **(C)** Validation of the stress-related genes ATF4 and CHOP in M2 polarized macrophages after treatment with GB111-NH_2. Cells were treated as described previously in panel **(A)** and normalized expression of ATF4 and chop are presented in bar graphs (n=3 biological replicates per group). P values below 0.05 were considered statistically significant and Student's t-test was used to determine statistical significance.

Supplementary Table 1. Quantitative PCR primers used in this study.

Gene Symbol	Sequence	Specie
Retnla F	CTGGGTTCTCCACCTCTTCA	Mus
Retnla R	GCTGGGATGACTGCTACTG	Mus
Arg-1 F	CTCCAAGCCAAAGTCCTTAGAG	Mus
Arg-1 R	AGGAGCTGTCATTAGGGACATC	Mus
IL-6 F	TAGTCCTTCCTACCCCAATTTCC	Mus
IL-6 R	TTGGTCCTTAGCCACTCCTTC	Mus
IL-1a F	CGAAGACTACAGTTCTGCCATT	Mus
IL-1a R	GACGTTTCAGAGGTTCTCAGAG	Mus
RPLP0 F	AGATTCGGGATATGCTGTTGGC	Mus
RPLP0 R	TCGGGTCCTAGACCAGTGTTC	Mus
TNFA F	CCAGACCCTCACACTCAGATC	Mus
TNFA R	CACTTGGTGGTTTGCTACGAC	Mus
MCP-1 F	TTAAAAACCTGGATCGGAACCAA	Mus
MCP-1 R	GCATTAGCTTCAGATTTACGGGT	Mus
IL1B F	TGGGCCTCAAAGGAAAGAAT	Mus
IL1B R	CAGGCTTGTGCTCTGCTTGT	Mus
ATF4 F	ATGGCGCTCTTCACGAAATC	Mus
ATF4 R	ACTGGTCGAAGGGGTCATCAA	Mus
CHOP F	CTGGAAGCCTGGTATGAGGAT	Mus
CHOP R	CAGGGTCAAGAGTAGTGAAGG T	Mus
IL-10 F	GCTCTTACTGACTGGCATGAG	Mus
IL-10 R	CGCAGCTCTAGGAGCATGTG	Mus
MRC-1 F	CGGAATTTCTGGGATTCAGCTTC	Mus
MRC-1 R	CTCTGTTCAGCTATTGGACGC	Mus
CCR 7 F	AGTCTTCCAGCTGCCCTACA	Hs
CCR 7 R	TCGTAGGCGATGTTGAGTTG	Hs
CD206 F	GTGGCCGGAGTAGTCATCAT	Hs
CD206 R	TCTTGAGGTAGGTGCACACG	Hs
IL-1α F	CCTGGGCATTCTTGTTTCAATT	Hs
IL-1α R	AGTTTTGATAACAGTGGTCTCATGGT	Hs
IL-1β F	GCCAATCTTCATTGCTCAAGTGT	Hs
IL-1β R	AGCCATCATTTCACTGGCGA	Hs
IL-6 F	TCCTGCAGAAAAAGGCAAAGA	Hs
IL-6 R	CATTTGTGGTTGGGTCAGGG	Hs
iNOS F	ACCTTTGATGAGGGGACTGG	Hs
iNOS R	GTTCTTCACTGTGGGGCTTG	Hs
MCP-1 F	AACCCAAGAATCTGCAGCTAACTT	Hs
MCP-1 R	AAGGCATAATGTTTCACATCAACAA	Hs
RPLP0 F	GTCGGAGGAGTCGGACGAG	Hs
RPLP0 R	GCCTTTATTTCCTTGTTTTGCAAA	Hs
TNF α F	CCCATGTTGTAGCAAACCCT	Hs
TNF α R	TTATCTCTCAGCTCCACGCC	Hs

Supplementary Table 2. Upregulated proteins GB111-NH_2 vs Control macrophages.

ID	log2FC	adj.P.Val
Mpeg1	3.03486666666667	1.21060987706768e-08
Aplp2	3.92322	1.21060987706768e-08
App	3.16570000000002	5.25755657223711e-08
Plau	5.34945000000002	7.04598033434308e-08
Lgmn	1.60151666666667	9.55474850164939e-07
Sqstm1	2.95790000000001	9.55474850164939e-07
Nrp2	1.90452333333334	3.43709791149043e-06
Gpnmb	1.91098666666667	4.25367207477558e-06
Sema4d	1.94495000000001	5.14779065218177e-06
Fth1	1.20405666666668	2.15826459037195e-05
Psap	1.69407333333334	2.58064223942571e-05
Pld3	1.80800666666667	3.17886111463495e-05
Ctsl	1.59325666666667	4.42920287255624e-05
Pltp	1.44390666666666	8.07057887972179e-05
Lipa	1.79880666666668	0.000138643904896723
Sdcbp	1.01668666666667	0.00026642085261956
Mmp12	2.99440000000001	0.000459752809136278
Tnfrsf1b	1.80835333333334	0.000710274808230225
Lilrb4	1.47129000000002	0.00071628074495902
Hmox1	1.03040000000001	0.00124573580977347
Apoe	1.69505000000001	0.00133435533836745
Ftl1;Ftl2	1.00706333333335	0.0013442663774812
Bst2	2.39221	0.0013571337205082
Hpse	1.23932666666667	0.00212597464209625
Cd36	1.08768000000001	0.00214980187213681
Rnf149	2.31942000000002	0.00488055903596125
Adam8	1.10065333333334	0.00803649279039081
Vamp4	1.04964000000001	0.0080478886653192
Tgfbi	1.08423000000001	0.0152160278439146
Tmem106a	1.18136000000001	0.0158821299824522
Tax1bp1	1.44203666666668	0.0158821299824522
Fn1	1.62751333333334	0.0158821299824522
Cd93	1.11056000000001	0.0232789596167197
Map1lc3b;Gm5612;Map1lc3a	1.37284333333335	0.049346782872378
Sqle	1.14800333333334	0.0765623152708027

Supplementary Table 3. Upregulated proteins 7-Keto GB111-NH_2 vs 7-Keto treated macrophages.

ID	log2FC	adj.P.Val
Nrp2	1.076446667	0.03586582
Pltp	1.12156	0.08430602
Csf1r	1.129453333	0.045793015
Cd36	1.139213333	0.05506421
Fth1	1.146726667	0.053288044
Lilrb4	1.153346667	0.012648641
Ctsl	1.23668	0.061759539
Lgals9	1.321953333	0.01656318
Gpnmb	1.4818	0.000784377
Lgmn	1.50516	0.001399592
Pld3	1.543653333	0.006495812
Lipa	1.726706667	0.01826412
Psap	1.772966667	0.001399592
Sqstm1	1.811986667	0.029429617
Bst2	2.024493333	0.054987252
Mmp12	2.316	0.03586582
Plau	2.5564	0.000162561
Mpeg1	2.607673333	0.000131752
Aplp2	3.099386667	0.03586582
App	3.332673333	0.000131752