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Supplemental Information

Obesity Activates a Program of Lysosomal-Dependent Lipid Metabolism in Adipose Tissue Macrophages Independently of Classic Activation

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Supplemental Tables & Figures

Table S1.

Functional Clusters of Genes Correlated with Phenotypes

Clusters	Functional Cluster	Overrepresentation	Corrected p-value
Adipose Tissue Programs Correlated with Body Mass	Lysosome	12.7	4.5×10^{-12}
	Vesicle	6.19	4.8×10^{-4}
	Inflammatory response	5.97	1.3×10^{-5}
	Cell/Lymphocyte activation	4.71	1.6×10^{-5}
	B-cell/NK cell signaling	*4.02*	3.3×10^{-5}
	Sh3 domain proteins	3.79	0.018
	Glycoproteins	3.74	0.00025
	Sh2 domain proteins	3.72	0.041
	Glycosidases	3.13	0.00077
	Chemotaxis	2.89	0.047
	Actin reorganization	2.88	0.04
	Immune response	2.51	0.0029
	Lipid/sphingolipid metabolism	2.5	0.0063
Adipose Tissue Programs Correlated with Fasting [Insulin]	Lysosome	10.72	1.7×10^{-11}
	Immune Effector	3.88	0.012
	Glycosylation	3.77	2.1×10^{-5}
	Wound Response	3.66	0.037
Adipose Tissue Programs Correlated with a Macrophage Transcriptional Program	Lysosome	17.02	4.8×10^{-16}
	Pigment Granule	8.26	1.0×10^{-9}
	Cell cycle	5.54	2.9×10^{-5}
	B-cell/NK cell signaling	5.23	3.9×10^{-8}
	Inflammatory response	4.84	2.0×10^{-4}
	Immune cell activation	4.79	8.4×10^{-8}
	Actin reorganization	4.53	0.0013
	Phagocytosis	4.25	5.5×10^{-5}
	Ubiquitinization	3.72	3.6×10^{-5}
	Lipid metabolism	3.59	0.001
	Glycosidase	2.94	2.2×10^{-4}
	Apoptosis	2.86	0.0016
	ATP binding/Kinase	2.79	6.6×10^{-6}
	Phosphotase	2.64	9.1×10^{-4}
	Lymphocyte homeostasis	2.54	0.004
	Antigen processing	2.34	0.024
	Zymogen	2.12	8.8×10^{-4}
	Phagocytosis	2.11	5.5×10^{-5}
	Vascular development	1.92	0.025
	Response to fungi	1.88	0.034
	Chemotaxis	1.8	0.044
	Immune system regulation	1.79	1.6×10^{-4}
Programs in ATMs Increased by Obesity	Lysosome	7.19	9.1 x 10-7
	ATP binding	5.95	2.3 x 10-5
	Mitosis	4.6	0.012
	Vesicle Transport	2.79	0.047

Table S1. Function clusters of genes whose expression is associated with phenotypes. Microarray expression analysis was performed on either adipose tissue from a group of 34 mice or from adipose tissue macrophages from lean or obese mice. Using an overrepresentation algorithm functional classes of genes were identified that were associated with each phenotype.

Figure S1.

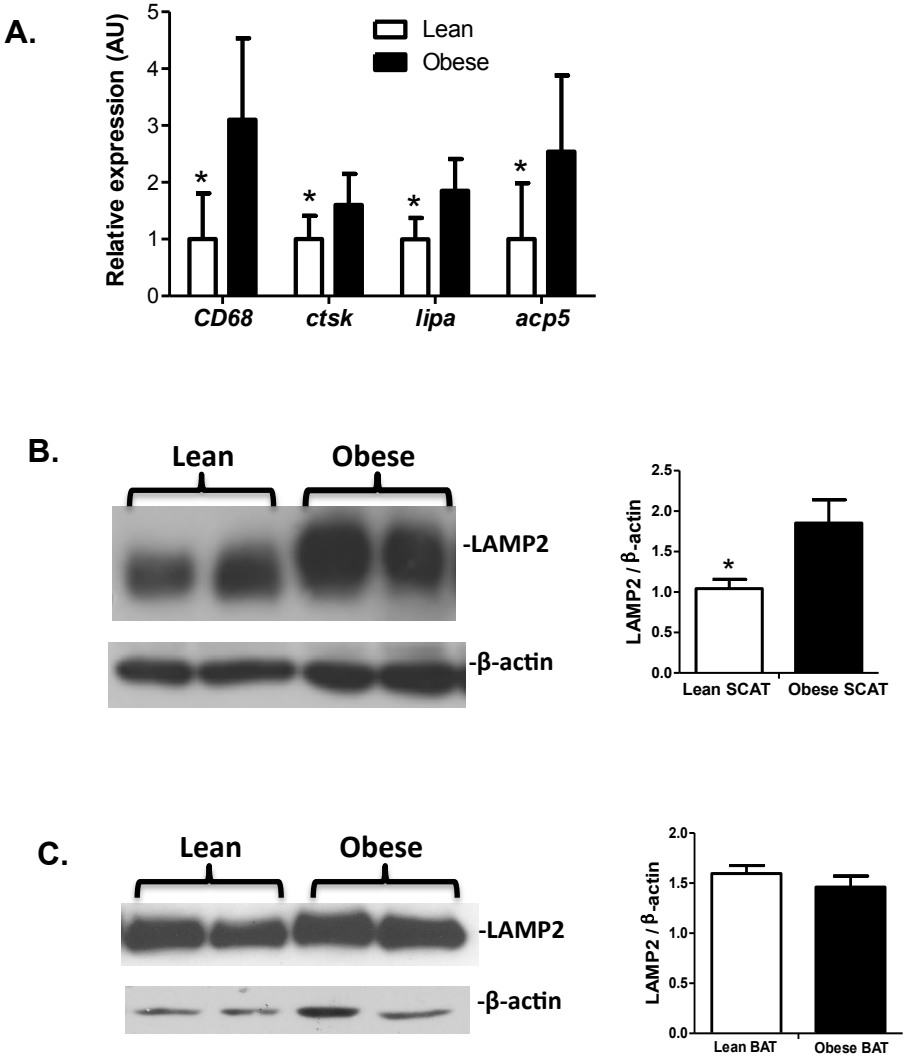

Figure S1. Obesity increases expression of lysosomal genes and proteins in human and murine adipose tissue related to Figure 1 (**A**) Expression of a macrophage-specific gene and lysosome genes in human mesenteric adipose tissue from lean (n=5) and obese (n=20) individuals. Obesity (C57BL/6J *Lep ob/ob*) increases the expression of lysosomal protein LAMP2 in (**B**) subcutaneous but not (**C**) intrascapular brown adipose tissue of mice (n=4; * p-value <0.05 lean vs obese). All values are means +/- SD.

Figure S2.

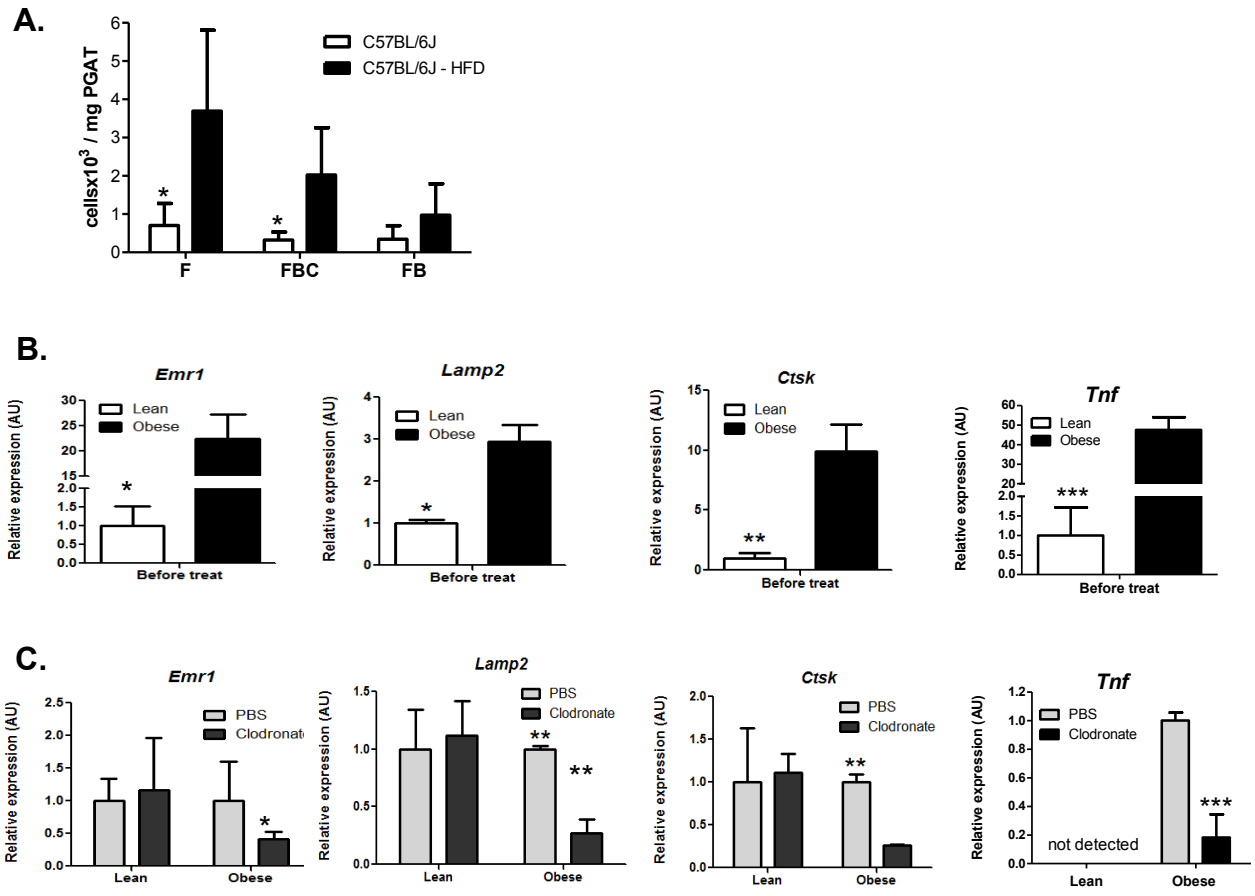

Figure S2. Effect of high fat diet-induced obesity on ATM content and lysosomal gene expression related to Figure 2 (**A**) Total F4/80+ macrophages (F) and macrophage sub-populations of CD11c**+** (FBC) and CD11c- (FB) (n=4). (**B)** Gene expression of macrophage-specific (*Emr1*) and lysosomal genes (*Lamp2, Ctsk*) were measured in the adipocyte fraction of adipose tissue, ie buoyant cells after digestion, (**B)** before and (**C**) after depletion of macrophages by liposome encapsulated clodronate or PBS (normalized to PBS liposome treatment; n=5; p-value * < 0.05, ** < 0.01, *** , 0.005). All values are means +/- SD.

Figure S3.

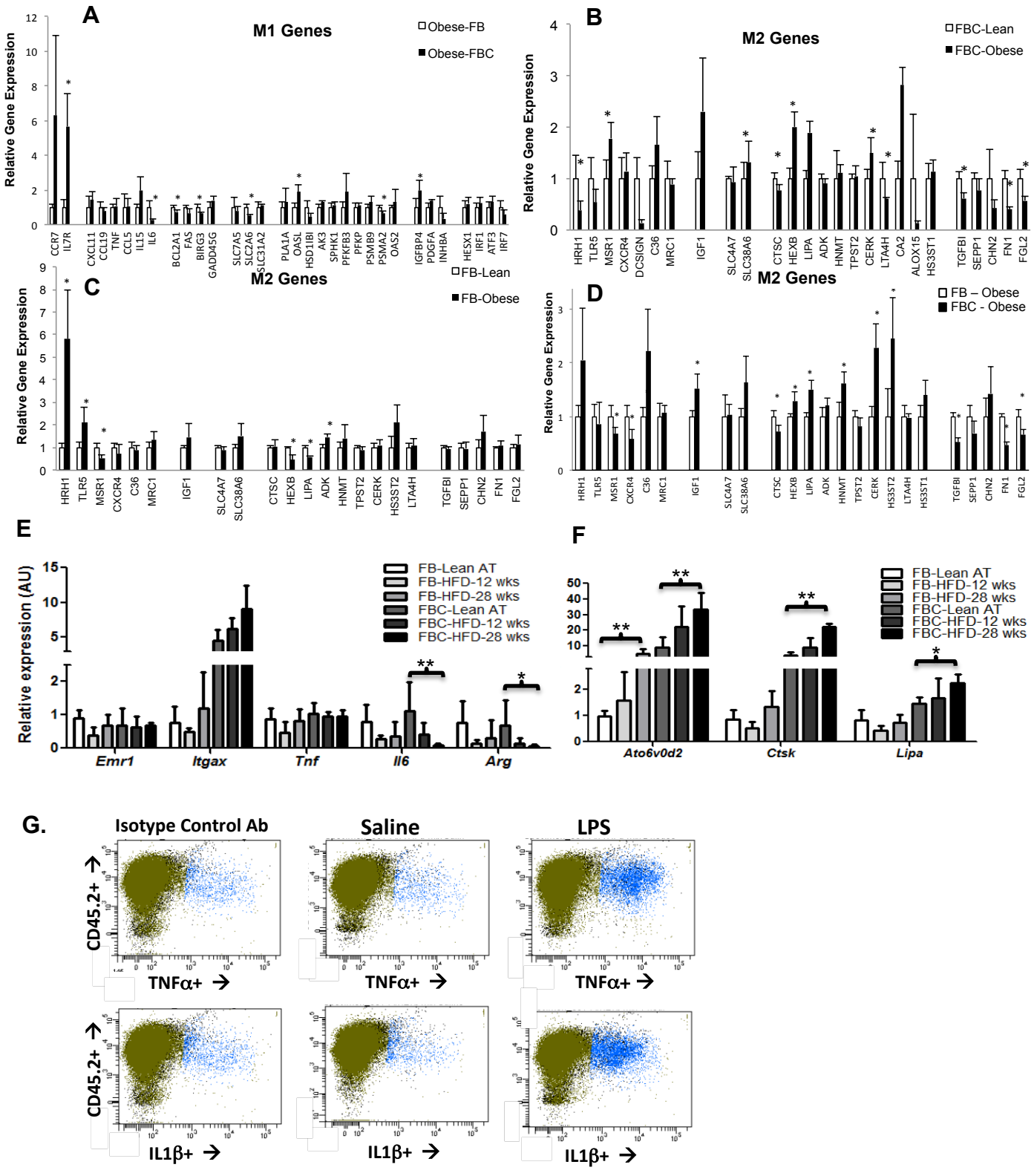
A
M1 Genes
Obese-FB
Obese-FBC
Relative Gene Expression
CCR7 IL7R CXCL11 CCL19 TNF CCL5 IL15 IL6 BCL2A1 FAS BIRG3 GADD45G SLC7A5 SLC2A6 SLC31A2 PLA1A OAS1 HSD11B1 AK3 SPHK1 PFKFB3 PFKP PSMB9 PSMA2 OAS2 IGFBP4 PDGFA INHBA HES1 IRF1 ATF3 IRF7
B
M2 Genes
FBC-Lean
FBC-Obese
Relative Gene Expression
HRH1 TLR5 MSR1 CXCR4 DCSIGN C36 MRC1 IGF1 SLC4A7 SLC38A6 CTSC HEXB LIPA ADK HNMT TPST2 CERK LTA4H CA2 ALOX15 HS3ST1 TGFBI SEPP1 CHN2 FN1 FGL2
C
M2 Genes
FB-Lean
FB-Obese
Relative Gene Expression
HRH1 TLR5 MSR1 CXCR4 C36 MRC1 IGF1 SLC4A7 SLC38A6 CTSC HEXB LIPA ADK HNMT TPST2 CERK HS3ST2 LTA4H TGFBI SEPP1 CHN2 FN1 FGL2
D
M2 Genes
FB – Obese
FBC - Obese
Relative Gene Expression
HRH1 TLR5 MSR1 CXCR4 C36 MRC1 IGF1 SLC4A7 SLC38A6 CTSC HEXB LIPA ADK HNMT TPST2 CERK HS3ST2 LTA4H HS3ST1 TGFBI SEPP1 CHN2 FN1 FGL2
E
Relative expression (AU)
FB-Lean AT
FB-HFD-12 wks
FB-HFD-28 wks
FBC-Lean AT
FBC-HFD-12 wks
FBC-HFD-28 wks
Emr1 Itgax Tnf Il6 Arg
F
FB-Lean AT
FB-HFD-12 wks
FB-HFD-28 wks
FBC-Lean AT
FBC-HFD-12 wks
FBC-HFD-28 wks
Ato6v0d2 Ctsk Lipa
G.
Isotype Control Ab
Saline
LPS
CD45.2+
TNFα+
IL1β+

Figure S3. Effects of Obesity on Inflammatory Polarization and Lysosomal Gene Expression by ATMs related to Figure 3 ATMs were purified by FACS from PGAT of obese *Lep ob/ob* or lean *Lep +/+* C57BL/6J male mice. (**A**) The expression of genes induced in classically activated (M1 polarized) macrophages were compared in purified F4/80+, CD11b+, CD11c- cells (FB's) and in F4/80+, CD11b+, CD11c+ (FBC's). The expression of genes induced in alternatively activated (M2 polarized) macrophages were compared (**B**) lean and obese FBs (**C**) lean and obese FBCs, and (**D**) obese FBs and FBCs ATMs were also purified by FACS from PGAT of lean low fat fed C57BL/6J male mice or obese high fat diet (HFD) C57BL/6J male mice that were HFD fed for 12 or 28 weeks. (**E**) The expression of macrophage (*Emr1, Itgax*) and inflammatory genes was measured in FB's and FBC's from 8 week old lean and HFD fed mice. (**F**) Expression of lysosomal genes were measured in the same FB and FBC populations (normalized to lean FB expression; n=4). (**G**) Splenocytes were isolated from mice 1 hour after intraperitoneal injection of saline or LPS and TNFα and IL1β content measured using FACS analysis. Typical FACS plots are shown (p value * <0.05, ** < 0.01). All values are means +/- SD.

Figure S4.

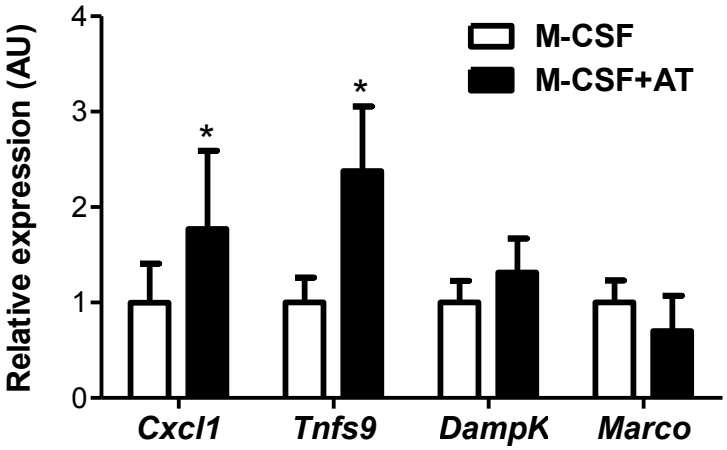

Figure S4. Adipose tissue induces expression of ATM-specific genes related to figure 4. Gene expression of ATM (*Cxcl1,Tnfs9*) and Kupffer cell (*Marco, Dampk*) enriched genes in bone marrow cells differentiated in the absence (white bars) or presence (black bars) of AT (n=4; * p-value<0.05) . All values are means +/- SD.

Figure S5

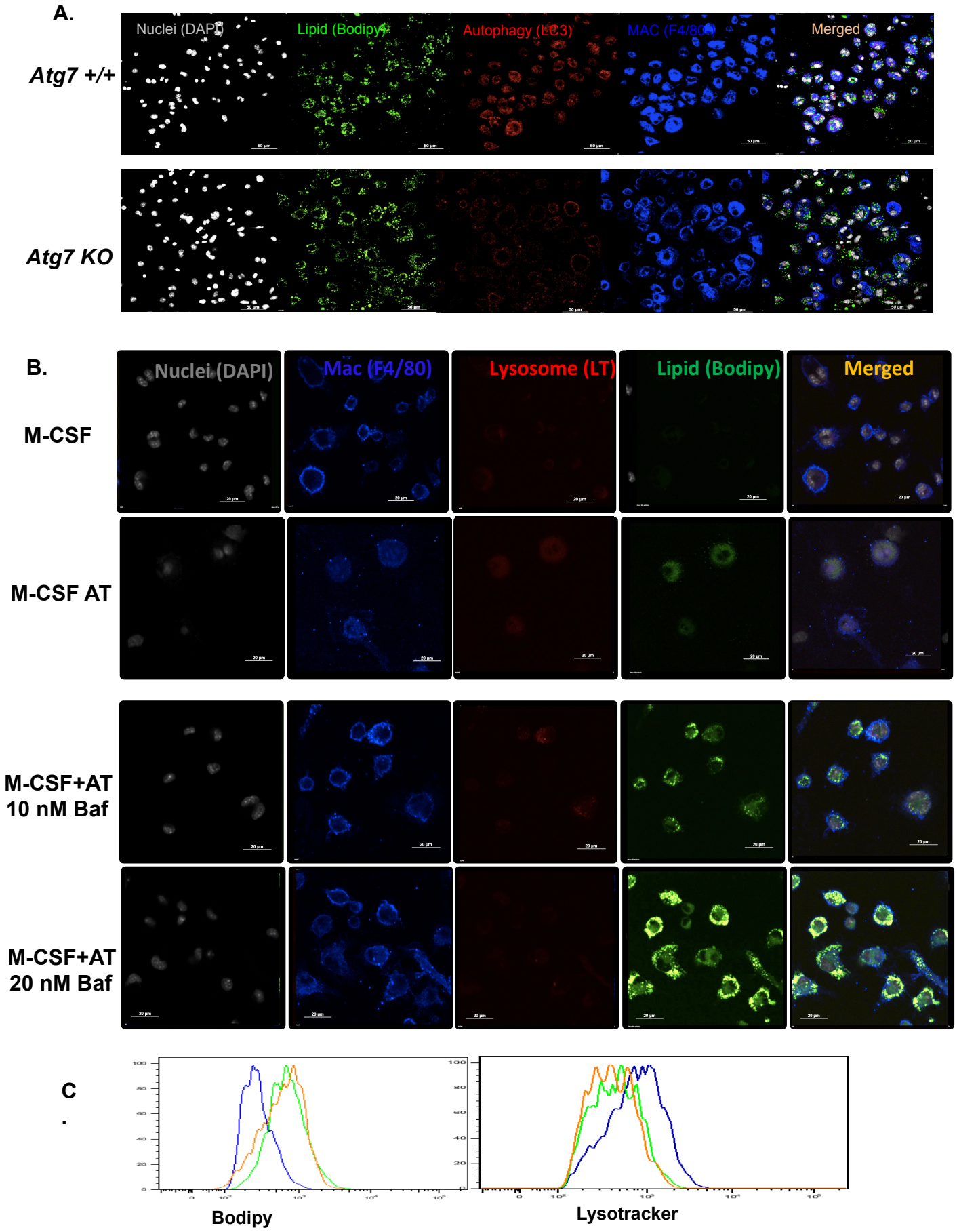
A.
Nuclei (DAPI)
Lipid (Bodipy)
Autophagy (LC3)
MAC (F4/80)
Merged
Atg7 +/+
Atg7 KO
B.
Nuclei (DAPI)
Mac (F4/80)
Lysosome (LT)
Lipid (Bodipy)
Merged
M-CSF
M-CSF AT
M-CSF+AT
10 nM Baf
M-CSF+AT
20 nM Baf
C.
Bodipy
Lysotracker

Figure S5. Inhibition of autophagy and lysosome function on lipid content of ATMs related to Figure 5 Bone marrow cells were differentiated in the presence of M-CSF alone or M-CSF and adipose tissue (AT) as described in Methods. (**A**) Immunofluorescent confocal images of lipid content in differentiated ATMs derived from mice in which autophagy is impaired in myeloid cells (*Atg7* KO) or possess intact autophagy (*Atg7 +/+*). (**B**) Immunofluorescent confocal images of lysosomes and lipid in differentiated ATMs and ATMs treated with bafliomycin A (Baf) at the indicated concentrations for 12 hours. Nuclei were identified with DAPI, macrophages (Mac) with an anti-F4/80 antibody, lysosomes with Lysotracker (LT), neutral lipid with Bodipy. (**C**) FACS histogram quantifying lipid and lysosome content by Bodipy and Lysotracker staining, respectively in ATMs (blue) and ATMs treated with 10nM (beige) or 20nM (green) of bafliomycin A for 12 hours.

Figure S6.

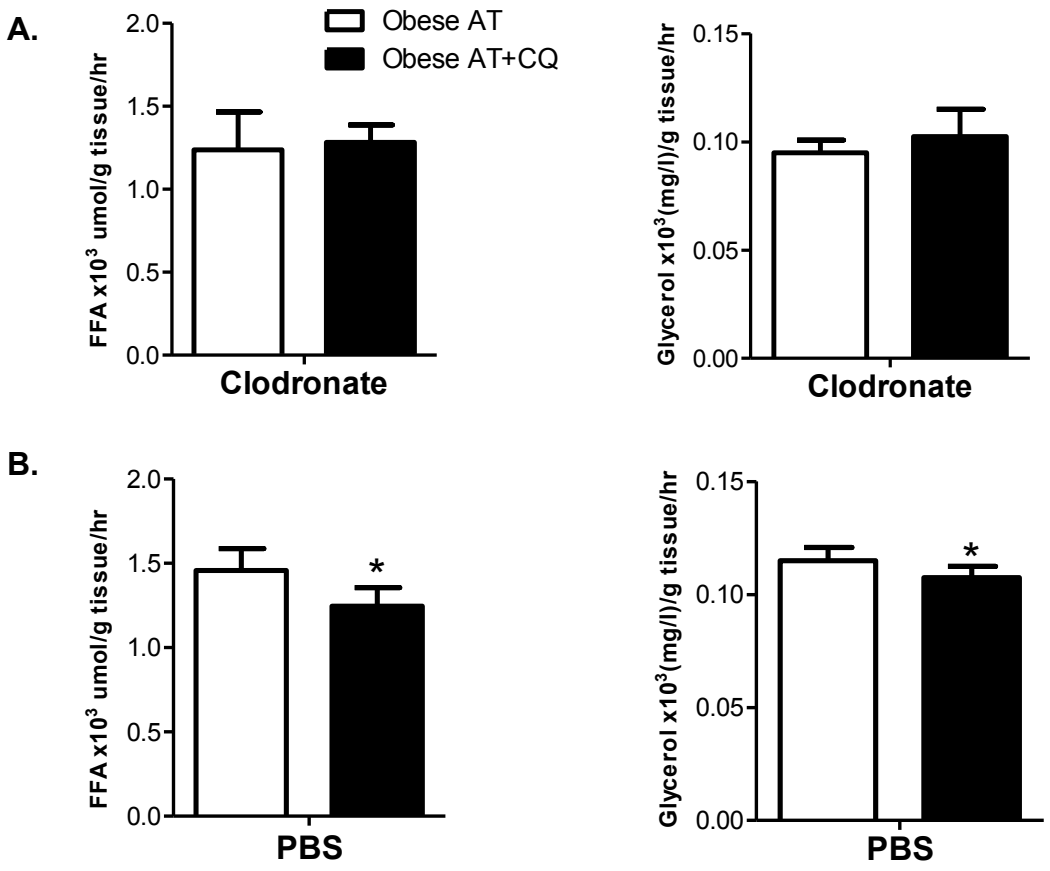

Figure S6. Depletion of macrophages from adipose tissue eliminates the anti-lipolytic effect of chloroquine related to Figure 6. Perigonadal adipose tissue from obese (C57BL/6 *Lep ob/ob*) mice were either (**A**) depleted of macrophages by treatment with liposomes containing clodronate or were (**B**) treated with liposomes containing PBS. The release of free fatty acids (FFA) and glycerol were measured in the presence (white bars) or absence (black bars) of chloroquine (CQ). (n=4, * p-value < 0.05). All values are means +/- SD.

Table S2

Primer Sequences used for qPCR

Mouse:

Arg1	Forward: 5'-CTCCAAGCCAAAGTCCTTAGAG-3'
	Reverse: 5'-AGGAGCTGTCATTAGGGACATC-3'
Adfp	Forward: 5'-CCCGTATTTGAGATCCGTGTT-3'
	Reverse: 5'-TAGGTATTGGCAACCGCAAT-3'
Atp6v0d2	Forward: 5'- AGCCAGCCTAACTCAGC -3'
	Reverse: 5'- GCTTCTTCCTCATCTCCGTGTC-3'
Atp6v1b2	Forward: 5'- AGCCTCGTCTCACCTACAAGA -3'
	Reverse: 5'- TCTCAGCGTATCTGGGAAACTT -3'
Apoe	Forward: 5'-CTG ACA GGA TGC CTA GCC G-3'
	Reverse: 5'-CGC AGG TAA TCC CAG AAG C-3'
Fabp4	Forward: 5'- GGGGCCAGGCTTCTATTCC -3'
	Reverse: 5'- GGAGCTGGGTTAGGTATGGG -3'
Atp7	Forward: 5'- CCTGCACAACACCAACACAC -3'
	Reverse: 5'- CACCTGACTTTATGGCTTCCC -3'
Cd36	Forward: 5'-GCGACATGATTAATGGCACA-3'
	Reverse: 5'-CCTGCAAATGTCAGAGGAAA-3'
Ccr2	Forward: 5'-TGCCATCATAAAGGAGCCA-3'
	Reverse: 5'-AGCACATGTGGTGAATCCAA-3'
Chop	Forward: 5'- CAG AGC CAG AAT AAC AGC CG-3'
	Reverse: 5'- GTC TCC AAG GTG AAA GGC AG -3'
Clec4f	Forward: 5'- AAGCAACAACTCTGGACACGAC -3'
	Reverse: 5'- TAGATGGCTCCCAAGCAACTG-3'
Cpt1a	Forward: 5'-TGC ACT ACG GAG TCC TGC AA -3'
	Reverse: 5'-GGA CAA CCT CCA TGG CTC AG-3'
Csf1r	Forward: 5'-CATACAGCATTACAACTGGACCTACC-3'
	Reverse: 5'-CAGGACATCAGAGCCATTCACAG-3'
Cxcl1	Forward: 5'- GGGACACCTTTTAGCATCTTTTG -3'
	Reverse: 5'- ATGGCTGGGATTCACCTCAAG-3'

Table S2

Dmpk	Forward: 5’- AACCTCTTCTTCCAGGGCTTC -3’
	Reverse: 5’- CCTTCGTGGGCTACTCCTACTG-3’
Emr1	Forward: 5’-CTTTGGCTATGGGCTTCCAGTC-3’
	Reverse: 5’-GCAAGGAGGACAGAGTTTATCGTG-3’
Fsn	Forward: 5’- TCTTTCTAACAACCACCCTCTGG -3’
	Reverse: 5’- CTTCACGACTCCATCACGAATG -3’
Igf2r	Forward: 5’- TGGCGAATGAAGTCTACCTGAA-3’
	Reverse: 5’- CTCCCACTCAAACACAAAGTCG -3’
Il1b	Forward: 5’- TGA AGC AGC TAT GGC AAC TG-3’
	Reverse: 5’- AGG TCA AAG GTT TGG AAG CA -3’
Il10	Forward: 5’-GCTCTTACTGACTGGCATGAG-3’
	Reverse: 5’-CGCAGCTCTAGGAGCATGTG-3’
Itgam	Forward: 5’- ATT CGG TGA TCC CTT GGA TT-3’
	Reverse: 5’- GTT TGT TGA AGG CAT TTC CC-3’
Itgax	Forward: 5’-CCTACTTTGGGGCATCTCTTTG-3’
	Reverse: 5’-GCACCTCTGTTCTCCTCCTCTC-3’
Lamp2	Forward: 5’- ATGTGCCTCTCTCCGGTTAAA-3’
	Reverse: 5’- GCAAGTACCCTTTGAATCTGTCA-3’
Lipa	Forward: 5’- GGAAACAGCAGAGGAAACACCT -3’
	Reverse: 5’- CACGGGAGCCAAGACTAAAAC -3’
Lpl	Forward: 5’-TTTGGCTCCAGAGTTTGACC-3’
	Reverse: 5’-TGTGTCTTCAGGGGTCCTTAG-3’
Marco	Forward: 5’- GAAGACAGACCGATTTTGACC -3’
	Reverse: 5’- ATCCATTGCCACAGCACATCTC-3’
Mrc2	Forward: 5’-TACAGCTCCACGCTATGGATT-3’
	Reverse: 5’-CACTCTCCCAGTTGAGGTACT-3’
Msr1	Forward:5’-AGAGGGCTTACTGGACAAACTG-3’
	Reverse: 5’-GGCTTTCCTGGTGCTCCTG-3’

Table S2

Nos2	Forward: 5’-AATCTTGGAGCGAGTTGTGG-3’
	Reverse: 5-CAGGAAGTAGGTGAGGGCTTG-3’
Ppib	Forward: 5’-CAGCAAGTTCCATCGTGTCATC-3’
	Reverse: 5’-CTCTTTCCTCCTGTGCCATCTC-3’
Pparg	Forward: 5’- GGAAGACCACTCGCATTCCTT -3’
	Reverse: 5’- GTAATCAGCAACCATTGGGTCA -3’
Ppib	Forward: 5’- CAG CAA GTT CCA TCG TGT CAT C -3’
	Reverse: 5’- CTCTTTCCTCCTGTGCCATCTC-3’
Plin2	Forward: 5’- TCTGCGGCCATGACAAGTG -3’
	Reverse: 5’- GCAGGCATAGGTATTGGCAAC-3’
Rps3	Forward: 5’- ATC AGA GAG TTG ACC GCA GTT G -3’
	Reverse: 5’- AAT GAA CCG AAG CAC ACC ATA G -3’
Tnf	Forward: 5’-CCAGACCCTCACTAGATCA-3’
	Reverse: 5’-CACTTGGTGGTTTGCTACGAC-3’
Tlr2	Forward: 5’-AAACAACTTACCGAAACCT3’
	Reverse: 5’-TGTAAATTTGTGAGATTGG-3’
Tlr4	Forward: 5’- ATGGCATGGCTTACACCACC -3’
	Reverse: 5’- GAGGCCAATTTTGTCTCCACA -3’
Tnfsf9	Forward: 5’- GAGCCCTGTTGTGTAGTGTTGG -3’
	Reverse: 5’- GCAGGCATAGGTATTGGCAAC
Human:	
ACP5	Forward: 5’- AGGCTTTTCCTCCAACCTGT-3’
	Reverse: 5’-TCACATACGTGGGCATCTGT-3’
CD68	Forward: 5’- GCTGGCTGTGCTTTTCTCG-3’
	Reverse: 5’-GTCACCGTGAAGGATGGCA-3’
CTSK	Forward: 5’- CCACGATGGTGCAGTGTAAC-3’
	Reverse: 5’-GCACAAACAAATGGGGAAAC-3’
LIPA	Forward: 5’-CCTGCTTCACGTCCCTATGT-3’
	Reverse: 5’- TGATGCTGCAAGAAAAGCAG-3’

Table S2 Primer sequences Sequences for primers and primer pairs used in quantitative RT-PCR.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals and animal care

All mice were obtained from the Jackson Laboratory (Bar Harbor, ME) except for C57BL/6J *Atg7 fl/fl* which were generated by and obtained from Masaaki Komatsu at Tokyo Metropolitan Institute of Medical Science . For microarray studies mice C57BL/6J, B6.Cg-*Lep* $^{ob/ob}$/J (C57BL/6J *Lep*$^{ob/ob}$), B6.Cg-*A*^{y}/J (C57BL/6J *Ay/a*), were obtained at 7 weeks of age. Mice were fed a low fat diet (Research Diets # D12490) unless otherwise noted. High fat diet animals were fed a food pellets that derived ~ 60% of its calories from fat (Research Diets # D12492) beginning at 8 weeks of age. Mice treated with insulin sensitizing agents pioglitazone (10mg/kg/day) or CL316243 (3mg/kg/day) began treatment at 20 weeks of age by including the drug in their food. Mice were sacrificed at 22-23 weeks of age and perigonadal adipose tissue collected. For all other studies mice male were obtained from Jackson Laboratory at 8-10 weeks of age. Mice made obese by high fat feeding for non-microarray studies were begun on a high fat diet at 8 weeks of age (Research Diets Inc. # D12492). Mice in non-microarray studies were sacrificed at 12-14 weeks of age. Mice in all studies were housed 3-5 animals per ventilated Plexiglas cages within a pathogen-free barrier facility that maintained a 12-hour light/12-hour dark cycle. All procedures were approved by the Columbia University IACUC.

Isolation stromal vascular cells and the adipocyte-rich fraction of adipose tissue

Adipose tissue was isolated from mice immediately after CO_2 asphyxiation. Tissues were minced into fine (<10-mg) pieces and centrifuged at 500 g for 5 minutes to remove connective tissue debris. Minced samples were placed in a digestion solution of DMEM containing Liberase 3 or Liberase TM (0.14 units/ml) (Roche Applied Science)/ BSA, 20 mg/ml, DNase I, 50 U/ml (Sigma-Aldrich). The samples were then incubated at 37°C on an orbital shaker (185 rpm) for 30-45minutes. Once digestion was complete, samples were passed through a sterile 250-μm nylon mesh (Sefar Filtration Inc.). The suspension was centrifuged at 500 g for 5 minutes. The pelleted cells were collected as the SVCs and top layer of lipid containing cells/material were collected as the buoyant adipocyte-rich fraction (ARF). The SVCs were resuspended in erythrocyte lysis buffer (BD Biosciences) and incubated at room temperature for 3 minutes. The erythrocyte-depleted SVCs were centrifuged at 500 g for 5 minutes and the pellet cells resuspended in FACS buffer (PBS, 0.2%, fatty acid depleted-BSA, 5 mM EDTA) at a concentration of 7 x 10^6 cells/ml for fluorescence activated cell sorting or purification, or in culture medium (DMEM (Invitrogen), 20% L929 cell- conditioned media, 10% fetal bovine serum (Invitrogen), 1% of Penicillin-Streptomycin liquid (Invitrogen)) at a concentration of 650,000 cells/ml for culture and live confocal microscopy studies. The ARF was washed with DMEM buffer and collected again as the buoyant top layer of lipid rich material after a 5 minute centrifugation at 500g at room temperature.

Quantitative RT-PCR.

Total RNA was extracted from the perigonadal (epididymal or parametrial) (PGAT), inguinal subcutaneous (SCAT) and brown adipose tissue (BAT) of individual mice using Trizol reagent (Invitrogen). RNA was isolated from SVCs and primary cells using RNeasy mini-kits (Qiagen). First-strand cDNA was synthesized using Superscript III reverse transcriptase and random hexamer (Invitrogen). Quantitative RT-PCR assays were carried out using DNA Engine Opticon 2 system instruments (Bio-Rad) and PCR SYBR Green I QuantiTect Master Mix (QIAGEN). Data were normalized to *Ppib* using the ΔΔC(t) method and are presented as relative transcript levels. All primers used are listed in Supplemental Table 1.

Hematoxylin and eosin staining and Oil Red O staining of purified cells

F4/80+, CD11b+, CD11c- (FB) and F4/80+, CD11b+, CD11c+ cells were purified from the stromal vascular fraction of perigonadal adipose tissue using an FACSAria cell sorter as described above (Becton Dickson). Purified cells were placed on microscope slides air dried. Differentiated bone marrow cells were cultured on chamber slides. Adherent cells were washed and air dried, fixed with zinc-formalin fixative (Anatech Ltd.) for 10 minutes and thereafter stained by hematoxylin and eosin stain or Oil Red O using standard methods .

Immunophenotyping and flow cytometry.

For standard FACS analysis, cells were collected in FACS buffer as described above and incubated in the dark for 30 min with of 0.5mg/ml FcBlock (BD Pharmingen) and then for an additional 30 min with fluorophore-conjugated antibodies or fluorescent dyes. The antibodies used were as follows: CD11b-PE and APC, CD11c-PE and PE-TR, CD3e-PE, CD19-PE, NK1.1-APC, TNFα-PE and IL-1β-PE (all eBioscience), CD45.2-Percp-Cy5.5, and Ly6G-PE and FITC, (all BD Pharmingen), F4/80-APC (AbD Serotec) and fluorescently tagged isotype controls. Fluorescent dyes used were Lysotracker and 4, 4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (Bodipy) (Invitrogen). After incubation with primary antibodies and/or fluorescent dyes, cells were washed twice in FACS buffer, resuspended in FACS buffer containing 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). For immune phenotyping, cells were analyzed using an LSRII flow cytometer (Becton Dickson) equipped with a dozen excitation lines. Data analysis was performed using Flowjo software (Tree Star). All samples were gated on DAPI− cells before quantification. For purification, cells were sorted using a BD FACSAria Cell Sorter into Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum. For FACS analysis of intracellular proteins, ie TNFα and IL-1β, fixation/permeabilization was performed on splenocytes or stromal vascular cells of PGAT using a commercially available kit according to the manufacturer's instructions (BD Cytofix/Cytoperm Fixation/Permeabilizaton Kit). Following permeabilization cells were incubated with the appropriate antibodies and analyzed as above. We used the following immunophenotyping to identify immune cell populations in adipose tissue: Immune cells: CD45.2+; Macrophages: F4/80+; FBC Macrophages: F4/80+, CD11b+, CD11c+; FB Macrophages: F4/80+, CD11b+, CD11c-; Dendritic Cells (DC): F4/80-, CD11b+, CD11c+; T-cells: CD3e+, NK1.1-; NKT: CD3e+, NK1.1+; NK cells: CD3e-, NK1.1+; B-cells CD19+, CD11b-.

Microarray gene expression.

For whole adipose tissue samples, total RNA was extracted from the perigonadal adipose tissue of individual mice using a commercially available acid-phenol reagent (Trizol; Invitrogen Inc.). RNA was isolated from sorted FBC (F4/80+, CD11b+, CD11c+) cells and FB (F4/80+, CD11b+, CD11c-) cells using RNeasy micro-kits (Qiagen), using a PicoPure RNA isolation kit then amplified two-rounds. Labeled cRNA was prepared according to the manufacter's instructions (Affymetrix) and hybridized to either Mouse U74v2 arrays (whole adipose tissue) or Mouse Genome 430 2.0 arrays (purified FB and FBC adipose tissue macrophages). Primary data extraction was performed with Microarray Suite 5.0 (Affymetrix) for U74v2 arrays and Expression Console (Affymetrix), Data were deposited in NCBI GEO database (series accession #: GSE8831). Functional class analysis was performed using DAVID Suite software (NIAID, NIH http://david.abcc.ncifcrf.gov).

Immunofluorescence histochemistry

Stromal vascular cells or bone marrow-derived cells were cultured on coated chamber slides (LAB-TEK) in DMEM/20% L929 cell-conditioned media/10% fetal calf serum/ 1% PCN & Streptomycin. For identification of lysosomes, live cells were washed with PBS and cultured in DMEM containing 10 uM Lysotracker (Invitrogen) for 30 minutes at 37ºC. Cells were washed with PBS three times at room temperature. Cells were fixed cells with Z-fix (Anatech Ltd) for 10 minutes and washed again with PBS three times at room temperature. To reduce background fluorescence the fixed samples were incubated 0.2 M glycine for 30 minutes at room temperature. All samples were incubated in blocking buffer (PBS solution containing 1% BSA and 5% goat serum) for 30 minutes at room temperature. To identify macrophages, samples were incubated with an anti-F4/80 mAb at 1 μg/ml (Invitrogen) or control antibody - Rat IgG2a (Invitrogen) at 1 μg/ml in blocking buffer for 30 minutes at room temperature. Samples were washed with PBS three times and then incubated with secondary Cy5 - labeled goat anti-rat IgG (Invitrogen) at 5ug/ml in blocking buffer for 1 hour at room temperature. Samples were washed and incubated with DAPI nuclear dye (1:8,000 v/v) and Bodipy (1:300 v/v) for 5 min at room temperature. After 3 further washes with PBS at room temperature, the slides were covered with glass coverslips affixed with Flucro-gel mounting solution (Electron Microscopy Sciences). Fluorescent confocal microscopy was performed using a Nikon AIR MP.

Western blot

Stromal vascular cells, adipocyte-rich fractions and purified F4/80+ cells were isolated from PGAT from lean (C57BL/6J *Lep*$^{+/+}$) and obese (C57BL/6J *Lep* $^{ob/ob}$) male 12-15 week old mice. Protein was extracted using Tissue Extraction Reagent I with protease inhibitor cocktail (Invitrogen). Protein concentration was measured using BIO-RAD Protein Assay (Bio-Rad). Samples were run in 8%, 12% or 15% SDS-polyacrylamide gels (Bio-Rad) under reducing conditions according to the manufacturer's instructions. Transfer of proteins to nitrocellulose membranes (Thermo) was carried out using standard protocols . Proteins were detected by immunoblotting with relevant antibodies and the ECL chemiluminescence detection system (Amersham Biosciences) according to the manufacturer's instructions. Primary antibodies against mouse TNFα (Abcam), LAMP-2 (Abcam), β-actin (Calbiochem) or α-Tubulin (Sigma) were commercially obtained. The intensity of each protein band was determined using Image Analysis, Quantity One (Bio-Rad) and normalized against β-actin of α-tubulin.

Ex vivo bone marrow cells differentiation

Bone marrow cells were collected from femurs of 4-8 week old C57BL/6J Lep$^{+/+}$ mice. Cells were washed in basic bone marrow culture medium, (MEM-α with 10% FBS, 1% Non-essential amino acids (Invitrogen) and 1% penicillin (Invitrogen)). Cells were counted and plated at 50-60 million cells per 175 ml tissue culture flask in basic bone marrow medium at 37 C, 5% CO_2. After two days in culture, non-adherent cells were collected, counted and plated in 25 mm tissue culture dishes at ~1.5 million cells per plate in 2 ml of culture medium supplemented with human M-CSF (30ng/ml) (R&D Systems). After two days of differentiation in the presence of M-CSF, media was removed and fresh culture medium supplemented with M-CSF (30ng/ml) was added. Porous tissue inserts (BD Bioscience) containing 100 mg of PGAT from C57BL/6J 12 week old male mice were placed in the wells. Controls contained no adipose tissue. After three days of further differentiation in the presence of adipose tissue the adherent cells were analyzed by immunohistochemistry, FACS analysis and qPCR. To generate ATMs with impaired autophagy, bone marrow was

isolated from mice that carried the LysM-Cre (B6.129P2-*Lyz2tm1(cre)Ifo*/J) (Jackson Laboratory, Bar Harbor ME) and the homozygous floxed allele *Atg7 fl/fl .* Lysosome function was inhibited by incubating differentiated cells with differentiation medium containing either chloroquine (20 μM), bafliomycin A (10nM or 20nM) for 12 hours.

Liposome-encapsulated clodronate preparation.

Liposomes were prepared as previously described . Briefly, 16 mg of cholesterol and 172 mg of phosphatidylcholine (Sigma-Aldrich) were dissolved in chloroform in a round-bottom flask. The chloroform was evaporated at 37°C in a rotary evaporator under vacuum until a thin lipid film formed. Two grams of clodronate (dichloromethylenediphosphonic acid disodium salt) (Sigma-Aldrich) were dissolved in 10 ml of PBS. The clodronate-PBS solution or the control-PBS solution was added to the lipid film and shaken at 250 rpm at room temperature for 30 minutes to disperse the lipid film. The solution was sonicated at room temperature for 15 minutes until no clumps of lipid were visible at room temperature in a water bath sonicator (50 watts). The liposomes were centrifuged at 49,400 g for 1 hour and resuspended in 5 ml of PBS.

Ex vivo adipose tissue studies.

Adipose tissue was removed from perigonadal fat pads of male lean (C57BL/6J $Lep^{+/+}$) or obese (C57BL/6J $Lep^{ob/ob}$) mice. Under sterile conditions, the tissue was cut into small pieces (<10 mg) in DMEM supplemented with 1% penicillin. Minced samples were centrifuged for 1 minute at 400 g at room temperature. The buoyant tissues were collected and cultured in 100 mg per ml of explant culture medium (DMEM with 10% FBS, 1% penicillin and 2% FA-free BSA) at 37°C in a 5% CO_2 incubator for 6 hours, after which the tissue was placed in fresh culture medium. After an additional 20 hours the medium was replaced with fresh medium with and without 20 uM chloroquine. To assess the rate of NEFA and glycerol release, medium was removed and the explants washed three times in PBS; the explants were cultured in fresh medium of DMEM containing 2% FA-free BSA for 2 hours. [NEFA] and [glycerol] were measured in the medium using enzymatic colometric kits (NEFA - Wako Diagnostics; glycerol - Sigma-Aldrich).

Adipose tissue explants depleted of macrophages were cultured with liposome-encapsulated clodronate as previously described . Briefly after 6 hours of culture, fresh medium with 10% liposomes containing either clodronate or PBS was added to explants. They were maintained for 20 hours at 37°C in a CO_2 incubator, then wash three times with PBS. The media was replaced with DMEM containing 10% FBS, 1% penicillin and 2% FA-free BSA with and without 20 uM chloroquine. The explant samples were cultured for an additional 20 hours and [NEFA] and [glycerol] measured in the medium as above.

Electron microscopy

Following sacrifice, animals were perfused with using 4% paraformaldehyde in 1M phosphate buffer at room temperature at least 15 minutes until the perfusion completed.. Adipose tissue is removed and further fixed with 2.5% glutaraldehyde in 0.1M Sorenson's buffer (pH 7.2) more than 48 hours at 4°C. Tissues were postfixed in 1% OsO4 also in Sorenson's buffer for one hour. After dehydration tissue is embedded in Lx-112 (Ladd Research Industries, Inc.). 60nm thick sections were cut on the MT-7000 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined under a JEOL JEM-1200 EXII electron microscope. Images were captured with an ORCA-HR digital camera (Hamamatsu) and recorded with an AMT Image Capture Engine.

Reference: Principles and Techniques of Electron Microscopy. Biological Applications, Third Edition, M.A. HAYAT

In Vivo lysosome inhibition experiments

Twelve week old obese C57BL/6J *Lep* $^{ob/ob}$ male mice were fasted for four hours and then anesthetized with ketamine (80 mg/kg, ip) and xylazine (10 mg/kg, ip) and placed in the supine position on a warm heating pad . The shaved abdomen was sterilely prepped with a betadine solution and the perigonadal adipose tissue depots were identified without compromise of the peritoneum. An evenly divided dose of 5 mg/kg of chloroquine (Sigma) in sterile PBS or PBS alone were injected into each PGAT. Mice recovered overnight with free access to food and water. 20 hours after surgery mice were once again fasted for four hours. Serum was collect to measure [NEFA] and [glycerol] after initial four hour fast (but prior to treatment) and after final fast.

Human adipose tissue

Twenty obese (BMI ranging from 34.8-61.3 kg/m2) Caucasian women with a mean age of 40.5 (26-50) years who were scheduled for Roux-en-Y gastric bypass (RYGB) surgery were included. Patients were eligible if they had no Diagnostic and Statistical Manual of Mental Disorders (DSM) IV diagnosis, were older than 18 years, understood the objective of the study, and gave informed consent. Biopsies of the mesenteric fat compartment were taken at the beginning of the surgical procedure after an overnight fast. The control group consisted of age and gender matched female subjects scheduled for elective cholecystectomy. They were lean (BMI 20-24.4 kg/m2) and had a normal glucose tolerance test, HOMA-IR and HbA1C. The samples were snap frozen in liquid nitrogen, and thereafter stored in -80ºC for subsequent analysis. The women participated in a study on the short term metabolic effects of bariatric surgery. The study was approved by the Medical Ethical Committee of the Academic Medical Center, (AMC) Amsterdam

Statistics

Data are presented as mean ± SD. All *P* values were calculated using 2-tailed distribution, 2-sample unequal variance Student's *t* test. All calculations were performed using Microsoft Excel and Statistica (Statsoft Inc.).

SUPPLEMENTAL REFERENCES

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