SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Lipoproteins

LDL was isolated from fresh human plasma by preparative ultracentrifugation as described (1). LDL was vortex aggregated for 30 sec (2). Levels of endotoxin in the LDL isolated from human plasma, quantified using a chromogenic limulus amebocyte lysate endotoxin assay kit (GenScript), were significantly below those necessary for activation of TLR4 (typically <0.05 ng/ml). acLDL and oxLDL were purchased from Alfa Aesar.

For analysis of plasma lipoproteins, samples (100 µl of pooled plasma from four mice) were separated by size-exclusion chromatography on a Superose 6 10/300 GL column (GE Healthcare) at a flow rate of 0.5 ml/min at 4°C in a buffer containing PBS, pH 7.4, 1 mM EDTA. Eluate was collected in 500-µl fractions. For lipoprotein analyses, fractions corresponding to different lipoproteins species were identified by measuring the cholesterol content of fractions 13–48 using the Cholesterol E kit (Wako).

Bone marrow isolation

Donor bone marrow was collected from the fibulas and tibias of 12-week-old, chow-fed WT or *Grn*$^{-/-}$ mice. Marrow was flushed with DMEM media supplemented with 2% FBS and 10 U/ml heparin. Cells were pooled in a Falcon tube and triturated before passing through a 40-µm cell strainer. Cells were pelleted at 600 x *g* for 7 min, incubated with red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 10 µM EDTA) for 5 min on ice, washed twice with DMEM media without serum, and resuspended in DMEM media without serum. 5 x 10^6 cells were immediately transplanted via tail vein injection in 200 µl of high-concentration glucose DMEM media without serum.

Plasma assays

Blood glucose was measured from tail blood using a glucometer (Freestyle). Plasma progranulin levels were measured by a sandwich ELISA (3) with a rabbit polyclonal, anti-mouse progranulin antibody (capture antibody), which recognizes an epitope between amino acids 198–214 (4), and a sheep polyclonal, anti-mouse antibody (detection antibody) (R&D Systems, AF2557). Plasma cytokine levels were measured using a multiplexed, antibody-based electrochemiluminescence assay from Meso Scale Discovery (mouse pro-inflammatory 7-plex ultra-sensitive kit, K15012C-1). White blood cell levels were measured after 8 weeks of western diet feeding. Mice were fasted overnight, and 50 µl of tail blood was collected into EDTA-containing tubes. Blood-cell counts were determined by an automatic analyzer (HEMAVET, Drew Scientific). Metabolic parameters were measured in mice that were fasted overnight. After 10 weeks on a western diet, blood was collected via heart puncture into EDTA-containing tubes (5 mM EDTA final). Blood was kept on ice until centrifugation at 3,000 rpm for 7 min at 4°C to obtain plasma. Plasma total cholesterol and triglycerides were measured by colorimetric assays

(Cholesterol E, Wako, and Triglyceride Infinity, Thermo Scientific). Measurements of plasma progranulin, cytokines, and lipids were determined in technical duplicates.

Macrophage isolation

BMDMs were isolated from femurs and tibias of age-matched, chow-fed WT mice and *Grn*$^{-/-}$ mice (Figs. 3, 4, S2, and S3) or *Grn*^{R493X} knock-in mice (Figs. 5 and S4). Bone marrow was flushed with DMEM/F12 media (Thermo Fisher), and cells were passed through 0.40-µm nylon cell strainer (Corning Falcon) and pelleted at 1200 rpm for 5 min at RT. Cells were resuspended and grown for 7 days in DMEM/F12 supplemented with 20% heat-inactivated FBS (Hyclone), 20% v/v L929 conditioned medium, 100 U/ml penicillin, 100 µg/ml streptomycin. Fresh media was added on day 3 or 4 of culture.

Real-time qPCR

Total RNA was isolated from 100 µl of whole blood using the RNeasy Protect Animal Blood kit (Qiagen), or from mouse liver (100 mg) and BMDMs (triplicate wells) using the RNeasy Mini kit (Qiagen). RNA was treated with DNAse I (Qiagen) and reverse-transcribed to obtain cDNA. qPCR was performed with SYBR Green Master Mix (Applied Biosystems) with a Bio-Rad CFX96 touch real-time PCR detection system (Figs. 3E and S3) or an Applied Biosystems Prism 7900HT sequence detection system (Fig. 1B). Primers for each gene (available upon request) were validated by analyzing template titration and dissociation curves. Results for qPCR were normalized to the housekeeping genes *Cyclo* or *Gapdh*, and evaluated by comparative CT method. mRNA levels are expressed relative to the saline-treated WT samples.

Immunofluorescence

BMDMs were plated on glass-bottom 12-well plates (Cellvis) at a density of 5 x10^5 cells per well. On the following day, cells were fixed with 4% paraformaldehyde for 45 min at room temperature. Cells were subsequently stained with a rat anti-LAMP1 antibody (1:500, Developmental Studies Hybridoma Bank, clone 1D4B) and a sheep anti-progranulin antibody (1:200, R&D Systems, AF2557), followed by goat anti-rat IgG Alexa568 and donkey anti-sheep IgG Alexa488 (1:1000, Thermo Fisher). Cells were counterstained with Hoechst (1:5000). Images were acquired using a spinning disk confocal (Yokogawa 491 CSU-X1) set up on a Nikon 492 Eclipse Ti inverted microscope with a 20x objective (Nikon) and an iXon Ultra 897 EMCCD camera (Andor). Cellular LAMP1 fluorescence intensity was determined using CellProfiler with >90 cells per genotype.

Foam cell formation

For the experiment shown in Fig. 4B, macrophages were plated onto poly-D-lysine coated glass-coverslip bottom dishes overnight and left untreated, or incubated with agLDL (250 µg/ml) or acLDL (50 µg/ml) for 12 h at 37°C and 5% CO_2. Cells were fixed with 3% PFA for 20 min and washed with PBS. They were subsequently stained with LipidTOX red (1:1000, Thermo Fisher) for 15 min at room temperature to stain neutral lipids, and washed with PBS. Images were acquired with the confocal microscope described above using a 40x 0.8 NA objective. For image quantification, MetaMorph software was used. Images were thresholded to exclude

fluorescence signal not associated with lipid droplets prior to quantification of total integrated LipidTOX red signal per field.

For the experiments shown in Fig. S3, macrophages were plated in 4-well glass chambers (MatTek) at a density of 1 x 10^6 cells per well in serum-containing media. The cells were switched to phenol-free, serum-free DMEM/F12 media treated with or without 25 µg/ml human oxLDL (Thermo Fisher, NC9892467), 50 µg/ml acLDL, or 500 µM oleic acid (conjugated to BSA) in serum-free media for 14 h. For cells in panel C, LysoTracker Green (1:20,000, Thermo Fisher) was also added at this time. After overnight treatment, LipidTOX 594 (1:1000, Thermo Fisher) was added to visualize lipid droplets, and cells were immediately imaged by confocal microscopy.

For biochemical lipid measurements, macrophages were plated in 6-well plates at a density of 2 x 10^6 cells per well in serum-containing media. After allowing the cells to attach, the media was switched to serum-free media for 3 h. Cells in duplicate wells were treated without or with 25 µg/ml oxLDL, 50 µg/ml acLDL, or 500 µM oleic acid (conjugated to BSA, 3:1 molar ratio oleic acid:BSA) in serum-free media for 20 h. After rinsing cell monolayers twice with PBS, lipids were extracted by shaking with 1 ml per well of hexane:isopropanol (3:2, v/v) for 10 min at RT, twice. Solvent was collected, pooled, and dried under nitrogen gas. After lipid extraction, proteins were extracted by adding 500 µl per well of 0.3 N NaOH + 1% SDS and shaking for 2–3 h at RT. Protein concentration was determined with the *DC* protein assay kit (Bio-Rad). Lipids were resuspended in chloroform and spotted on a TLC plate (Whatman, WC4865-821). Neutral lipids were resolved in a solvent system containing hexane:diethyl ether:acetic acid (80:20:1, v/v/v). To visualize lipids, the TLC plate was dipped in 10% cupric sulfate in 8.4% phosphoric acid and charred at 140°C for 10 min. Lipids were quantified by densitometry of the charred TLC plate with ImageJ. Data were normalized by protein content.

Delivery of lysosomal contents

Lysosome labeling of macrophages plated on poly-D-lysine coated glass-coverslip bottom dishes was accomplished via an overnight pulse with 1 mg/ml biotin-fluorescein-dextran. Cells were chased for 2 h in medium and subsequently incubated with streptavidin-Alexa546-agLDL for 90 min. Next, cells were incubated with 200 µM biotin in media for 10 min to bind any unoccupied streptavidin sites prior to cell permeabilization. Cells were then fixed with 1% PFA for 15 min, washed, and permeabilized with 1% (v/v) Triton X-100 in PBS containing 200 µM biotin for 10 min to remove unexocytosed lysosomal contents. Images were acquired using a Zeiss LSM 510 laser scanning confocal microscope with a 40x 0.8 NA objective. For image quantification, MetaMorph software, Universal Imaging/Molecular Devices Corporation was used. All images subjected to comparative quantification were acquired on the same day, using the same microscope settings. Each experiment was repeated at least three times. For every experiment, >30 randomly chosen fields with a total >200 cells per condition were imaged and subjected to quantification. To quantify the amount of lysosome exocytosis, we obtained a single plane for each field at wavelengths appropriate for streptavidin-Alexa546-agLDL (red) and biotin-fluorescein-dextran (green). We determined a threshold in the red channel (agLDL) that would include nearly all of the observable agLDL in the images. We then measured the total fluorescein fluorescence intensity within the thresholded area for each field. We used the same threshold level for each image within an experimental data set. By this procedure, the total

fluorescein signal intensity within the thresholded regions per field was measured. Data were normalized by the amount of biotin-fluorescein-dextran delivered to lysosomes, as determined by confocal imaging of non-permeabilized cells.

Actin measurements

To visualize F-actin, macrophages plated on poly-D-lysine-coated glass-coverslip bottom dishes were incubated with Alexa546-agLDL for 1 h, washed with PBS and fixed for 20 min with 3% PFA. Cells were subsequently washed with PBS, and incubated with 0.02 U/ml of Alexa488-phalloidin in 0.5% (w/v) saponin in PBS for 1 h at room temperature. Images were acquired using a Zeiss LSM 510 laser scanning confocal microscope with a 40x 0.8 NA objective. For image quantification, MetaMorph software was used. All images subjected to comparative quantification were acquired on the same day using the same microscope settings. Each experiment was repeated at least three times, and >100 cells were examined per condition in each experiment. The procedures used to quantify the amount of F-actin in the vicinity of agLDL were described in detail (5). Briefly, we obtained stacks of confocal images for each field at wavelengths appropriate for Alexa546-agLDL (red) and Alexa488-phalloidin (green). We determined a threshold in the red channel (agLDL) that would include nearly all of the observable agLDL in the images. We then measured the total Alexa488-phalloidin fluorescence intensity within the thresholded area for each field. By this procedure, the total phalloidin fluorescence intensity within the thresholded regions per field touching agLDL was measured.

References

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Supplementary Table

Table S1. Plasma Parameters and Blood Cell Counts

	Tx-WT (n=13)	**Tx-KO** (n=12)	***p***
Metabolic Parameters			
Body weight (g)	24.0 ± 2.3	24.2 ± 2.0	0.72
Blood glucose (mg/dl)	80.3 ± 10.4	80.6 ± 11.5	0.90
Inflammatory Mediators (pg/ml)			
TNF	3.1 ± 0.9	2.4 ± 0.4	0.04*
IL-1β	4.0 ± 1.8	3.4 ± 2.2	0.28
IL-6	50.3 ± 18.7	44.9 ± 18.3	0.60
IL-10	65.7 ± 49.2	44.2 ± 13.2	0.15
CXCL1/KC	157.2 ± 6.4	140.2 ± 43.9	0.58
IL-12 p70	264.6 ± 193	186.4 ± 71.7	0.21
Leukocytes (10^3/µl)			
White blood cells	13.22 ± 2.7	14.5 ± 3.57	0.56
Neutrophils	1.93 ± 0.76	1.79 ± 0.60	0.72
Lymphocytes	10.86 ± 2.1	12.33 ± 2.92	0.31
Monocytes	0.38 ± 0.13	0.39 ± 0.20	0.80
Eosinophils	0.042 ± 0.02	0.038 ± 0.039	0.15

All measurements are from *Ldlr*$^{-/-}$ mice transplanted with WT or *Grn*$^{-/-}$ bone marrow (Tx-WT and Tx-KO, respectively) after 8 weeks of western diet feeding. Values are mean ± SD; *p<0.05, as determined by Mann-Whitney U test.

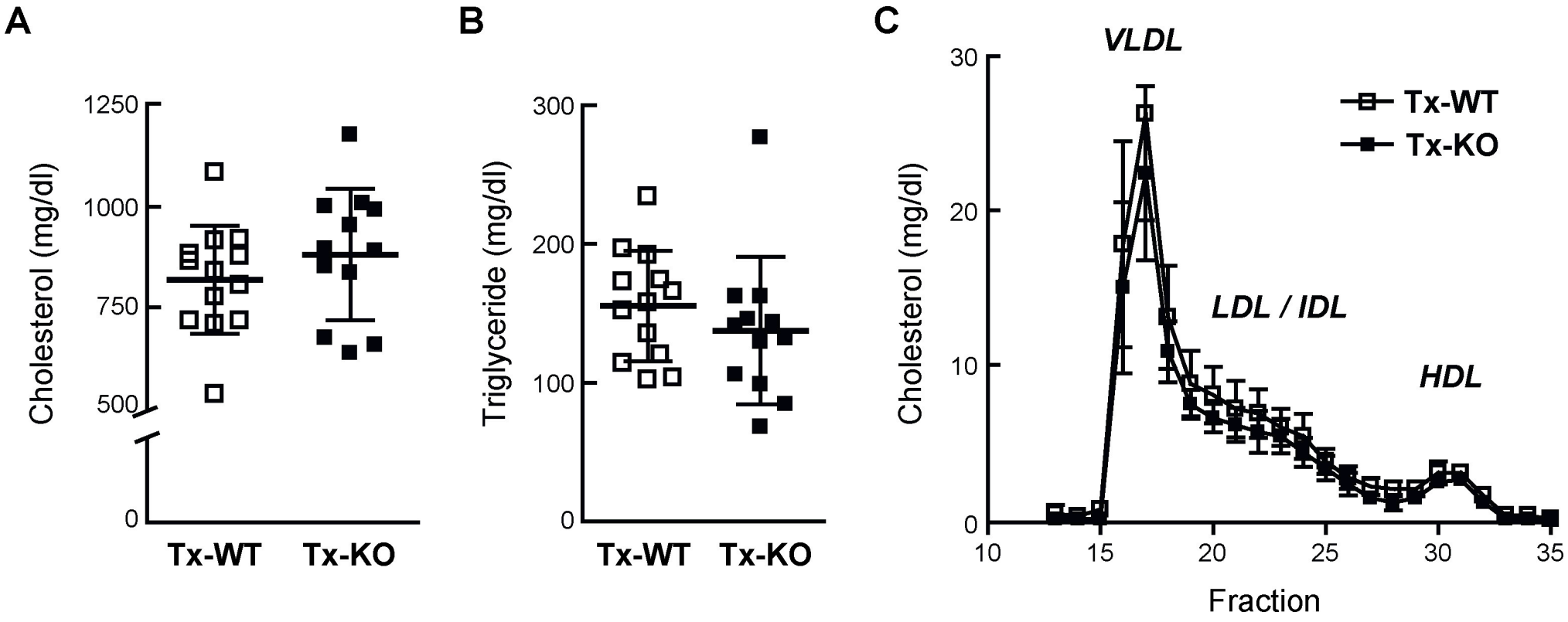
Supplementary Figure Legends

Fig. S1. Similar levels of plasma lipids in *Ldlr*$^{-/-}$ mice transplanted with WT or *Grn*$^{-/-}$ bone marrow.
(A–B) Plasma cholesterol (A) and triglyceride (B) levels in Tx-WT and Tx-KO mice after 10 weeks of western diet feeding (n=12–13 mice per group). (C) Cholesterol distribution in plasma lipoproteins separated by fast protein liquid chromatography (n=3 pools of 4 mice each). LDL, low-density lipoprotein; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; VLDL, very low-density lipoprotein. Values are mean ± SD.

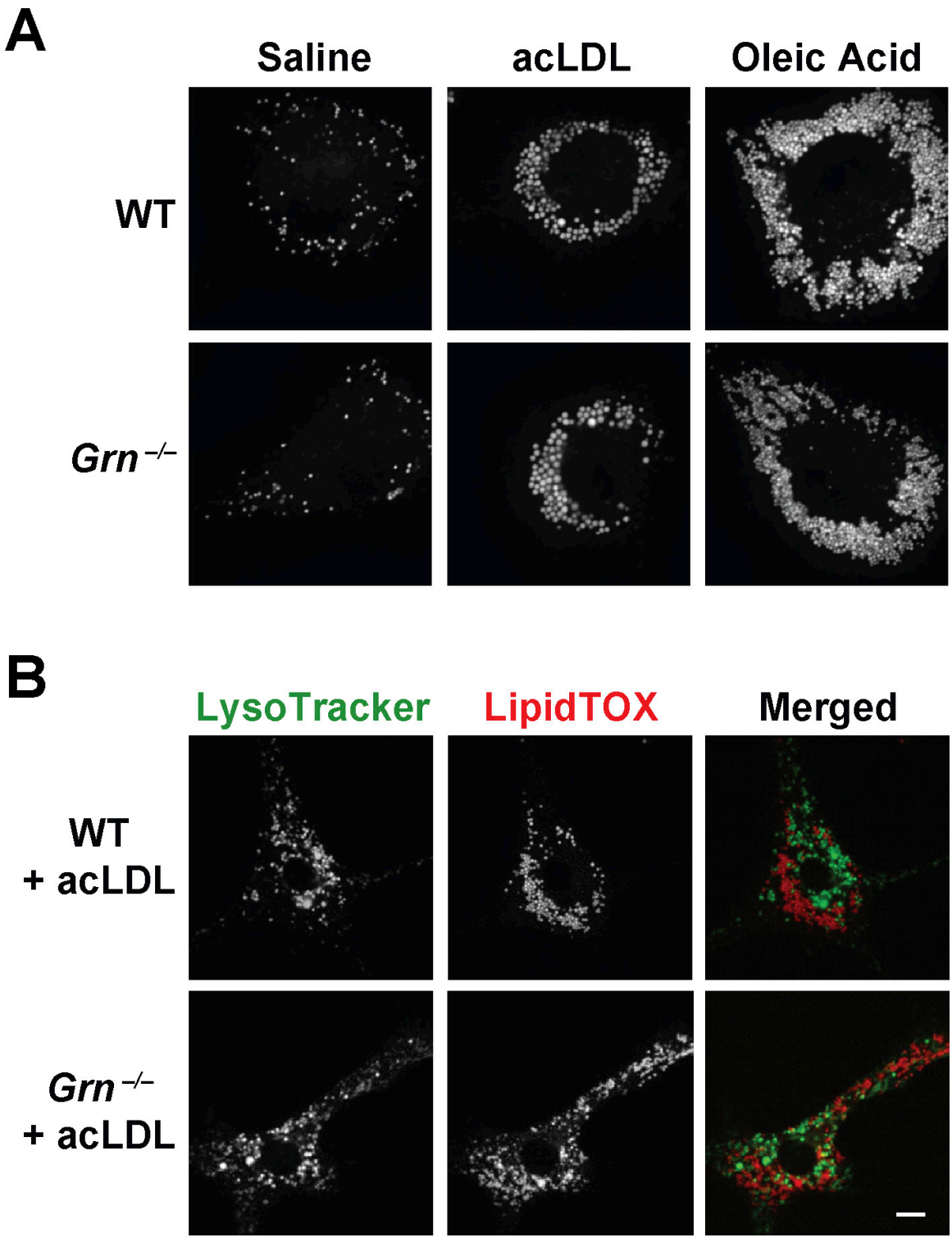
Fig. S2. *Grn*$^{-/-}$ macrophages take up and store acLDL and oxLDL normally *in vitro*.
(A) Similar neutral lipid levels in WT and *Grn*$^{-/-}$ BMDMs after treatment with 25 μg/ml oxLDL, 50 μg/ml acLDL, or 500 μM oleic acid for 14 h. Lipids were analyzed by imaging with the neutral lipid dye BODIPY. (B) Double-staining with LysoTracker (green) and LipidTOX (red, neutral lipids) indicates no accumulation of neutral lipids in lysosomes of *Grn*$^{-/-}$ macrophages. Scale bar, 5 μm. acLDL, acetylated LDL.

Fig. S3. agLDL does not trigger a pro-inflammatory response in cultured macrophages.
WT and *Grn*$^{-/-}$ BMDMs were treated with agLDL (250 μg/ml) or acLDL (100 μg/ml) for 7 h, and gene expression was determined by qPCR. Values are mean ± SD. ****p<0.0001, as determined by two-way ANOVA with Tukey post hoc test. n.s., not significant.

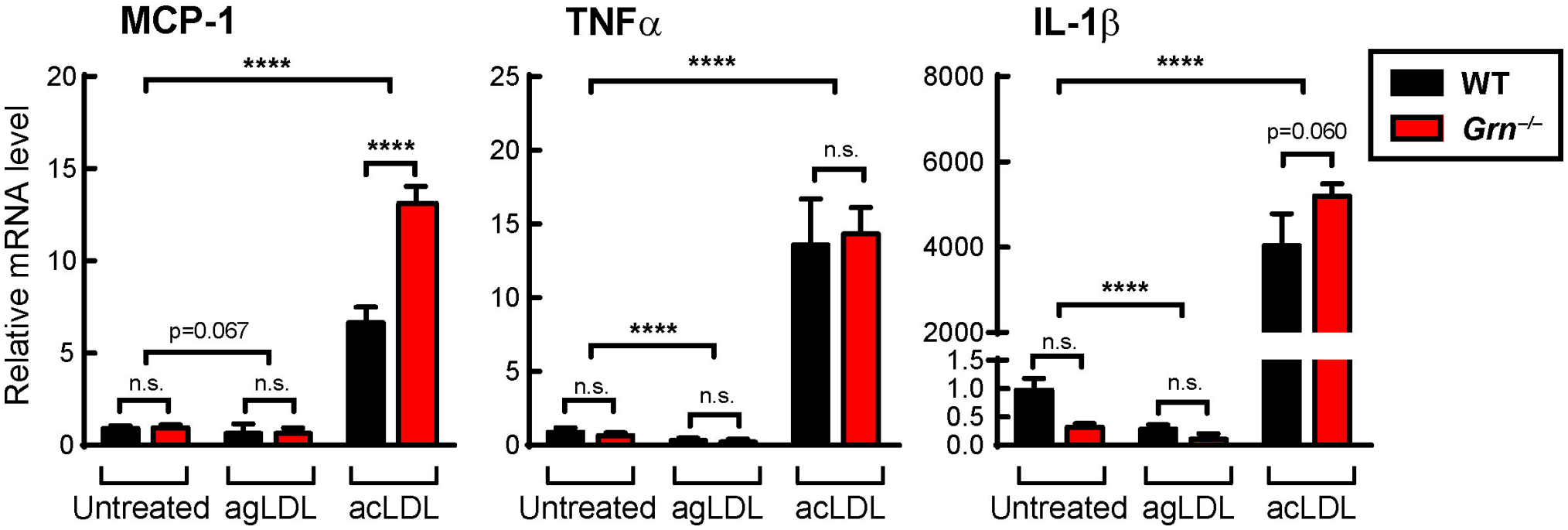
Fig. S4. Lysosome loading of biotin-fluorescein-dextran is marginally decreased in *Grn*$^{-/-}$ macrophages.
WT and *Grn*$^{-/-}$ BMDMs were incubated in media containing biotin-fluorescein-dextran (1 mg/ml) overnight, followed by a 2-h chase. Lysosome loading of biotin-fluorescein-dextran was assessed by confocal microscopy, and the fluorescence per field was quantified (right). Values are mean ± SD. ***p<0.001, as determined by student's t test. Scale bar, 20 μm.



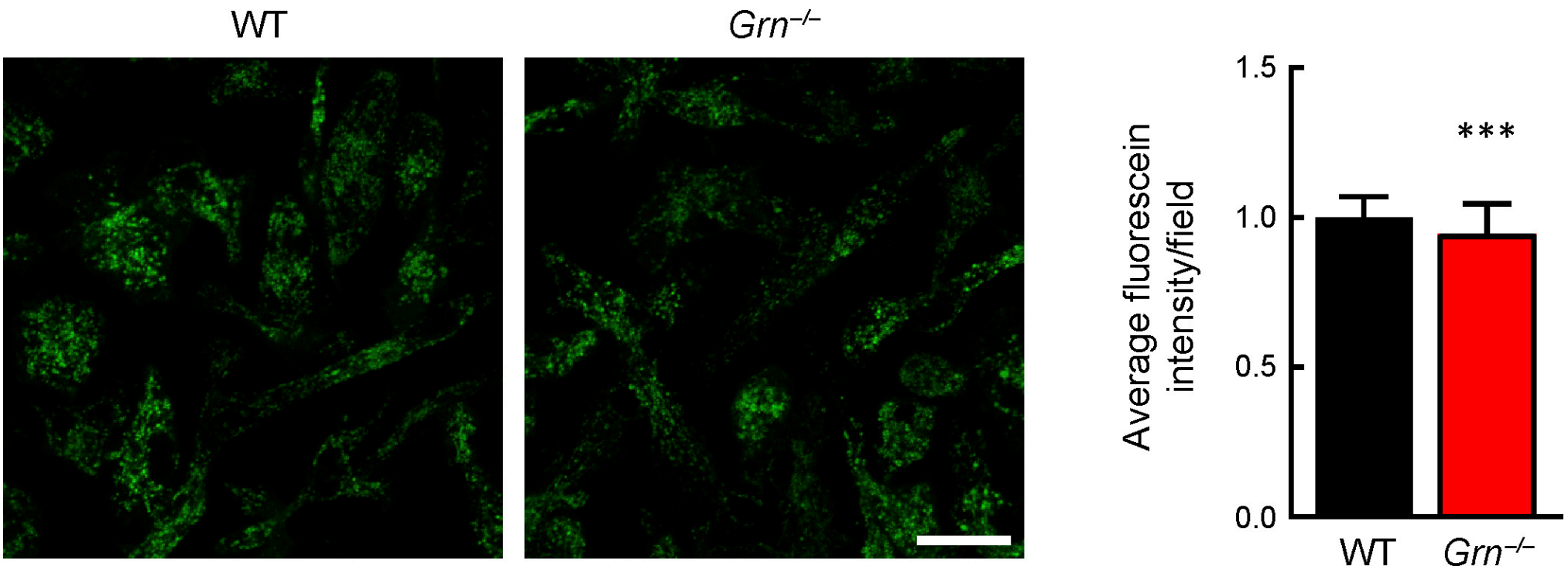
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4