**Basic Sciences**

Induction of Lysosomal Biogenesis in Atherosclerotic Macrophages Can Rescue Lipid-Induced Lysosomal Dysfunction and Downstream Sequelae

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**Objective**—Recent reports of a proatherogenic phenotype in mice with macrophage-specific autophagy deficiency have renewed interest in the role of the autophagy-lysosomal system in atherosclerosis. Lysosomes have the unique ability to process both exogenous material, including lipids and autophagy-derived cargo such as dysfunctional proteins/organelles. We aimed to understand the effects of an atherogenic lipid environment on macrophage lysosomes and to evaluate novel ways to modulate this system.

**Approach and Results**—Using a variety of complementary techniques, we show that oxidized low-density lipoproteins and cholesterol crystals, commonly encountered lipid species in atherosclerosis, lead to profound lysosomal dysfunction in cultured macrophages. Disruptions in lysosomal pH, proteolytic capacity, membrane integrity, and morphology are readily seen. Using flow cytometry, we find that macrophages isolated from atherosclerotic plaques also display features of lysosome dysfunction. We then investigated whether enhancing lysosomal function can be beneficial. Transcription factor EB (TFEB) is the only known transcription factor that is a master regulator of lysosomal biogenesis although its role in macrophages has not been studied. Lysosomal stress induced by chloroquine or atherogenic lipids leads to TFEB nuclear translocation and activation of lysosomal and autophagy genes. TFEB overexpression in macrophages further augments this prodegradative response and rescues several deleterious effects seen with atherogenic lipid loading as evidenced by blunted lysosomal dysfunction, reduced secretion of the proinflammatory cytokine interleukin-1β, enhanced cholesterol efflux, and decreased polyubiquitinated protein aggregation.

**Conclusions**—Taken together, these data demonstrate that lysosomal function is markedly impaired in atherosclerosis and suggest that induction of a lysosomal biogenesis program in macrophages has antiatherogenic effects. *(Arterioscler Thromb Vasc Biol. 2014;34:1942-1952.)*

**Key Words:** atherosclerosis ■ autophagy ■ inflammasome ■ lipid metabolism ■ lysosomes ■ macrophages

Every year over 30% of all deaths in the United States are attributable to cardiovascular disease stemming from myocardial infarction, stroke, or ischemic heart failure.1 Progressive plaque formation, or atherosclerosis, is the pathogenic mediator of the vast majority of such cases and is primarily caused by the failure of the vascular system to handle increased circulating lipid. Currently, the mainstay of treatment focuses on preventing lipid accumulation through use of drugs, such as statins. However, no treatment strategy directly addresses the pathogenic signaling processes that occur in atherosclerosis.2

As lipid is delivered to the vessel wall, cholesterol clearance is largely handled by macrophages owing to their immense phagocytic capacity. In cases of lipid abundance, macrophage cholesterol stores can rise significantly leading to cellular dysfunction, local inflammation in the vessel wall, and the development of instability in plaque architecture.3 It is believed that atherosclerotic progression and the resulting inflammatory response can be ameliorated if plaque macrophages handled lipid more efficiently.

The autophagy-lysosomal system is a highly evolutionarily conserved cellular process with critical roles in the degradation and recycling of long-lived/damaged intracellular material including accumulated lipids.4,5 Recent work has shown that although macrophages normally display a robust autophagic response, foam cell macrophages appear to develop a dysfunction in autophagy.6 This deficiency contributes to a hyperinflammatory state and abnormalities in lipid trafficking.

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Leading to dramatic increases in atherosclerosis. Given the essential role of lysosomes in mediating the overall degradation capacity of cells including autophagosome processing, it is possible that progressive dysfunction in the lysosomal apparatus itself underlies such deleterious effects in atherosclerotic macrophages.

Several lines of evidence raise the possibility that lysosomal dysfunction is a critical step in foam cell formation and plaque development. Genome-wide association studies have correlated polymorphisms in lysosomal acid lipase (LIPA), the enzyme responsible for hydrolyzing cholesterol esters, with atherosclerotic progression. Using microscopy, Jerome and colleagues have shown that in early atherosclerosis, lipid flux is maintained such that cholesterol esters are effectively hydrolyzed in the lysosome and shuttled to cytoplasmic stores as lipid droplets. With disease progression, this process begins to break down, leading to inefficiencies in lysosomal degradative capacity and eventual dysfunction. Most recently, cholesterol crystals (CC) either derived extracellularly or via intralysosomal conversion of oxidized low-density lipoprotein (oxLDL) have been shown to activate the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome by disrupting lysosomal integrity. Thus, the notion that modified lipids can have deleterious effects on lysosome function, and subsequent atherosclerosis is an attractive one deserving of investigation.

In the initial part of our studies, we use oxLDL or CC, modified lipids known to be poorly hydrolyzed in lysosomes and commonly encountered in the plaque, to recapitulate the foam cell phenotype in primary mouse peritoneal macrophages in vitro. We use several independent methods, including lysosomal morphology, pH, and proteolytic capacity, to determine the effects of such lipids on macrophage lysosome function. Subsequently, we use recently developed methods of reproducibly isolating macrophages from atherosclerotic mouse aortas to determine the extent of lysosomal dysfunction in plaque macrophages.

The recently discovered transcription factor EB (TFEB) is the only known transcription factor that drives the expression of a majority of lysosomal and autophagy genes. A member of the MiT/TFE helix–loop–helix subfamily, TFEB, initiates a lysosomal biogenesis program, thus stimulating the overall degradative capacity of cells. TFEB has also recently been demonstrated to increase lysosomal lipid catabolism, lipolysis, and cellular fatty acid oxidation. Thus, this provides an exciting new way to address whether foam cell formation and downstream sequelae can be reversed by enhancing lysosomal function. However, it has never been studied in the context of macrophage biology and atherosclerosis. In the latter part of our studies, we show that inducing a lysosomal biogenesis program is possible by overexpressing TFEB in macrophages. Furthermore, we demonstrate that this strategy has salutary effects on atherosclerotic macrophages by reducing lipid-mediated lysosome dysfunction, increasing cholesterol efflux, blunting inflammasome hyperactivation, and increasing the clearance of cytoplasmic inclusions.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Atherosclerotic Macrophages Have Morphologically Abnormal Lysosomes

The lysosome receives extracellular cargo (via endocytosis) and cytosolic material (via autophagy) for degradation. Failure of the lysosome to process its content efficiently leads to an accumulation of undigested material inside the lumen. For example, nearly all models of lysosomal storage disorders demonstrate noticeably enlarged lysosomes. Thus, an assessment of lysosomal morphology is an important first step in an evaluation of lysosomal function. We used immunofluorescence to visualize the lysosomes of peritoneal macrophages (PMACs) treated with atherogenic lipids. LAMP1 (lysosomal-associated membrane protein 1), a glycoprotein abundantly expressed on the lysosomal membrane, is a commonly used marker of lysosomal morphology. Incubation of PMACs with either oxLDL or CC for a 24-hour period caused a significant and persistent increase in the size of LAMP1+ vesicles as assessed by confocal microscopy (Figure 1A and 1B).

Atherosclerotic Macrophages Have Dysfunctional Lysosomes

Optimal lysosome function requires the ability to maintain an acidic pH and sequester a range of proteolytic enzymes operating at low pH away from the cytosol. A tool that permits the monitoring of pH-sensitive indices of lysosomal function is the lysosomotropic dye LysoTracker Red. Decreases in LysoTracker intensity relative to baseline suggest disruptions in lysosomal function, integrity, or quantity. We found that oxLDL-treated PMACs developed a progressive loss of LysoTracker signal with time (Figure 1C). Although a similar result was observed in PMACs treated with CC (Figure 1D), 2 points are worthy of mention. First, in contrast to oxLDL, the significantly larger size of CC precluded equal uptake by all cultured macrophages thus resulting in a bimodal LysoTracker distribution (ie, cells with either dramatic losses of fluorescence signal or unaffected cells comparable with untreated control; Figure 1D). Second, CC-treated macrophages developed a loss of fluorescence signal significantly more than oxLDL treatment for a similar 24-hour time-frame (Figure 1C and 1D). Because oxLDL has recently been proposed to result in increased in situ formation of intralysosomal CC and lysosome dysfunction, we also compared the lysosomal effects of longer term oxLDL incubation with those of CC. Interestingly, when cells were exposed to 72-hour
oxLDL, the effect on lysosomes was on-par with 24 hours of incubation with CC or the classic lysosomal inhibitor bafilomycin (Figure IA in the online-only Data Supplement).

Two primary factors can underlie the observed reduction in LysoTracker Red intensity after atherogenic lipid treatment: either a loss of lysosomal acidity leads to poor retention of the dye or a disruption in membrane integrity leads to lysosomal leakage and loss of lysosomes. We desired to evaluate these in the following experiments.

Atherogenic Lipids Increase Lysosomal pH

To determine lysosomal pH more accurately, we turned to a derivative lysosomotropic dye, LysoSensor Yellow/Blue. Although LysoSensor still diffuses and is selectively retained in lysosomes, it exhibits a dual emission spectra. At high pH (>6.0), the dye fluoresces at a peak wavelength of 460 nm, whereas at low pH, the peak emission is 530 nm. Fluorometric measurement of the signal intensity at both wavelengths provides an elegant method of distinguishing samples on the basis of pH level. As shown in Figure 1E, oxLDL and CC both led to reductions in the 530/460 nm fluorescence emission ratio, indicating a significant rise in lysosomal pH with atherogenic lipid treatment.

Atherogenic Lipids Increase Lysosomal Membrane Permeability

An intact lysosomal membrane is essential for the maintenance of a lysosomal proton gradient and the retention of the various intraluminal proteins and enzymes. Disruption of the lysosomal membrane has been proposed to contribute to the pathogenesis of several lysosomal storage diseases and to the activation of the inflammasome complex.13,22 We sought to measure the ability of oxLDL and CC to affect membrane porosity by fluorescence activated cell sorting (FACS) analysis of macrophages loaded with DQ-ovalbumin (10 μg/mL) and treated with oxLDL or CC. G and H, FACS analysis of PMACs loaded with DQ-ovalbumin and treated with oxLDL or CC. Mean fluorescence intensity for each peak was determined and expressed as a percentage of control (untreated cells). Representative results of ≥3 independent experiments are shown. B and E, Graphs show the mean±SEM (*P<0.05). DAPI indicates 4’,6-diamidino-2-phenylindole.

Figure 1. Atherogenic lipids alter lysosome morphology and function. A and B, Confocal microscopy of peritoneal macrophages (PMACs) loaded with oxidized low-density lipoprotein (oxLDL, 50 μg/mL) or cholesterol crystal (CC, 500 μg/mL), and stained with LAMP1 (lysosomal-associated membrane protein 1) antibody. Lysosome diameter was quantified from n=25 cells. C and D, Fluorescence activated cell sorting (FACS) analysis of PMACs treated with oxLDL or CC and stained with LysoTracker Red (200 nmol/L). E, Measurement of lysosomal pH after specified lipid treatment with 50 nmol/L LysoSensor Yellow/Blue. Changes in pH were quantified as the ratio of emission at 530 nm to emission at 460 nm (n=8–10 wells for each treatment). F, FACS analysis of PMACs loaded with 10 kDa tetramethylrhodamine (TMR)-dextran (25 μg/mL) followed by treatment with oxLDL or CC. G and H, FACS analysis of PMACs loaded with DQ-ovalbumin (10 μg/mL) and treated with oxLDL or CC. Mean fluorescence intensity for each peak was determined and expressed as a percentage of control (untreated cells). Representative results of ≥3 independent experiments are shown. B and E, Graphs show the mean±SEM (*P<0.05). DAPI indicates 4’,6-diamidino-2-phenylindole.
the lysosomal membrane, we also used larger 70-kDa dextran molecules. Significant loss of fluorescence was again seen in a portion of CC but not in oxLDL-treated macrophages (Figure IB in the online-only Data Supplement). These data suggest that lysosomal membrane integrity is predominantly affected by CC and based on the leakage of both 10- and 70-kDa dextrans, the degree of lysosomal membrane compromise seems to be significant.

Atherogenic Lipids Diminish the Proteolytic Capacity of Lysosomes

The effect of oxLDL and CC on lysosomal pH and membrane integrity would be predicted to alter the degradative capacity of lysosomes. We used FACS to measure lysosome function via fluorochrome-conjugated ovalbumin (DQ-ova). On endocytosis, DQ-ova is delivered to the late endosome/lysosome and is subject to proteolysis by lysosomal enzyme leading to a quantifiable fluorescence. Figure 1G shows loss of DQ-ova fluorescence on 24-hour incubation with oxLDL with similar but more dramatic defects seen with CC (Figure 1H). Akin to the LysoTracker experiments demonstrating comparable effects of prolonged oxLDL with shorter courses of CC (Figure IA in the online-only Data Supplement), 72-hour exposure of macrophages to oxLDL led to dramatic decreases in DQ-ova signal on-par with 24 hours of CC or bafilomycin (Figure IC in the online-only Data Supplement).

Plaque Macrophages Display Lysosome Dysfunction

The experiments performed above were an in vitro assessment of lipid-loaded PMACs. Do macrophages present in atherosclerotic plaques in vivo develop lysosomal dysfunction? To answer this question, we needed a method to isolate resident macrophages reproducibly from mouse tissue, including the aorta. Recently, the surface markers merTK and CD64 in combination have been shown to label mature resident tissue macrophages specifically.23 Thus, the use of selective antibodies to these markers can be used with FACS to isolate and study resident macrophages from any tissue of the mouse (Figure 2A).

We first used ApoE-/- mice rendered atherosclerotic after 2 months of Western diet and isolated macrophages from the spleen, liver, and aorta. LysoTracker Red signal of CD45+/merTK+/CD64+ macrophages from atherosclerotic aortas clearly showed diminished intensity when compared with spleen and liver macrophages from the same mice (Figure 2B) indicative of dysfunctional lysosomes in plaque macrophages. We then compared resident macrophages of aortas from wild-type and atherosclerotic (ApoE-null) mice fed a Western diet for 2 months. Similarly, macrophages derived from the atherosclerotic aortas displayed a reduction in LysoTracker Red intensity (Figure 2C). The observed dysfunction seemed to be a result of atherosclerotic progression as aortic macrophages from young adult (6 weeks old) ApoE-/- mice fed a chow diet (where no overt atherosclerosis can be seen) had comparable LysoTracker Red staining to spleen and liver macrophages from the same mice (Figure IIA in the online-only Data Supplement) and wild-type control aortas (Figure IIB and IIC in the online-only Data Supplement).

Lysosomal Stress Mediated by Chloroquine or Atherogenic Lipids Leads to a Compensatory Lysosomal Biogenesis Transcriptional Response

Work in the past few years has led to the discovery of the transcription factor TFEB as the only known master regulator of lysosomal biogenesis with an ability to increase the transcription of numerous lysosomal and autophagy genes in coordinated fashion.15,16 Increases in TFEB expression can directly lead to upregulation of a cohort of genes involved in autophagy/lysosome formation and the acid hydrolases involved in degradation of macromolecules.15,16 Furthermore, the induction of lysosomal stress by potent lysosomal...
inhibitors, such as chloroquine, can initiate a compensatory increase in lysosomal biogenesis via TFEB activation and nuclear translocation. Although TFEB has to date not been studied in macrophages, it would be surmised to have an important role given the highly prodegradative nature of this cell type.

In light of our data demonstrating the development of lysosomal dysfunction in atherosclerotic macrophages, we first evaluated the effects of such stressors on the lysosomal biogenesis transcriptional response. Indeed, such a response seems to be conserved in macrophages as chloroquine treatment for 3 and 12 hours leads to the induction of a panel of lysosomal and autophagy TFEB gene targets (Figure 3A, top) concomitant with nuclear translocation of TFEB (Figure 3B). Interestingly, a similar albeit more blunted response occurs after exposure of macrophages to oxLDL and CC (Figure 3A and 3B, lower panels). Because the nuclear translocation of TFEB seems to occur similarly in the presence of chloroquine and atherogenic lipids (Figure 3B), the more significant transcriptional response observed with chloroquine is unlikely to simply be a result of chloroquine’s relatively higher potency in inhibiting lysosomes. For example, incubation of PMACs with oxLDL and CC for longer periods (12 and 24 hours) demonstrates a gradual reduction in TFEB nuclear staining, suggesting desensitization (Figure III in the online-only Data Supplement), whereas TFEB seems to remain in the nucleus at 24 hours with both chloroquine and bafilomycin (Figure IV, top in the online-only Data Supplement).

**Overexpression of the TFEB Can Induce a Robust Lysosomal Biogenesis Program in Macrophages**

Given the inability of macrophages to mount a robust compensatory transcriptional response to atherogenic lipids, we desired to determine whether TFEB overexpression, a condition that is known to activate lysosomal biogenesis in other cell types clearly can rescue lipid-induced lysosomal pathology. Tissue-specific overexpression of TFEB in mice has been successfully demonstrated using a loxP-Cre method. Backcrossing these mice with ones expressing Cre under the control of Lysosomal-M promoter (LysM-Cre), we were able to obtain macrophage-specific expression of TFEB (Figure 4A). PMACs from these mice showed increases in TFEB and target-gene expression (Figure 4B). We also detected concomitant increases in LAMP1 expression by FACS analysis, suggesting a TFEB-mediated stimulation of the lysosomal pool (Figure 4C). Furthermore, in contrast to control cells, TFEB overexpression resulted in unabated translocation to the nucleus at baseline that was independent of modulation by lysosomal stressors, such as chloroquine, bafilomycin, or oxidized LDL (Figure IV in the online-only Data Supplement). Because incubation of macrophages with atherogenic lipids leads to a profound lysosomal dysfunction by FACS analysis, we first tested whether

**Figure 3.** Lysosomal stress promotes transcription factor EB (TFEB) nuclear translocation and transcriptional activation of lysosomal-autophagy genes. **A.** Quantitative polymerase chain reaction of peritoneal macrophages (PMACs) either untreated (0 time-point) or treated with chloroquine, oxidized low-density lipoproteins (oxLDL), or cholesterol crystals for 3 and 12 hours. The transcription of a cohort of autophagy and lysosomal genes is expressed as fold over untreated cells (n=2–4 wells for each treatment). Graphs show the means±SEM (P<0.05). **B.** Confocal microscopy of PMACs treated for 3 hours with the lysosomal inhibitor chloroquine (10 μmol/L), oxLDL (50 μg/mL), or cholesterol crystals (500 μg/mL), and stained with TFEB antibody and DAPI (4’,6-diamidino-2-phenylindole; nuclei). Representative results of ≥3 independent experiments are shown. LC3 indicates microtubule-associated protein 1A/1B-light chain 3; LIPA, lysosomal acid lipase.
TFEB can ameliorate this process. Figure 4D shows the effect of CC on loss of LysoTracker staining in control PMACs. In contrast, the reduction in LysoTracker fluorescence is less severe in TFEB-overexpressing (TFEB-Tg) macrophages, suggesting relative preservation of lysosomal function. We next turned our attention to a variety of functional assays aimed at interrogating the effects of TFEB overexpression in atherosclerotic macrophages.

**TFEB Overexpression Enhances Cholesterol Efflux**

A prominent sequelae of lysosome dysfunction in atherosclerotic macrophages is an impairment in cholesterol efflux. Lipophagy of cholesteryl esters present in macrophage lipid droplets followed by lysosomal hydrolysis via LIPA is a mechanism by which free cholesterol is generated and primed for cellular efflux. We loaded control and TFEB-Tg macrophages with acetylated LDL to generate foam cells and assessed the degree of cholesterol efflux to an ApoAI acceptor. Although there is little increase in cholesterol efflux in the early timepoints, TFEB leads to a significant increase in efflux at 24 hours (Figure 5A) that persists to 48 hours (Figure VA in the online-only Data Supplement). Of note, this TFEB-enhanced efflux is ApoAI dependent because incubation of loaded cells without ApoAI acceptor has no appreciable effect (Figure VB in the online-only Data Supplement). The longer time-frame is in keeping with previous data showing that cholesterol efflux generated from the lysosomal pool occurs with slower kinetics. Importantly, the enhanced cholesterol efflux clearly involves LIPA because TFEB overexpression selectively upregulates LIPA mRNA and enzyme activity (Figure 5B and 5C), whereas a well-known LIPA inhibitor Lalistat-2 (Figure VC in the online-only Data Supplement) leads to diminished cholesterol efflux in both control and TFEB-Tg macrophages (Figure 5D). Finally, we tested the effect of autophagy deficiency on TFEB’s induction of cholesterol efflux by repeating similar assays in ATG5-deficient (ATG5-KO) and dual ATG5-KO/TFEB-Tg macrophages (Figure 5E). In agreement with previous reports, ATG5 deficiency leads to reduced cholesterol efflux. Intriguingly, the absence of autophagy in TFEB-Tg macrophages blunted but did not completely abrogate the ability of TFEB to stimulate cholesterol efflux (Figure 5E). This suggests that effects of TFEB effects are likely mediated by the broader lysosomal biogenesis response that only partly includes the induction of autophagy genes.

**TFEB Overexpression Reduces Inflammasome Activation**

Recent work has shown that an intact autophagy-lysosomal system and lysosomal membrane integrity are critical factors in suppression of the inflammasome complex and production of interleukin (IL)-1β. In addition, CC in the atherosclerosis system are important activators of inflammasomes and hypersecretion of IL-1β in plaque macrophages. Given the salutary effects of TFEB on macrophage lysosomal function, we interrogated the inflammasome system in the setting of TFEB overexpression. In control PMACs, the combination of lipopolysaccharide and CC synergistically hyperactivates IL-1β production without significant effects on pro–IL-1β protein levels consistent with...
inflammasome activation (Figure 6A). In contrast, TFEB-Tg macrophages displayed dramatically lower IL-1β secretion in the presence of lipopolysaccharide and CC, again independent of pro–IL-1β modulation (Figure 6A). We noted a similar beneficial response to TFEB overexpression in macrophages exposed to lipopolysaccharide and ATP, another potent activator of the inflammasome complex (Figure 6B). As autophagy deficiency has been shown to also synergistically activate the inflammasome complex, we again desired to parse the role of TFEB in stimulating both lysosomal and autophagy genes by conducting similar assays in ATG5-KO and dual ATG5-KO/TFEB-Tg macrophages (Figure 6C). The presence of TFEB once again led to significantly diminished IL-1β secretion even in the absence of autophagy, suggesting that stimulation of TFEB of the lysosomal pool is most likely the predominant mechanism by which inflammasome signaling is dampened.

**TFEB Overexpression Reduces Inclusion Body Formation**

Finally, inclusion body formation is also a consequence of an impaired autophagy-lysosomal system. In the absence of an intact lysosomal machinery, aged and misfiled proteins aggregate in cytoplasmic precipitates that can be toxic to the cell. In support of this, treatment of peritoneal macrophages with atherogenic lipids (oxLDL and CC) can increase inclusion body formation (Figure 6D, control) likely by disrupting lysosomal function. Consistent with the induction of a prodegradative response, we find that TFEB overexpression is also able to reduce the size and number of inclusion bodies induced by atherogenic lipids (Figure 6D and 6E). Of note, in the absence of atherogenic lipids, TFEB overexpression actually results in a diffuse cytoplasmic (rather than punctuate) increase in p62 levels (Figure 6F). This rise is consistent with p62 being a transcriptional target of TFEB (Figure 4B) and is an effect distinct from the ability of TFEB to reduce the extent of inclusion bodies in atherogenic lipid-treated macrophages. Because p62 is a critical chaperone for removing protein aggregates via the autophagy-lysosomal apparatus, it is likely that transcriptional stimulation of TFEB of p62 is a necessary precursor to efficient removal of p62-enriched inclusion bodies.

**Discussion**

The autophagy-lysosomal system is crucial in the processing and clearance of endocytosed material from the cell periphery, as well as intracellular contents, including long-lived/dysfunctional organelles and proteins. We and others have shown that this system is involved in the macrophage response to lipid in the atherosclerotic plaque and that defective autophagy increases the rate of atheroma progression. Our understanding of the mechanism by which autophagy becomes dysfunctional in the plaque is rudimentary. The observation that p62 accumulates in both atherosclerotic plaques and lipid-loaded peritoneal macrophages in the absence of any changes in autophagic flux suggests that the defect does not involve autophagy per se but rather the final component of the system, the lysosome. Using a series of complementary experiments, we have shown that atherosclerotic macrophages do indeed develop lysosomal dysfunction, including lysosomal engorgement, increased lysosomal pH, decreased proteolytic capacity, and increased membrane porosity. Most importantly, we have demonstrated that TFEB,
the transcriptional master regulator of lysosomal biogenesis and function, can be harnessed in macrophages to rescue many of the downstream functional consequences. Specifically, induction of lysosomal biogenesis in macrophages rescued CC-induced lysosome dysfunction, tempered inflammasome activation, enhanced cholesterol efflux, and reduced inclusion body formation. A summary of these findings is outlined in Figure 7.

The exact mechanism by which the atherogenic lipids oxLDL and CC perturb lysosomal function is not known. Oxidized LDL is taken up by macrophage scavenger receptors and is trafficked to the endolysosomal compartment. Oxidized LDL can then bind and inactivate cathepsins with high affinity, inactivate other proteases including the Na⁺/K⁺-Gases, and produce a form of apolipoproteinB that is highly resistant to hydrolysis. OxLDL has also been demonstrated in endothelial and smooth muscle cells to inhibit activity and expression of the enzyme crucial to cholesterol ester hydrolysis, LIPA. Most recently, the formation of cholesterol microcrystals and ensuing disruption of lysosomal integrity has directly been linked to the buildup of oxLDL in the lysosomal compartment. Such a mechanism would favor the notion that the mechanism of lysosomal dysfunction mediated by oxLDL and larger CC lies in a continuum (with the oxLDL pool eventually precipitating as insoluble crystals). Our data support this premise as the degree of lysosomal dysfunction induced by prolonged exposure to oxLDL eventually matched that seen with shorter courses of CC.

Once cholesterol takes on a crystalline form in lysosomes (either by phagocytosis of exogenous material or in situ formation), the mechanism by which lysosomes are rendered dysfunctional are more apparent. Extensive studies on the lysosomal handling of other types of crystalline material have been performed. Silica crystals are known to induce lysosome permeability with the reactive crystalline surface being postulated to perforate the lysosomal membrane and leakage of its contents directly. Interestingly, the sharp edges of crystalline material have been shown to penetrate the lysosome membrane of cells within the intimal layer of atherosclerotic human coronary vessels. Our data demonstrating a reduction in tetramethylrhodamine-conjugated dextran with CC

**Figure 6.** TFEB overexpression reduces cholesterol crystal (CC)-mediated inflammasome activation and cytoplasmic inclusion body formation. **A–C,** ELISA of secreted interleukin (IL)-1β in the media of (A) control and TFEB-overexpressing (TFEB-Tg) peritoneal macrophages (PMACs) treated with lipopolysaccharide (LPS, 200 ng/mL) ± CC (500 μg/mL) for 24 hours, (B) LPS±ATP for 2 hours, or (C) ATG5-deficient (ATG5-KO) and ATG5-KO/TFEB-Tg PMACs treated with LPS±cholesterol crystals for 24 hours. Cell lysates from respective treatments in **A** and **B** were also subjected to immunoblot for pro–IL-1β. **D,** Confocal microscopy of control and TFEB-Tg PMACs loaded with CC (500 μg/mL) and stained with DAPI (4',6-diamidino-2-phenylindole) and antibodies against polyubiquitinated proteins and p62. **E,** The number of p62+ aggregates were quantified in macrophages (n=25) imaged in **D.** **F,** Total p62 levels were determined by measurement of total fluorescence intensity. Graphs in **A–C** reflect n=2 to 4 wells for each treatment, and all graphs show the mean±SEM (**P<0.05; NS, not significant). Representative results of ≥3 independent experiments are shown.
support other recent studies, where the leakage of fluorescent dextrans was linked to lysosome perforation and loss of its contents.13,14

Our data also suggest that an additional consequence of atherogenic lipid loading seems to be a loss of lysosomal acidification (or rise in lysosomal pH). Regardless of the source of lipid (ie, free cholesterol in crystalline form or that derived from the cholesteryl esters of oxLDL), it is clear that lysosomal cholesterol content increases. The accumulation of lysosomal free cholesterol can directly cause an increase in lysosomal membrane cholesterol content.38 Intriguingly, translocation and attenuation of the activity of V-ATPase, the proton transporter responsible for lysosomal acidification, is dependent on lysosomal membrane cholesterol levels.38 This could be a major contributor to the loss of acidification observed. It is important to note that we observed no significant differences in the rate of pH increase for a 24-hour period when comparing oxLDL or CC. Yet, one would have expected CC to have a significantly larger effect on lysosomal pH given their ability to also increase lysosomal porosity and destabilization. Detailed temporal comparison of the metabolism of macrophage of these lipids with correlation to pH changes could help answer some of these questions.

An exciting and promising aspect of our study is the ability of the transcription factor TFEB to induce lysosomal biogenesis in macrophages and in turn to rescue several of the deleterious responses instigated by atherogenic lipid loading. TFEB has wide ranging effects on numerous processes related to enhanced autophagy and lysosomal function.15 Importantly, TFEB is translocated to the nucleus in several models of lysosomal storage disorders and under conditions of lysosomal stress, initiating the expansion of the lysosomal compartment of the cell and the degradation of accumulated lysosomal contents.15,24,25 Our finding that atherogenic lipids lead to the nuclear translocation of TFEB and the induction of a lysosomal biogenesis transcriptional program indicates that macrophages have the capacity to undergo similar regulation as other cell types.

Taken together, these observations make TFEB an attractive therapy for alleviating conditions of lysosomal distress. For example, adeno-associated virus–mediated overexpression of TFEB provides neuroprotection and enhanced clearance of α-synuclein in an in vivo model of Parkinson disease.39 We have shown that atherosclerosis behaves as an acquired lysosomal storage disorder. It is plausible that augmentation of lysosomal degradative capacity by TFEB in macrophages of atherosclerotic plaques can lead to significantly reduced lesion size, inflammation, and instability.

Given the pleiotropic effects of TFEB on the autophagy-lysosomal system, the exact nature of the salutary effects on macrophage function remains unclear. TFEB not only increases lysosome number but also increases the expression of numerous lysosomal enzymes, including proteases such as the cathepsins and lipases such as LIPA (Figure 5B).15–18 Increased LAMP1 levels in TFEB-overexpressing macrophages (Figure 4C) would indicate an increase in the lysosome pool consistent with at least partial expansion of nascent lysosomes. However, the degree to which an increased quantity of nascent lysosomes or an increased efficiency of the current lysosome pool play a role in macrophages is difficult to ascertain. Furthermore, although autophagy and lysosomal degradation are intrinsically linked, is there an independent contribution of autophagy in mediating effects of TFEB in macrophages? Our initial evaluation of this has involved characterizing peritoneal macrophages from macrophage-specific autophagy-deficient (ATG5-KO) mice and ones with ATG5-KO in the presence of TFEB overexpression (Figures 5E and 6C). Interestingly, autophagy seemed to play only a partial role in TFEB-mediated cholesterol efflux and
a negligible role in mitigating inflammasome activation. This would support the notion that stimulation of lysosomal biogenesis imparts the more critical aspect of TFEB action than its enhancement of autophagy.

Dissecting the role of lysosomes in atherogenesis and macrophages of the atherosclerotic plaque is in its incipient stages. Our study contributes to the emerging body of literature that lysosomes lie at a critical nexus in lipid metabolism and inflammatory signaling. Additional studies of TFEB and lysosomal biogenesis in vivo are sure to generate profound insights into the biology and future therapy of atherosclerosis.

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None.

References


Lysosomes play an essential role in the degradation of both extracellular and intracellular materials. This is especially true in cells with high degradative capacity, such as macrophages. Recent studies have implicated disruptions in the macrophage autophagy-lysosomal pathway and atherosclerosis, but it is not clear how this disruption occurs during atherosclerotic progression. An attractive possibility is that endocytosed atherogenic lipids render lysosomes dysfunctional with downstream consequences. We now provide evidence for this in-cultured primary macrophages, as well as macrophages from the atherosclerotic plaque. These observations led us to hypothesize that stimulation of cellular mechanisms to increase lysosomal function/number might have salutary effects. Indeed, overexpression of transcription factor EB, the only known master regulator of lysosomal biogenesis and a prodegradative response reverses the sequelae of lipid-induced lysosomal dysfunction. These findings highlight the importance of macrophage lysosomes in the pathogenesis of atherosclerosis and provide impetus for harnessing the lysosomal biogenesis response as an atheroprotective measure.

**Significance**

Lysosomes play an essential role in the degradation of both extracellular and intracellular materials. This is especially true in cells with high degradative capacity, such as macrophages. Recent studies have implicated disruptions in the macrophage autophagy-lysosomal pathway and atherosclerosis, but it is not clear how this disruption occurs during atherosclerotic progression. An attractive possibility is that endocytosed atherogenic lipids render lysosomes dysfunctional with downstream consequences. We now provide evidence for this in-cultured primary macrophages, as well as macrophages from the atherosclerotic plaque. These observations led us to hypothesize that stimulation of cellular mechanisms to increase lysosomal function/number might have salutary effects. Indeed, overexpression of transcription factor EB, the only known master regulator of lysosomal biogenesis and a prodegradative response reverses the sequelae of lipid-induced lysosomal dysfunction. These findings highlight the importance of macrophage lysosomes in the pathogenesis of atherosclerosis and provide impetus for harnessing the lysosomal biogenesis response as an atheroprotective measure.
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Supplemental Material:

Supplemental Figures I, II, III, IV, and V
Supplemental Figure I. Comparison of oxidized LDL and Cholesterol Crystals in Altering Lysosomal Function, Membrane Permeability, and Proteolytic Capacity. FACS analysis of PMACs treated with oxidized LDL (oxLDL, 50 μg/mL), cholesterol crystals (CC, 500 μg/mL), or Bafilomycin (200 nM) for the indicated times and stained with LysoTracker Red (200 nM). (B) FACS analysis of PMACs loaded with 70 kDa TMR-dextran (25 μg/mL) and subsequently treated with oxLDL or CC. (C) FACS analysis of PMACs loaded with DQ-ovalbumin (10 μg/mL) and treated with oxLDL, CC, or Bafilomycin for the indicated times. Bar graphs show mean fluorescence intensity for each peak expressed as a percentage of control. In (A,C), control signifies CC-free cells (subpopulation with higher fluorescence intensity peak) within the CC-treated population; for (B), control signifies untreated cells.
Supplemental Figure II. Lack of Lysosomal Dysfunction in Macrophages from Young ApoE-null Aortas Without Atherosclerosis. (A,B) FACS analysis of spleen, liver, and aortic resident macrophages isolated from pooled tissue of 6-week old (A) ApoE-/- (n=3) and (B) wild type (n=3) mice on a Chow Diet and stained with LysoTracker Red (200 nM). (C) Superimposition of histograms from aortic resident macrophages isolated from 6-week old wild-type and ApoE-/- mice displayed in (A) and (B). Bar graphs show mean fluorescence intensity for each peak expressed as a percentage of LysoTracker staining in macrophages from wild-type aortas.
Supplemental Figure III. Lysosomal Stress Promotes Transient TFEB Nuclear Translocation. Confocal microscopy of PMACs treated with the lysosomal inhibitor chloroquine (10 μm), oxidized LDL (50 μg/mL), or cholesterol crystals (500 μg/mL) for the indicated times and stained with TFEB antibody and DAPI nuclear stain.
Supplemental Figure IV. TFEB Overexpression in Macrophages Leads to Persistent TFEB Nuclear Localization. Confocal microscopy of Control or TFEB-overexpressing (TFEB-Tg) PMACs treated with the lysosomal inhibitors Bafilomycin (100 nM), chloroquine (10 μM), or oxidized LDL (50 μg/mL) for 24 hours and stained with TFEB antibody and DAPI nuclear stain.
Supplemental Figure V

**A**  
**ApoAI-induced Cholesterol Efflux**

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**B**  
**Basal Efflux**

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**C**  
**LIPA Activity Assay**

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Supplemental Figure V. TFEB Overexpression Enhances Cholesterol Efflux.

(A) Cholesterol efflux to ApoA1 (100 µg/ml) in control and TFEB-overexpressing macrophages loaded with Acetylated LDL (timepoints are indicated). (B) Basal cholesterol efflux in the absence of ApoA1. (C) Lysosomal Acid Lipase (LIPA) activity from control peritoneal macrophages treated for 24 hours with increasing doses of the inhibitor Lalistat. All graphs reflect n=2-4 wells for each treatment and show the mean +/- SEM (*p<0.05).
Induction of Lysosomal Biogenesis in Atherosclerotic Macrophages Can Rescue Lipid-Induced Lysosomal Dysfunction and Downstream Sequelae

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* These authors contributed equally to this work.

Materials and Methods

Animals
Animal protocols were approved by the Washington University Animal Studies Committee. C57BL6 and ApoE-/- (C57BL/6 background) mice were purchased from Jackson Laboratories. Mice with tissue-specific transgenic expression of TFEB and ATG5-deficiency were as previously described. TFEB transgenic mice were crossed with Cre-recombinase transgenic mice under the control of the Lysosomal-M promoter (gift of Dr. Herbert Virgin, Washington University School of Medicine) and the tissue-specific ATG5-deficient mice to generate macrophage-specific TFEB overexpressing, ATG5-deficient, or dual ATG5-deficient/TFEB overexpressing mice. Animals were housed in pathogen-free barrier facility and either fed a standard chow diet or a western-type diet containing 0.15% cholesterol providing 42% calories as fat (Harlan TD 88137).

Primary macrophage culture
Mice were injected with a 4% solution of thioglycollate (Sigma T9032). 4 days post-injection, the peritoneum was lavaged with DMEM/10% FBS. The ensuing cell suspension was pelleted, washed, counted, and plated in DMEM/10% FBS to obtain peritoneal macrophages (PMACs).

Generation of Atherogenic Lipids
Cholesterol crystals were generated by ethanol precipitation of cholesterol powder as described. Briefly, cholesterol powder (Sigma C8667) was dissolved in 100% ethanol at a concentration of 10 mg/ml by heating to 60°C. Crystals were allowed to form at -20°C, sedimented by centrifugation, resuspended in PBS at 50 mg/ml, and sonicated extensively. Oxidized-LDL was generated by Cu-oxidation of LDL as described. Briefly, LDL (Sigma L7914) was dialyzed to remove EDTA, incubated with
10 \mu M CuSO$_4$ for 6 hours, dialyzed to remove excess CuSO$_4$, and diluted to a stock concentration of 1 mg/ml in DMEM.

**Assessment of Lysosomal Function by FACS**

For all experiments, PMACs were plated on low adherence plates (Greiner 665102). To assess lysosomal pH and permeability, cells were treated with oxidized LDL (50 \mu g/mL) or cholesterol crystal (500 \mu g/mL) for the indicated timepoints. Bafilomycin (200nM) was used as a positive control. Cells were rinsed with PBS and incubated with CellStripper (Corning 25056) at 37\degree for 15 minutes. Cells were mechanically dissociated with the CellStripper and collected. Any residual cells were collected after an additional incubation of the plates with 0.25% trypsin (Cellgro 25050) for 10 minutes at 37\degree. Cells were pelleted, washed twice with HBSS and resuspended in FACS buffer (HBSS with 0.6% BSA, 5mM EDTA). Cells were treated with LysoTracker Red (200mM, Life Technologies L7528) for 15 minutes at 37\degree after incubation with lipids or pre-treated with 10kDa or 70kDa tetramethyl-rhodamine (TMR)-conjugated dextran (25 \mu g/mL, Life Technologies D1817 & D1818) for 2 hours at 37\degree before incubation with lipids. Cells were washed with FACS buffer and analyzed using the BD Biosciences LSR II flow cytometer.

For the assessment of lysosomal proteolysis, PMACs were plated and treated with either oxidized LDL (50 \mu g/mL) or cholesterol crystals (500 \mu g/mL) for the indicated time-points. Two hours before collection, media was removed and replaced with media containing 10 \mu g/mL DQ-ovalbumin (Life Technologies MP12053). Cells were then collected and analyzed by FACS as above. For the assessment of lysosomal number, control or TFEB-overexpressing PMACs were plated, adherent cells were collected with CellStripper, fixed in 4% paraformaldehyde, stained with PE-conjugated anti-LAMP1 antibody (BioLegend 121612), and analyzed by FACS as above. All FACS plots were quantified using FlowJo software by determining the geometric mean fluorescence intensity for each histogram, setting the control or untreated population as 100%, and normalizing each experimental point relative to control.

**Microscopy**

Peritoneal macrophages were plated on coverslips and treated with oxidized LDL (50 \mu g/mL) or cholesterol crystals (500 \mu g/mL) for the indicated timepoints. Immunofluorescence microscopy was performed as previously described $^6$. Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with PBS/0.1% Triton X-100, blocked in PBS/0.1% Tween containing 5% goat serum, and stained with either anti-LAMP1 mAb (Abcam ab24170), anti-TFEB mAb (MyBioSource MBS120432), anti-polyubiquitinated protein mAb (clone FK2, Millipore 04-263), or anti-p62 mAb (Abcam ab56416). Coverslips were then sequentially labeled with species-specific fluorochrome-conjugated secondary antibodies and DAPI (Sigma D9542) and visualized with the Zeiss LSM700 confocal microscope. Quantification of lysosome diameter, p62 fluorescence intensity, and p62 dots were conducted using Zeiss microscope software Zen.

**Isolation and FACS of Mouse Tissue Macrophages**

2
Analyses were conducted on tissues from pooled (n=3) wild type and ApoE-null mice either in early adulthood (6-week old, chow diet) where there is no evidence of atheroma formation or after Western-type diet feeding (2 months of Western Diet starting at 2 months of age) where overt atherosclerosis spanning the aorta of ApoE-null mice is seen. Liver, spleen, and the aorta (extending from the aortic root to the abdominal aorta at the level of the renal arteries) were carefully dissected from PBS-perfused wild type and apoE-null mice, cleaned of all surrounding connective tissue and fat, and rinsed in PBS. The liver and aorta were minced and digested for 60 minutes at 37° in buffer consisting of RPMI, 2.5 μg/mL Liberase (Roche 05401127001), 125 μg/mL DNase 1 (Sigma D4527), and 0.8 mg/mL hyaluronidase (Sigma H3506). Single cell suspensions were washed and stained with the following antibodies at 1:200 concentration: CD45 (eBioscience 4805182), CD64 (BD Pharmigen 558539), biotinylated merTK (R&D BAF591), and streptavidin conjugated PerCP-Cy5.5 (BioLegend 405214). As described above, LysoTracker Red was used to label the lysosomes, samples were analyzed using the BD Biosciences LSR II flow cytometer, and quantified using FlowJo software.

**Quantitative RT-PCR and Western Blotting**

RNA was extracted using TRIzol Reagent (Life Technologies 15596). 1 μg of RNA was used to create a cDNA template (iScript cDNA Synthesis Kit, BioRad 1708891). Real time quantitative PCR was performed using PowerSYBR Reagent (Applied Biosciences 4368711) and Applied Biosystems 7500 Fast Real-Time PCR System. The following mouse forward and reverse primers were used as previously described: 7, 8.

- **TFEB** (GTCTAGCAGCCACCTGAACGT; CAGGTACACAGCCTCCATGGT),
- **Beclin** (AATCTAAGGAGTTGCCGTATAC; CCAGTGCTTCAATCTTGCC),
- **LAMP1** (ACATCAGCCCAATGACACA; GGCTAGAGCTGGCATTC),
- **LAMP2A** (CCAAATTGGGATCCTAAACCTA; TGGTCAAGCAGTGTTTATTAATTCC),
- **LAMP2B** (GGTGCTCCTTTCCGGCTTGATT; ACCACCCATACAAGAGCAGGACT),
- **LC3** (CGTCCTGGGACAAGACAGGAG),
- **p62** (CGCCTCCAGGCCAAGACAGGAG),
- **ATP6V0D2** (CAGGGTGTAATCAAATGGGAC, AGGTCTCACCAGTCACT),
- **ABCA1** (TGGCCTCCAGGCCAAGAGC, GGTCTCACCAGTCACT),
- **ABCG1** (AGGTAAAAGCCCTTCCAAG; AGTTCTTGACCCAGAGCA),
- **LXRα** (TGCTGATTGGTCTCTGCACTTGC, TGTGTTGACCCCTCTTTGGA),
- **LXRβ** (ACAGTTCTGTGGCACTCCCAG; TTAATAGTTGGTACGTGGCGGA),
- **LIPA** (CTAGAATCTGCCAGCAAGCC; AGATTCTGGCAGCCAGCC),
- **GAPDH** as control (ACTCCCTCCCTCCAGG, TCTTGTGCTGAGCTGG).

Western blotting including protein separation, transfer and the immunoblotting used to detect pro-IL-1β was performed as described using IL-1β pAb (AF-401-NA) from R&D Systems.

**IL-1β Secretion**

Peritoneal macrophages elicited from control or macrophage-specific TFEB overexpressing, ATG5-null, or dual ATG5-null/TFEB over-expressing mice were harvested and plated as described above. Macrophages were treated with
lipopolysaccharide (LPS, 200ng/mL) versus LPS + cholesterol crystals (500 µg/mL) for 24 hours or LPS versus LPS + ATP (5 mM) for 2 hours. Media was collected and levels of IL-1β secreted into the media were determined by ELISA per manufacturer's instructions (R&D Systems, MLB00C).

**Cholesterol Efflux**

$^{3}$H-labeled Acetylated LDL ($^{3}$H-AcLDL) was first made by incubating 1 mg AcLDL (Invitrogen L35354) with 25 µCi $^{3}$H-cholesterol at 37°C for 24 hours. Peritoneal macrophages were loaded with $^{3}$H-AcLDL (100 µg/ml) for 24 hours, washed in DMEM/0.2% BSA, and equilibrated in same medium for an additional 12 hours. Cholesterol efflux was initiated by incubating cells with 100 µg/ml ApoAI (Sigma A0722) for the indicated times (ranging from 6 to 48 hours). Media and cell lysates were collected and the amount of labeled $^{3}$H-cholesterol in both compartments were determined by scintillation counting. Cholesterol efflux is reported as a percentage of $^{3}$H-cholesterol in the media/(media $^{3}$H-cholesterol + cellular $^{3}$H-cholesterol).

**Lysosomal Acid Lipase (LIPA) Activity Assay**

LIPA activity was determined using the fluorogenic substrate 4-methyl-umbelliferyl-oleate (4-MUO) as previously described with some modifications. Wildtype peritoneal macrophages treated with either the Lysosomal Acid Lipase inhibitor Lalistat-2 (Gift of Dr. Paul Helquist, University of Notre Dame) or macrophages elicited from control and macrophage-specific TFEB overexpressing mice were scraped in lysis buffer (10 mM Tris pH 8; 50 mM NaCl; 1% Triton X-100) containing protease inhibitors and protein concentrations quantified using the BCA reagent (Pierce). 4-MUO was reconstituted in DMSO (100 mg/ml) and diluted 1:100 in 4% Triton X-100. A 1:3:0.2 mixture of 4-MUO substrate, assay buffer (200 mM Sodium Acetate pH 5.5), and cell lysate (200 µl final volume) was incubated for 30 minutes at 37°C, reaction stopped with 1M Tris pH 8 (100 µl), and fluorescence intensity was obtained via a fluorometer (excitation 360 nm/emission 460 nm). Enzyme activity is reported as nmol/mg of cell lysate/hr.

**Statistical Analyses**

Statistical significance of differences was calculated using the Student unpaired t test for parametric data. Graphs containing error bars show the mean +/- standard error of the mean (SEM).

**References**


5. Steinbrecher UP, Witztum JL, Parthasarathy S, Steinberg D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis*. 1987;7:135-143


