Free Cholesterol Accumulation in Macrophage Membranes Activates Toll-Like Receptors and p38 Mitogen-Activated Protein Kinase and Induces Cathepsin K


Abstract—The molecular events linking lipid accumulation in atherosclerotic plaques to complications such as aneurysm formation and plaque disruption are poorly understood. BALB/c-Apoe<sup>−/−</sup> mice bearing a null mutation in the Npc1 gene display prominent medial erosion and atherothrombosis, whereas their macrophages accumulate free cholesterol in late endosomes and show increased cathepsin K (Ctsk) expression. We now show increased cathepsin K immunostaining and increased cysteine proteinase activity using near infrared fluorescence imaging over proximal aortas of Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> mice. In mechanistic studies, cholesterol loading of macrophage plasma membranes (cyclodextrin–cholesterol) or endosomal system (AcLDL+U18666A or Npc1 null mutation) activated Toll-like receptor (TLR) signaling, leading to sustained phosphorylation of p38 mitogen-activated protein kinase and induction of p38 targets, including Ctsk, S100a8, Mmp8, and Mmp14. Studies in macrophages from knockout mice showed major roles for TLR4, following plasma membrane cholesterol loading, and for TLR3, after late endosomal loading. TLR signaling via p38 led to phosphorylation and activation of the transcription factor Microphthalmia transcription factor, acting at E-box elements in the Ctsk promoter. These studies suggest that free cholesterol enrichment of either plasma or endosomal membranes in macrophages leads to activation of signaling via various TLRs, prolonged p38 mitogen-activated protein kinase activation, and induction of Mmps, Ctsk, and S100a8, potentially contributing to plaque complications. (Circ Res. 2009;104:455-465.)

Key Words: cathepsin K ★ p38 ★ Toll-like receptor

Clinical complications of atherosclerosis usually result from the formation of thrombus on a ruptured or eroded atherosclerotic plaque.1 Whereas early fatty streak formation involves lipid accumulation in macrophage foam cells in arteries, the growth of plaques and their complications are thought to involve a modified inflammatory response comprising both innate and acquired immune systems.2 Nonetheless, LDL cholesterol lowering remains the cornerstone for the treatment of established atherosclerosis and accumulation of cholesterol and oxidized lipids likely continue to drive the inflammatory response even in advanced plaques. The molecular mechanisms linking cellular lipid accumulation and inflammatory gene expression remain poorly understood.

Murine models have been useful for analyzing the role of different genes in plaque development,3 and recently there has been intense interest in the use of Apoe<sup>−/−</sup> mice to monitor plaque complications such as intraplaque hemorrhage, or apparent rupture involving the brachiocephalic artery.4 We observed a high frequency of medial degradation and atherothrombosis associated with lesions in the proximal aorta of Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> mice,5 and the incidence was higher in mice in the BALB/c compared with C57BL6 genetic background. The Npc1 mutation results in prominent accumulation of unesterified cholesterol in late endosomes6 and peritoneal macrophages from chow-fed Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> mice displayed marked accumulation of free cholesterol (FC) and, to a lesser extent, cholesteryl ester.5 Using microarrays, we discovered that Apoe<sup>−/−</sup>, Npc1<sup>−/−</sup> peritoneal macrophages have increased expression of cathepsin K (Ctsk), as well as several matrix metalloproteinase (MMP) genes. Cathepsin and MMPs have important roles in plaque development, medial breakdown and aneurysm formation.7,8

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Cathepsin K protein (CATK) is a potent elastase and Ctsk<sup>-/-</sup>, Apoe<sup>-/-</sup> mice show reduced lesion area, increased intimal collagen accumulation and decreased medial elastin fiber disruption compared to Apoe<sup>-/-</sup> controls,<sup>9,10</sup> suggesting an important role of Ctsk in plaque growth and complications. The present study was undertaken to elucidate the mechanisms linking macrophage cholesterol accumulation to increased expression of Ctsk and various Mmps.

**Materials and Methods**

**Reagents**

Aggregated (Agg)LDL and moderately oxidized (Ox)LDL,<sup>11,12</sup> and cyclodextrin–cholesterol complex (CD-chol)<sup>13</sup> were prepared as described in the online data supplement, available at http://circres.ahajournals.org.

**Mice**

BALB/cNeter, Npc<sup>1<sup>+/+</sup></sup>/J, C3H/HeJ, Tlr<sup>4<sup>del</sup></sup> (strain C57BL/10ScNj), Tlr<sup>4<sup>-/-</sup></sup> (strain C57BL/10ScSnJ), Tlr<sup>3<sup>-/-</sup></sup> (strain 129S1/Sv/C57BL6), Tlr3<sup>+/+</sup> (strain B6129SF1/J) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). Apoe<sup>-/-</sup>, Npc1<sup>-/-</sup> mice on BALB/cJ background were bred as described.<sup>5</sup> Mouse macrophages were derived from myeloid precursor cells from spleen (see the online data supplement).

**Cell Culture**

Peritoneal and bone marrow–derived macrophages were cultured as described.<sup>17</sup> Human THP-1 and mouse RAW264.7 cells were purchased (American Type Culture Collection). Spleen-derived macrophages were derived from myeloid precursor cells from spleen (see the online data supplement).

**RNA Analysis**

Total RNA was isolated using the RNasy Mini kit (Qiagen, Valencia, Calif). Real-time quantitative PCR assays were performed as described.<sup>5</sup>

**Small Interfering RNA Knockdown**

RANK and scrambled small interfering (si)RNAs were purchased from Applied Biosystems; Toll-like receptor (TLR)3, -4, -7, and -8 were from Invitrogen. siRNA was transfected into macrophages with Oligofectamine (Invitrogen).

**Immunofluorescent Staining of Aortic Cross-Sections**

Paraffin-embedded sections of the proximal aorta were immunostained with affinity-purified rabbit polyclonal CATK antibody or mouse monoclonal α-actin antibody as described<sup>18</sup> (see the online data supplement).

**Molecular Imaging**

Ex vivo fluorescence reflectance imaging was performed using Image Station 4000 MM Pro and Molecular Imaging Software (Kodak) as described.<sup>19</sup>

**Promoter Luciferase Assay**

Human CTSK promoter (–928 to +2 bp) was cloned into a pGL3 vector (Promega) and mutated as described.<sup>20</sup> CTSK promoter-luciferase and TK-Remilla luciferase were transfected into RAW264.7 cells with Lipofectamine 2000 (Invitrogen). Cells were collected and assayed 24 hours after loading using the Dual-Luciferase Reporter assay system and a TD20/20 luminometer (Promega).

**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation (ChiP) assays were performed as described (see the online data supplement).<sup>16</sup>

**Results**

We previously found increased Ctsk expression and secretion in Npc1<sup>-/-</sup> macrophages and increased elastase activity in aortic homogenates of Apoe<sup>-/-</sup>, Npc1<sup>-/-</sup> mice.<sup>5</sup> To confirm in vivo relevance, we used an affinity-purified antibody<sup>18</sup> and found strong CATK immunostaining in the macrophage-rich regions of atherosclerotic plaques in Apoe<sup>-/-</sup>, Npc1<sup>-/-</sup> mice (Figure 1A, white arrows), but not in Apoe<sup>-/-</sup> controls. To confirm an increase in CATK activity, we performed near-infrared fluorescence (NIRF) imaging using peptide substrates that fluoresce following cleavage by cysteinyl proteinases. This procedure revealed a marked increase in signal over the proximal aortic region of Apoe<sup>-/-</sup>, Npc1<sup>-/-</sup> mice compared to Apoe<sup>-/-</sup> controls (Figure 1B and 1C).

**Cholesterol Loading of Endosomes and Plasma Membrane Induces Ctsk Expression**

Npc1<sup>-/-</sup> macrophages have a defect in the trafficking of cholesterol from late endosomes to the plasma membrane and endoplasmic reticulum (ER) and accumulate FC in late endosomes.<sup>21</sup> We used different methods of cholesterol loading in concanavalin A (ConA)-elicited Npc1<sup>+/+</sup> peritoneal macrophages to determine whether increased Ctsk expression resulted from decreased trafficking of cholesterol to the ER, or from cholesterol accumulation in late endosomes. When Npc1<sup>-/-</sup> cells take up LDL or acetylated (Ac)LDL, there is defective suppression of LDL receptors and HMG-CoA reductase; however, these SREBP target genes are effectively repressed by 25-OH cholesterol (Figure 1, A, in the online data supplement).<sup>22</sup> 25-OH-chol treatment of Npc1<sup>+/+</sup> or Npc1<sup>-/-</sup> macrophages (Figure 2A and 2B) did not reduce Ctsk expression levels suggesting that induction does not result from defective SREBP function. Liver X receptor target genes are poorly regulated in Npc1<sup>-/-</sup> cells following cholesterol loading of the endosomal system.<sup>23</sup> However, LXR activation with compound T0901317 (T0) in macrophages did not affect Ctsk expression. AcLDL loading of Npc1<sup>+/+</sup> macrophages, which results in uptake into lysosomes and re-esterification of lipoprotein-derived cholesterol in the ER,<sup>24</sup> had no effect on Ctsk expression. However, FC loading of Npc1<sup>+/+</sup> macrophages by treatment with AcLDL+ACAT inhibitor 58035 resulted in a 2-fold induction of Ctsk mRNA. Addition of U18666A, which causes a block in the exit of cholesterol from late endosomes and thus mimics many aspects of the Npc1<sup>-/-</sup> phenotype,<sup>25</sup> caused a further increase in Ctsk in FC-loaded cells, indicating that effects of FC loading reflect endosomal rather than ER cholesterol accumulation. Because AcLDL+58035 treatment results in FC loading of the ER and induces the unfolded protein response,<sup>14</sup> we also tested the effects of unfolded protein response inducers thapsigargin and tunicamycin to ensure that FC-induced ER stress was not involved in Ctsk induction. These treatments did not increase Ctsk expression (shown for tunicamycin, Figure 2A) although CHOP protein was induced (supplemental Figure I, B). These experiments
suggested that Ctsk induction results from FC loading of late endosomes rather than altered sterol-induced ER regulatory events.

Prolonged loading of human THP-1 macrophages with aggregated LDL or OxLDL causes accumulation of FC in late endosomes/lysosomes resembling the phenotype of Npc1−/− cells.11,12 This effect is not observed in mouse peritoneal macrophages.11 Loading of human THP-1 macrophages with aggregated LDL or OxLDL led to a 2- to 3-fold increase in Ctsk expression (Figure 2C). These findings further support the hypothesis that cholesterol loading of late endosomes leads to Ctsk induction and show relevance in human macrophages.

Because the Npc1 mutation results in defective trafficking of both cholesterol and glycolipids,6 and aggregated LDL and OxLDL are complex preparations containing many different bioactive lipids, we tested whether cholesterol itself could lead to induction of Ctsk. CD-chol loading of Npc1−/− macrophages resulted in a robust, dose-related induction of Ctsk (Figure 2A and 2D). Ctsk was also induced in CD-chol–loaded THP-1 macrophages (Figure 2C) but not 293 cells (data not shown). CD-chol loading similarly induced Ctsk in the presence of 58035. Thus, Ctsk induction can be directly related to the effects of FC loading. Because CD-chol loading enriches the plasma membrane with FC,21 whereas AcLDL+U18666A treatment loads late endosomes, it appears that cholesterol enrichment of either endosomal or plasma membranes can induce Ctsk.

A Role of p38 Mitogen-Activated Protein Kinase in the Induction of Ctsk

We repeated microarray experiments using Affymetrix arrays with more complete coverage of the genome. This revealed additional genes upregulated in Npc1−/− cells.11,12 We confirmed the upregulation of newly discovered genes and Ctsk with real-time PCR in peritoneal macrophages elicited in various ways and in bone marrow–derived cells. Several genes upregulated in Npc1−/− cells are known p38 targets: Ctsk, S100a8 and Mmp8 (supplemental Table). Activation of p38 was confirmed by detection of increased phospho-p38 levels in Npc1−/− macrophages (Figure 3A). Elevated phospho-p38 levels were also observed in OxLDL loaded THP-1 cells (Figure 3B) and CD-chol–loaded mouse peritoneal macrophages (Figure 3C). In a time-course study, CD-chol loading led to an early peak (~0.5 hour), a variable decrease and then a prolonged late-phase (~8 hours) of phospho-p38 induction. Interestingly, AcLDL+U18666A loading of macrophages caused predominantly late-phase p38 phosphorylation (Figure 3D), possibly attributable to a delay in hydrolysis of AcLDL CE.

We next assessed the role of p38 in Npc1−/− and cholesterol-induced Ctsk expression. Treatment of Npc1−/− cells with two different p38 inhibitors reduced Ctsk expression (Figure 4A). In
contrast, treatment with c-Jun NH2-terminal kinase (JNK) or nuclear factor (NF)-κB inhibitors increased Ctsk mRNA levels (Figure 4A and supplemental Figure II), whereas ERK inhibitors had no effect (data not shown). Prolonged treatment with the p38 inhibitor SB202190 reduced Ctsk mRNA in Npc1−/− cells by more than 50% (Figure 4B). As shown by Western blotting in macrophage cell lysates, Npc1−/− or CD-chol–induced CATK protein levels were also greatly reduced by the p38 inhibitors (supplemental Figure III). Mmp8, S100a8, and Mmp14 mRNA levels were all increased in Npc1−/− cells, and reduced by treatment with the p38 inhibitors (Figure 4C). The cells treated with inhibitors appeared healthy by microscopy and the p38 inhibitors did not affect p38 phosphorylation as expected.

To confirm a role of p38 in Ctsk induction, we used p38α-deficient macrophages obtained from p38flox/flox X Ly-5MCre mice. Basal levels of Ctsk were reduced in these macrophages, and the effects of FC loading (AcLDL+58035 or CD-chol) on Ctsk expression were abolished (Figure 4D). CD-chol loading also induced the expression of Mmp8 and S100a8 and these effects were abolished in p38−/− macrophages (Figure 4E). To determine the specificity of these p38-dependent responses, we measured transcript levels of other sterol-regulated genes (Figure 4E). Apart from a modest reduction of Abca1 mRNA in p38−/− macrophages, also previously observed in tumor necrosis factor–treated macrophages,17 the cholesterol-mediated responses of these genes were not affected by p38 deficiency. These findings indicate that a novel signaling pathway can be initiated by cholesterol accumulation in plasma or endosomal membranes, leading to activation of p38 and induction of Ctsk, Mmp8, S100a8, and Mmp14.

Cholesterol-Mediated Induction of Ctsk Requires Microphthalmia Transcription Factor
Ctsk is highly induced during macrophage differentiation into multinucleated osteoclasts. Microphthalmia transcription factor (MITF) and NFATc1 are transcription factors necessary for osteoclast differentiation, and are transcriptionally induced and regulated by p38-mediated phosphorylation.26 We measured the expression of Mitf, Nfatc1, and Ctsk following CD-chol loading over time and related these changes to p38 phosphorylation (Figure 5A). Nfatc1 and cathepsin S (Ctss) were not induced by cholesterol loading. There was an early peak of Mitf induction. The induction of Ctsk was initiated after Mitf induction and during the late-phase p38 phosphorylation. Addition of p38 inhibitor following 7 hours of CD-chol loading resulted in substantial inhibition of Ctsk induction (data not shown), suggesting that prolonged p38 activation was required for Ctsk induction. Interestingly,
time course studies showed that NF-κB and JNK activation accompanied the early phase of p38 activation (<5 hours) but were not sustained during the late phase (data not shown). Thus, NF-κB and JNK repression of \(Ctsk\) (Figure 4A) may explain the delay in \(Ctsk\) induction by CD-chol.

To assess the role of MITF in \(Ctsk\) induction, we carried out cholesterol loading of splenic macrophages from mice carrying a dominant negative mutation of \(Mitf\) (\(mi/mi\)). The \(mi/mi\) mutation abolished the response to CD-chol loading (Figure 5B). Three E boxes in the human \(CTSK\) promoter are responsible for MITF transactivation during osteoclast differentiation.\(^{20}\) Transfection of a human \(CTSK\) promoter–luciferase construct into RAW macrophages resulted in significant induction of gene expression in response to CD-chol loading, and mutations in any of the 3 E-box elements abolished the induction (Figure 5C). These experiments indicate an essential role for MITF in cholesterol-mediated \(CTSK\) induction and suggest that regulation occurs via binding of E-box elements in the promoter.\(^{20}\) This was confirmed by ChIP analysis,\(^{16}\) showing a modest increase in binding of MITF, no change in binding of PU.1, and a marked increase in binding of phospho-p38 to the \(Ctsk\) promoter in mouse splenic macrophages (Figure 5D; note binding of p38 was only detected after CD-chol loading). These findings are similar to previous studies in which receptor activator of NF-κB ligand (RANKL) was shown to induce \(Ctsk\) expression via p38-mediated phosphorylation of MITF on the \(Ctsk\) promoter.\(^{16}\)

**RANK or Small GTPase Rab Do Not Mediate \(Ctsk\) Induction**

We next carried out experiments to elucidate the signaling pathways acting upstream of p38/MITF-mediated induction of \(Ctsk\). RANKL and CSF-1 are cytokines that commit myeloid precursor cells to the osteoclast lineage.\(^{27}\) Another secreted protein, osteoprotegerin (OPG), competes with RANK for RANKL binding and blocks RANK/RANKL signaling in cells. We treated bone marrow–derived macro-
phages from Npc<sup>++/+</sup> and Npc1<sup>+/−</sup> mice with OPG to assess the potential role of RANK/RANKL signaling in cholesterol-mediated Ctsk induction. Whereas OPG abolished Ctsk induction by exogenous RANKL and CSF-1 in both Npc1<sup>++/+</sup> and Npc1<sup>+/−</sup> cells, there was no effect of OPG in untreated Npc1<sup>+/−</sup> cells (supplemental Figure IV, A). Also, OPG treatment did not affect CD-chol–induced Ctsk expression in Npc1<sup>++/+</sup> macrophages, and injection of OPG into the peritoneal cavity of ConA-injected Npc1<sup>++/+</sup> mice did not affect Ctsk induction (data not shown). Moreover, a 70% decrease in RANK mRNA by siRNA knockdown did not reduce Ctsk mRNA (supplemental Figure IV, B). These experiments suggested that the RANK-RANKL signaling pathway is unlikely to be involved in Npc1<sup>+/−</sup> or CD-chol–induced Ctsk expression. Although RabS have been suggested to rescue the lipid trafficking defect in Npc1<sup>+/−</sup> cells, overexpression of either wild-type or dominant negative forms of Rab7, Rab9, and recycling endosome–associated Rab11 did not affect Ctsk promoter activity in CD-chol–loaded RAW macrophages (data not shown). We also assessed the possible involvements of autocrine or paracrine factors in the induction of Ctsk with a media transfer experiment. However, levels of Ctsk in bone marrow–derived cells from either Npc1<sup>++/+</sup> or Npc1<sup>+/−</sup> mice were not affected by conditioned media (supplemental Figure V).

Figure 4. Npc1 mutation– or cholesterol-mediated Ctsk induction is inhibited by chemical p38 inhibition or genetic p38 deletion. mRNA levels measured in ConA-elicited peritoneal macrophages by quantitative PCR and normalized to ribosomal 36B4. A and C. Cells treated with different inhibitors (10 μmol/L) or DMSO for 24 hours. *P<0.05 vs wild type (wt)/DMSO; #P<0.05 vs Npc1<sup>+/−</sup>/DMSO. B. Cells treated with 10 μmol/L SB202190 for the indicated time. D and E. Wild-type or p38α<sup>−/−</sup> cells treated with 50 μg/mL AcLDL ±10 μg/mL ACAT inhibitor SB5835 for 18 hours, 5 mmol/L CD-chol (2.5:1, mol:mol) for 24 hours, or untreated (CTR). *P<0.05 vs wild-type/CTR. #P<0.05 vs wild-type/CD-chol, $P<0.05 vs p38α<sup>−/−</sup>/CTR.
Toll-Like Receptor Signaling Mediates Cholesterol-Induced Ctsk Expression

Multiple TLRs can activate p38 signaling.29 MyD88 and TRIF are adaptor proteins that link TLR activation to downstream mitogen-activated protein (MAP) kinase signaling.29 To evaluate the role of TLRs in cholesterol-mediated Ctsk induction, we assessed Ctsk expression in bone marrow–derived Myd88−/−, Trif−/− macrophages and wild-type controls. The expression of Ctsk and other p38 targets was reduced in untreated Myd88−/−, Trif−/− cells compared to controls, and the cholesterol-mediated induction was abolished (Figure 6A). In addition, late-phase p38 phosphorylation was abolished in Myd88−/−, Trif−/− macrophages (Figure 6B). These data indicate that TLRs or interleukin (IL)-1 receptor initiate cholesterol-induced signaling leading to sustained p38 phosphorylation and Ctsk induction. IL-1
Figure 6. TLR signaling mediates cholesterol-induced Ctsk expression. A, Reduced expression of Ctsk and other genes in basal and CD-chol-loaded bone marrow-derived macrophages from Myd88<sup>-/-</sup>, Trif<sup>-/-</sup> mice. B, Inhibition of late-phase p38 phosphorylation in Myd88<sup>-/-</sup>, Trif<sup>-/-</sup> cells compared to wild-type (C57BL/6J) controls. C, Induction of Ctsk expression by TLR3, -4, and -7 ligands in wild-type peritoneal macrophages. D, Synergistic effect of Poly (I:C) and CD-chol, but not LipidA, on Ctsk expression. *P<0.05 vs control (CTR); #P<0.004 vs C57BL/6J, same treatment. All treatments were for 24 hours.
(5 ng/mL) treatment did not induce Ctsk in mouse peritoneal macrophages. To determine whether TLR activation was sufficient for Ctsk induction, we treated mouse peritoneal macrophages with activators of various TLRs (Figure 6C). TLR3 [poly(I:C), 2.5 μg/mL], TLR4 (lipid A, the active component of lipopolysaccharide, 100 ng/mL) and TLR7 (gardiquinmod, 10 μg/mL) ligands increased Ctsk mRNA by 2- to 4-fold, whereas TLR2 (PGN) and TLR9 (CpG) ligands did not. Lipid A and poly(I:C) had additive effects on Ctsk mRNA induction (Figure 6D). Whereas lipid A did not have any additional effect on CD-chol–induced Ctsk expression, the combination of poly(I:C) and CD-chol synergistically increased Ctsk mRNA (Figure 6D). Importantly, the CD-chol preparation contained <0.125 endotoxin units/mL. Moreover, CD-chol showed greater induction of Ctsk than lipid A, whereas the induction of inflammatory genes such as IL-6 or
tumor necrosis factor by CD-chol was only approximately one-tenth of that observed with lipid A treatment, indicating that LPS contamination could not explain the Ctsk response.

To assess the role of various TLRs in Ctsk induction in Npc1−−/− macrophages, we used siRNA knockdowns (Figure 7A). As determined by real-time PCR, the knockdowns were effective (63% to 79%; legend of Figure 7) and specific for each TLR. Knockdown of TLR3 had the largest effect on Ctsk expression. However, knockdown of all TLRs localized to the endosomal system (TLR3, 7 and 8)30 as well as TLR4 led to some reduction in Ctsk, indicating activation of signaling via multiple TLR family members in Npc1−−/− cells.

To determine whether TLR4 is necessary for cholesterol-induced Ctsk expression, we loaded peritoneal macrophages from Tlr4−−/− mice with CD-chol or AcLDL+U18666A. The response to lipid A was abolished, whereas the response to CD-chol loading was significantly but only partially reduced by the Tlr4 mutation (32% to 44% in different experiments) (Figure 7B). Although baseline Ctsk expression was higher in Tlr4−−/− macrophages (possibly reflecting decreased NF-κB and JNK expression), AcLDL+U18666A resulted in no further increase in Ctsk expression, suggesting that TLR4 was also involved in the response to endosomal cholesterol loading. Similar results were obtained using macrophages from C3H/HeJ mice that carry a spontaneous mutation in Tlr4 (data not shown). CD-chol induction of Ctsk was unaltered but poly (I:C) and AcLDL+U18666A-mediated expression were abolished in Tlr3−−/− macrophages (Figure 7C). These data suggest that Ctsk induction by CD-chol loading of plasma membrane depends partially on TLR4, whereas AcLDL+U18666A loading of the endosomal compartment leads to TLR3 and TLR4 signaling.

Discussion

Our studies have elucidated a novel signaling pathway in macrophages, initiated by FC accumulation in plasma or endosomal membranes and leading to activation of TLRs, notably TLR3 and -4, and sustained phosphorylation of p38 MAP kinase. Several p38 targets that were induced by cholesterol loading, such as Ctsk,9,10 various Mmps,5 and S100a8,31 participate in atherogenesis or its complications, suggesting the relevance of this signaling pathway to the mechanisms of accelerated, complicated atherosclerosis of Apoe−/−, Npc1−−/− mice.5

Although the Npc1 mutation is rare, our findings may be relevant to the common forms of chronic atherosclerosis that involve overloading of macrophages with For example, chronic loading of human macrophages with AggLDL or Ox-LDL, a situation that is likely to be physiologically relevant to human atherogenesis,32 also leads to accumulation of unesterified cholesterol in late endosomes,11,12 resulting in p38 activation and Ctsk induction (Figure 3A and 3B). Moreover, the enrichment of plasma membrane cholesterol using CD-chol led to marked activation of p38 signaling and Ctsk induction. This may simulate the effects of cellular membrane enrichment occurring as a result of contact between cells and atherogenic plasma lipoproteins with a high cholesterol/phospholipid ratio.32 Although CD-chol loading also leads to increased sterol in the endocytic recycling compartment,33 reversal of CD-chol induction of Ctsk by mutant forms of ABCA1 that are localized to plasma membrane34 suggest that the plasmalemma is the relevant compartment (M. Ishibashi, A.R. Tall, unpublished studies, 2007).

Previous studies have indicated an important role of TLRs in atherogenesis, providing a link between hyperlipidemia and expression of a variety of inflammatory and chemokine genes.35 Deficiency of Myd88 in Apoe−/− mice led to a major reduction in atherosclerosis and inflammatory gene expression. Similarly, deficiency of TLR4 in Apoe−/− mice led to reduced atherosclerosis but the effect was much smaller than that of Myd88 deficiency, suggesting involvement of different TLRs upstream of Myd88. TLR2 has also been implicated in atherogenesis.35 Interestingly, our studies indicate an important role of TLR3 in the response to endosomal cholesterol loading with AcLDL+U18666A as well as the Npc1−−/− mutation. In fact, multiple TLRs that reside in endosomes appeared to be involved in the induction of Ctsk in Npc1−−/− cells, ie, TLR3, 7, and 8, as well as TLR4. Endosomes play a critical role in sorting and silencing signaling receptors such as TLRs. Inhibition of the endosomal pathway blocks post-endosomal cholesterol sorting and increases TLR4 signaling36 and mislocalization of late endosomes results in the sustained activation of MAP kinases.37 In Npc1−−/− fibroblasts, TLR4 signaling fails to shut off, and active TLR4 accumulates in endosomes and increases cytokine secretion.38

Our studies suggest spontaneous activation of TLR signaling by cholesterol loading of endosomes, but indicate that cholesterol accumulation may also enhance the responses to exogenous TLR ligands (Figure 6D). A variety of endogenous ligands of TLR4 as well as LPS may be involved in atherogenesis.39 Although TLR3 or 7 have not yet been implicated in atherogenesis, necrotic cells release endogenous RNA and heat shock protein-70 that stimulate TLR3 and 7.40,41 Poly(I:C) and endosomal cholesterol loading synergistically induced Ctsk (Figure 6D), suggesting that macrophage phagocytosis of virally infected apoptotic cells may also result in a similar synergy between RNA-mediated and cholesterol-induced responses.

In summary, our study provides new insights into the mechanisms linking macrophage cholesterol accumulation and inflammatory responses mediated by p38 activation. Our findings add to the growing evidence for involvement of TLRs and implicate TLR3, as well as other TLRs, in mediating signaling from cholesterol-loaded endosomes. There is increasing evidence for involvement of MMPs and cathepsins in aneurysm formation and possibly plaque destabilization.7,8 Although there is likely to be redundancy among individual MMPs or cathepsins, our studies suggest a common underlying mechanism involving p38 activation in plaque complications.

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Disclosures

None.

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Supplement Material

Methods

Reagents

Chemical reagents were purchased from Sigma-Aldrich and tissue culture reagents from Invitrogen, except where specified. LDL and acetylated LDL (AcLDL) were purchased from Biomedical Technologies, Inc (MA). Aggregated LDL (AggLDL) was made by vortexing (1 min) and sonicating LDL with a Branson sonifier (10 min, 70% duty cycle) on ice. Moderately oxidized LDL (OxLDL) was made by dialyzing LDL against 0.9% NaCl containing 20 µM CuSO₄ at 37°C for 2 hours. Cyclodextrin-cholesterol complex (CD-chol) was prepared by sonicating cholesterol in medium containing cyclodextrin. Acyl-coenzyme A-cholesterol acyltransferase (ACAT) inhibitor 58035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide was from J. Heider, formerly of Sandoz (East Hanover, NJ). MAP kinase inhibitors (Calbiochem), Toll-like receptor (TLR) ligands (Invivogen) were purchased. A molecular imaging agent for cysteinyl proteinase activity (ProSense 680) was from VisEn Medical, MA.

Mice Littermates $Apoe^{/-}$, $Npc^{+/+}$ were used as control for $Apoe^{/-}$, $Npc^{/-}$ mice. $p38^{\text{flax/flax}}$ mice were backcrossed four generations with C57BL6/J mice. $p38^{\text{flax/flax}}$ mice were crossed with LysMCre/C57BL6 mice to generate $p38^{\text{flax/flax}}$, LysMCre+/- mice. These mice were intercrossed. $p38^{\text{flax/flax}}$, LysMCre+/- mice were used as source for $p38$ deficient peritoneal macrophages and littermates $p38^{\text{flax/flax}}$, LysMCre-/- mice were used as controls. $Myd88/Trif^{/-}$ mice were backcrossed for 10 generations with C57BL6/J mice.
Wild type C57BL6/J mice were used as controls for Myd88/Trif^-/^- mice. Mitf^mi/mi mice were also on C57BL6/J background.

**Cell culture**- Mouse peritoneal macrophages were isolated by peritoneal lavage with PBS 3 days after intraperitoneal injection with 40 ug concanavalin A (ConA), or 1ml of 3.85% thioglycollate (Thio), or no injection (Resi). Bone marrow cells were flushed from femurs and tibias with PBS. These cells were cultured in DMEM/10% FBS. Bone marrow cells derived from Myd88/Trif^-/^- mice were cultured in 20% L-cell-conditioned DMEM/10% FBS medium for 7 days before treatment.

**Spleen-derived macrophages**- Spleen-derived precursors were harvested in DMEM, dissociated with a 23-1/2 gauge needle, passed through a 40-micron filter, and resuspended in DMEM/10% heat-inactivated low endotoxin FBS/ PenStrep /1X glutamate supplement/50 ng/ml recombinant colony-stimulating factor 1 (CSF-1). Cultured cells (1 spleen/plate) were incubated 3 days (7%CO_2, 37ºC,), flushed out of plates and seeded onto 10 cm tissue culture plates at 2-3 million cells/plate. The cells expressed macrophage markers within 2-3 days.

**Immunofluorescent staining of aortic cross-sections**- Aortic sections were dewaxed, rehydrated, blocked with 10% goat serum in PBS, and incubated overnight at 4 ºC with the first antibody in 1% BSA in PBS: affinity purified rabbit polyclonal anti-cathepsin K (1:50), mouse monoclonal antibody against SMC α-actin (1:100, Biomed, Foster City, CA). Mouse IgG at the same dilutions were used as negative controls. Sections were then incubated with the appropriate secondary antibody: goat anti-rabbit Cy3-conjugated and goat anti-mouse Cy2-conjugated antibody (1:200, Rockland, Gilbertsville, PA) for cathepsin K and VSMC staining.
Microarray Gene Expression Profiling-RNA was extracted and pooled from thioglycollate-elicited peritoneal macrophages derived from BALB or BALB- Npc<sup>-/-</sup> mice (5 each group). Labeling, hybridization and data analysis were provided by Gene Expression Center at University of Wisconsin using Affymetrix Mouse Genome 430 2.0 Genechips.

Chromatin immunoprecipitation assay-spleen derived macrophages were plated at density of 2X10<sup>6</sup> cells per 10 cm dish and treated with or without 5 mM CD-cholesterol (2.5:1, M:M) for 18 hours. Cells were cross-linked with 1% final concentration of formaldehyde at 37ºC for 10 min. Soluble chromatin was prepared following sonication with a Branson 250 digital sonifier (Branson Ultrasonics, Danbury, CT) to an average DNA length of 200-1000 bp. ~5 x 10<sup>5</sup> cell equivalent (one-sixth) of the sheared soluble chromatin was pre-cleared with tRNA-blocked Protein G-agarose, and 10% of the pre-cleared chromatin was set aside as input control. Immunoprecipitation was carried out with 5 µg of antibodies as indicated in the figures overnight at 4 °C. Immune complexes were pulled down using Protein G-agarose, washed, and eluted twice with 250 µl of elution buffer (0.1 M NaHCO<sub>3</sub>, 1% SDS), and cross-linking was reversed in 200 mM NaCl at 65 ºC overnight with 20 µg of RNase A (Sigma). DNA was purified following proteinase K treatment (Invitrogen) with the Qiagen PCR purification kit using the manufacturer's instructions. Samples were analyzed by real-time PCR either by SYBR Green super mix (Bio-Rad). The threshold for the promoter being studied was adjusted by that of input values and represented as relative abundance. All qPCR reactions were analyzed by melt curve analysis and agarose gels to confirm the presence of a single specific band.
**Media Transfer** - Bone marrow cells of \( Npc1^{+/+} \) and \( Npc1^{-/-} \) mice were flushed from femurs and tibias with PBS. These cells were grown in DMEM/10%FBS for 24 hours as donors and media were collected as conditioned media. Another group of bone marrow cells were collected 24 hours after donors. These were recipient cells. The recipient cells were plated and grew in DMEM/10%FBS or conditioned media from \( Npc1^{+/+} \) or \( Npc1^{-/-} \) donors. mRNA from recipient cells were collected for \( Ctsk \) expression four days after plating.

**Statistics**

Results from at least three independent experiments, performed in triplicate, were used to calculate mean±S.D unless specified in the legend. Statistical significance was determined by 2-tailed Student’s \( t \) test.

**Online Table I. Fold changes of genes induced in \( Npc1^{-/-} \) macrophages as determined by Taqman real-time PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Thio</th>
<th>ConA</th>
<th>Resi.</th>
<th>BM day 0</th>
<th>BM day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctsk</td>
<td>2.5</td>
<td>14</td>
<td>12</td>
<td>3.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Mmp14</td>
<td>2.4</td>
<td>5</td>
<td>3.4</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Mmp8</td>
<td>3.5</td>
<td>4.4</td>
<td>1.7</td>
<td>8.9</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mmp9</td>
<td>N.S.</td>
<td>N.S.</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>S100a8</td>
<td>5.8</td>
<td>5.5</td>
<td>2.6</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>S100a9</td>
<td>13</td>
<td>15</td>
<td>1.7</td>
<td>4.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Ym1</td>
<td>13</td>
<td>18</td>
<td>52</td>
<td>45</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
The expression levels of genes are normalized to ribosomal protein 36B4. The ratio of expression levels between $Npc1^{-/-}$ and wild type are shown. N>3, P<0.05. Thio, thioglycollate-elicited peritoneal macrophages; ConA, conconavalin A-elicited macrophages; Resi., resident peritoneal macrophages; BM, bone marrow derived cells. N.S., no significant change. N.D. not determined. ↓, decreased.
Online Figure I

A

![Graph showing Hmgcr/36B4 (Arbitrary unit) for Npc1+/+ and Npc1-/- mice with control and 25 OH-chol treatment.]

B

![Western blot showing CHOP and actin protein levels for CTR and tunicamycin treated samples.]

**Online Fig I. Positive controls for 25-hydroxylcholesterol and tunicamycin treatment.** A. ConA-elicted peritoneal macrophages from *Npc1*+/+ or *Npc1*−/− mice were treated with 10 μM 25-OH-chol for 24 hours. The levels of HMG-CoA reductase mRNA were measured by real-time PCR and normalized to 36B4. B. ConA-elicted peritoneal macrophages from wt mice were treated with 2ug/ml tunicamycin for 18 hours. CHOP and actin protein levels were measured by western blot.
Online Figure II

Online Fig II. A peptide inhibitor of JNK also increased Ctsk mRNA. ConA-elicited peritoneal macrophages from Npc1\(^{+/+}\) or Npc1\(^{-/-}\) mice were treated with JNK inhibitor I (Calbiochem) or negative control for 24 hours. Cells were then harvested for RNA analysis.
Online Fig III. *Npc1*~<sup>-/-</sup> or CD-cholesterol (CD-chol)-induced cathepsin K protein (CATK) levels were reduced by p38 inhibitors. A. Thioglycollate-elicited peritoneal macrophages from *Npc1*<sup>+/+</sup> or *Npc1*<sup>-/-</sup> mice were treated with 10 uM p38 inhibitors SB203580 or SB202190 for 48 hours. *, P<0.05 compared with *Npc1*<sup>+/+</sup>/DMSO; #, P<0.05 compared with *Npc1*<sup>-/-</sup>/DMSO; &, P=0.06 compared with *Npc1*<sup>-/-</sup>/DMSO; n= 3. B. Thioglycollate-elicited peritoneal macrophages from *Npc1*<sup>+/+</sup> mice were loaded with 5 mM CD-chol (2.5:1, M:M) at the absence or presence of p38 inhibitors for 72 hours. *, P<0.05 compared with CTR; #, P<0.05 compared with CD-chol. CATK levels were measured by western blots in duplicates and normalized to β-actin. Data from three independent experiments were used to calculate mean±S.D.
Online Fig IV. RANKL-RANK pathway was not involved in Npc1 deletion induced Ctsk induction. A. Osteoprotegerin treatment does not reverse the induction of Ctsk in Npc1<sup>−/−</sup> bone marrow cells. Bone marrow derived cells were flushed out from femur bone cavity with PBS or PBS containing 50ng/ml Osteoprotegerin. The cells were then plated in DMEM/10%FBS. The cells were maintained for 4 days with indicated treatments. The doses for treatments are, MCSF, 10ng/ml, Rank ligand, 25 ng/ml, Osteoproteterin, 50 ng/ml. B. Knockdown of RANK did not result in decreased Ctsk expression in Npc1<sup>−/−</sup> macrophages. ConA elicited Npc1<sup>−/−</sup> macrophages were transfected with scrambled or RANK siRNA. 72 hours after transfection, cells were harvested for RNA analysis.
Online Figure V

Online Fig V. The level of Ctsk was determined by cell genotype not factors in the media. Fresh bone marrow derived cells from Npc1<sup>+/+</sup> or Npc1<sup>-/-</sup> mice were grown in normal cell media (DMEM/10%FBS) or conditioned media from Npc1<sup>+/+</sup> or Npc1<sup>-/-</sup> cells for 4 days. Then cells were harvested for RNA analysis.