Retinal Cell Biology

Oxidized Lipoprotein Uptake Through the CD36 Receptor Activates the NLRP3 Inflammasome in Human Retinal Pigment Epithelial Cells

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Purpose. Accumulation of oxidized phospholipids/lipoproteins with age is suggested to contribute to the pathogenesis of AMD. We investigated the effect of oxidized LDL (ox-LDL) on human RPE cells.

Methods. Primary human fetal RPE (hf-RPE) and ARPE-19 cells were treated with different doses of LDL or ox-LDL. Assessment of cell death was measured by lactate dehydrogenase release into the conditioned media. Barrier function of RPE was assayed by measuring transepithelial resistance. Lysosomal accumulation of ox-LDL was determined by immunostaining. Expression of CD36 was determined by RT-PCR; protein blot and function was examined by receptor blocking. NLRP3 inflammasome activation was assessed by RT-PCR, protein blot, caspase-1 fluorescent probe assay, and inhibitor assays.

Results. Treatment with ox-LDL, but not LDL, for 48 hours caused significant increase in hf-RPE and ARPE-19 ($P < 0.001$) cell death. Oxidized LDL treatment of hf-RPE cells resulted in a significant decrease in transepithelial resistance ($P < 0.001$ at 24 hours and $P < 0.01$ at 48 hours) relative to LDL-treated and control cells. Internalized ox-LDL was targeted to RPE lysosomes. Uptake of ox-LDL but not LDL significantly increased CD36 protein and mRNA levels by more than 2-fold. Reverse transcription PCR, protein blot, and caspase-1 fluorescent probe assay revealed that ox-LDL treatment induced NLRP3 inflammasome when compared with LDL treatment and control. Inhibition of NLRP3 activation using 10 μM isoliquiritigenin significantly ($P < 0.001$) inhibited ox-LDL induced cytotoxicity.

Conclusions. These data are consistent with the concept that ox-LDL play a role in the pathogenesis of AMD by NLRP3 inflammasome activation. Suppression of NLRP3 inflammasome activation could attenuate RPE degeneration and AMD progression.

Keywords: CD36, oxidized LDL, NLRP3, pyroptosis

The pathogenesis of AMD is now generally believed to be mediated, at least in part, by low levels of chronic inflammation.[1] We and others have postulated that inflammatory signals generated by the NLRP3 inflammasome contribute to the development and/or progression of AMD by mediating RPE cell death and initiating or exacerbating an immune cascade.[2–4] In support of this concept, we have demonstrated the presence of NLRP3 activation in human eyes with AMD, but not in age-matched controls.[2]

The inflammasome NLRP3 is an intracellular sensor of metabolic distress and is composed of NLRP3, an adaptor protein ASC, and pro-caspase-1.[2–5] The activation of this multiprotein complex leads to the proteolytic cleavage of pro-caspase-1 to active caspase-1, which in turn can induce cell death by pyroptosis and/or the processing and release of the proinflammatory cytokines IL-1β and/or IL-18.[6,7] Thus far, the mechanism of NLRP3 inflammasome activation has been most extensively characterized in macrophages. Lysosomal destabilization induced by cholesterol crystals, amyloid-β,[6–8] cell stress signals from the endoplasmic reticulum, and oxidized mitochondrial DNA have all been shown to induce NLRP3 inflammasome activation in macrophages.[9,10]

The hallmark of AMD is the accumulation of insoluble deposits, called drusen, beneath the RPE layer and within the Bruch's membrane.[11–13] While the mechanism of drusen deposition is not clearly understood, analysis of drusen composition reveals that they are enriched in lipids and lipid-carrying lipoproteins.[12,14] The impairment of lipid metabolism with age has been suggested to contribute to lipid accumulation and AMD pathogenesis.[13,15] In addition, polymorphisms associated with genes related to lipid metabolism have been shown to be a risk factor for AMD.[16,17] Consistent with this notion, treatment of AMD patients who displayed numerous soft drusen with high dose of the cholesterol lowering drug, atorvastatin, resulted in the regression of drusen deposits and an associated improvement in vision.[18]

Studies suggest that the accumulated lipids and lipoproteins that become oxidized contribute to the dysfunction and death of RPE in AMD.[19–21] Although the mechanism of lipid and lipoprotein oxidation in AMD is not precisely known, oxidative

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events and lipoxygenase may mediate this process.[22,23] Oxidized LDL binds to the extracellular matrices, leading to further buildup.[24,25]

We speculate that the uptake of oxidized lipids by human RPE leads to NLRP3 inflammasome activation, thereby contributing to AMD pathogenesis. Transmembrane glycoprotein CD36, expressed by RPE cells,[26] has been shown to mediate the uptake of oxidized lipids[27,28] and has been implicated in the pathogenesis of atherosclerosis.[29] The work reported here demonstrates that the uptake of ox-LDL by human RPE through the CD36 receptor leads to NLRP3 inflammasome–mediated cell death and cytokine release, which we speculate contributes to the pathogenesis of AMD.

Methods

Cultured RPE

Human fetal (hf) eyes were obtained from Novogenix Laboratories, LLC (Los Angeles, CA, USA), in accordance with the institutional review board. We isolated and cultured hf-RPE as described previously.[30] First passage (P1) cells were maintained in media (media composition provided in supplementary data) containing 5% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA) for 2 to 4 weeks to allow for polarization and were subsequently used for experiments.

Human ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were propagated as described previously.[2] Confluent monolayers of cells were maintained in 1% FBS until the experiments were performed. For experiments, cells were serum starved overnight in DMEM/F12 medium supplemented with 2 mM L-glutamine and 100 U/mL penicillin/100 μg/mL streptomycin.

Lipoprotein Treatment

Human fetal RPE or ARPE-19 cells, grown at postconfluence for 2 to 4 weeks, were serum-starved overnight and treated for 24, 48, and 72 hours with LDL or ox-LDL (Biomedical Technologies, Alfa Asear, LLC, Ward Hill, MA, USA) suspended in serum-free media. Conditioned media and cell lysates were collected.

Measurement of Transepithelial Resistance (TER)

Human fetal RPE cells were cultured for at least 3 weeks on laminin-coated 0.4 μm transwells. Prior to lipoprotein treatment, RPE cells were serum-starved overnight and then treated for 48 hours with 500 μg/mL of LDL or ox-LDL suspended in serum-free media. Control hf-RPE cells were grown in serum-free media without lipoproteins. We measured TER at 0, 24, and 48 hours post lipoprotein treatment using a commercial volt ohm meter (EVOM^2; World Precision Instrument, Sarasota, FL, USA).

Assessment of Cell Death

Death of RPE was quantified by measuring the release of lactate dehydrogenase (LDH) into the media, as described previously.[2,31] ARPE-19 maintained for 1 to 2 weeks were serum-starved overnight and then incubated for 48 hours with 50, 100, and 300 μg/mL LDL or ox-LDL and controls received serum-free media only. Conditioned media were collected and LDH levels were measured. Human fetal RPE cells were maintained for 2 weeks in a 96-well plate and then treated with 100, 300, and 500 μg/mL LDL or ox-LDL to measure LDH release as above.

Visualization of the Cytoskeleton

Human fetal RPE cells were maintained for at least 3 weeks on laminin-coated 0.4 μm transwells. After overnight serum starvation, cells were treated with LDL or ox-LDL at 500 μg/mL for 48 hours. The cells were washed and fixed in 4% paraformaldehyde for 5 to 10 minutes, rinsed in PBS, and blocked in blocking buffer (10% goat serum, 0.05% Triton X-100, 0.01% sodium azide in PBS) for 1 hour at room temperature (RT). The cytoskeleton was visualized by incubating the cells with AlexaFluor 488 phalloidin (Life Technologies, Carlsbad, CA, USA) at 1:100 in antibody dilution buffer (1:4 dilution of the blocking buffer in PBS). The cells were then washed in PBS, and the transwell membranes were mounted using mounting medium (ProLong Gold; Life Technologies) and sealed. Images were taken using a fluorescent microscope (Eclipse E800; Nikon Corp., Tokyo, Japan).

Lysosomal Localization of ox-LDL

Human fetal RPE and ARPE-19 cells were grown for at least 1 week on laminin-coated 12-mm coverslips and were incubated with 10 μg/mL DiI-ox-LDL (Biomedical Technologies, Alfa Aesar, LLC, MA, USA) at 37°C with 5% CO_2 for 15 hours. The cells were washed and fixed with 4% paraformaldehyde, followed by blocking (as above) for 1 hour at RT. The cells were then incubated overnight at 4°C with anti–lysosomal membrane associated protein (LAMP)-1 antibody, washed and treated with respective secondary antibody for 2 hours at RT, rinsed in PBS, mounted using mounting medium (Life Technologies); and imaged using a fluorescent microscope (Nikon Corp.).

Real Time PCR (RT-PCR)

We treated ARPE-19 cells with 100 μg/mL LDL or ox-LDL for 24 hours and hf-RPE cells were treated with 500 μg/mL LDL or ox-LDL for 24 hours. We isolated RNA and RT-PCR was performed as described previously.[32] Real-time PCR reactions were performed using a master mix (SYBR Green; Roche, Basel, Switzerland) and PCR platform (Light Cycler 480 II; Roche). Primers used were listed in Supplementary Table S1.

Protein Immunoblot

We treated ARPE-19 cells with 100 μg/mL LDL or ox-LDL for 48 to 72 hours and hf-RPE cells were treated with 500 μg/mL LDL or ox-LDL for 48 to 72 hours. Cell lysates were prepared as described previously.[2] Proteins were separated using SDS-PAGE or gel (Tris-Tricine; Bio-Rad Laboratories, Hercules, CA, USA) under reducing conditions, transferred to nitrocellulose membrane and blocked with blocking buffer (Odyssey; LI-COR Biosciences, Lincoln, NE, USA) for 1 hour at RT. The membranes were incubated overnight at 4°C with primary antibodies prepared in blocking buffer (LI-COR Biosciences) with 0.1% Tween. After washing, the membranes were treated with the appropriate secondary antibodies, prepared in the buffer mentioned above for 45 minutes at RT. After washing, the membranes were scanned, and fluorescent band intensity was quantified using an infrared imaging system (Odyssey CLx Imaging System; LI-COR Biosciences).

CD36 Receptor-Blocking Assay

We grew ARPE-19 cells for 2 weeks on coverslips and incubated them with 20 or 40 μg/mL of control IgA or CD36 IgA antibody for 1 hour at 4°C. The cells were briefly warmed at 37°C and incubated with 10 μg/mL DiI-ox-LDL at RT for 30 minutes in the presence of the respective antibodies. Cells

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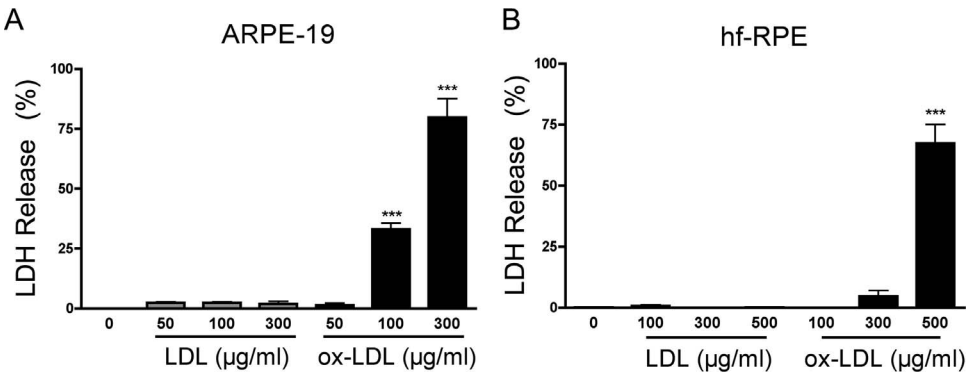

Figure 1. Ox-LDL induces RPE cytotoxicity in a dose-dependent manner. (**A**) We treated ARPE-19 cells with 50, 100, and 300 μg/mL LDL or ox-LDL or serum-free media; conditioned media were collected after 48 hours and LDH release was measured. Growth of ARPE-19 cells in 100 μg/mL or 300 μg/mL ox-LDL led to a significant increase in LDH release. (**B**) We treated hf-RPE cells with 100, 300, and 500 μg/mL LDL or ox-LDL or serum-free media; conditioned media were collected after 48 hours and LDH was measured. Growth of hf-RPE cells in 500 μg/mL ox-LDL led to a significant increase in LDH release. *** $P < 0.001$.

were washed, fixed for 5 minutes in 4% paraformaldehyde, rinsed in PBS, and mounted using mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). The coverslips were imaged using fluorescent microscope (Nikon Corp.) and analyzed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) to quantify the number of internalized DiI-ox-LDL particles.

The role of the CD36 receptor was assessed in ARPE-19 cells using a CD36 receptor–blocking antibody. Cells were preincubated with 40 μg/mL of control or CD36 IgA as mentioned above. The cells were then treated with 100 μg/mL ox-LDL in the presence of control or CD36 IgA and maintained for 48 hours at 37°C. Media from all the experimental conditions were collected, and LDH release was measured as described above.

Detection of Active Caspase-1 Using a Fluorescent Probe Assay

We seeded ARPE-19 cells into 48-well plates at a density of 5.0 to 7.5×10^4 cells per well and maintained for at least 2 weeks. The cells were serum-starved overnight and then treated with 100 μg/mL LDL or ox-LDL treatment in the presence or absence of fluorescent-labeled inhibitor of caspase-1 (FLICA, FAM-YVAD-FMK; Immunochemistry Technologies, Bloomington, MN, USA) for 48 hours at 37°C with 5% CO_2. The cells were washed, fixed, and imaged using a microscope (Nikon Eclipse TE2000-S; Nikon Corp.).

Statistical Analysis

All the data are represented as the mean ± SEM of at least three independent experiments. Statistical significance was analyzed by 1-way analysis of variance, followed by a post Tukey's comparisons test using commercial software (Prism 4; GraphPad, La Jolla, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Ox-LDL Leads to RPE Cell Death, Cytoskeletal Alteration, and Impaired Barrier Properties

To test the effects of ox-LDL treatment on RPE cell viability, ARPE-19 cells and primary hf-RPE cells were treated with different doses of LDL or ox-LDL for 48 hours (Fig. 1). We found that ARPE-19 cells that were exposed only to serum-free media or LDL did not show any LDH release (Fig. 1A). In contrast, 100 and 300 μg/mL ox-LDL treatment led to significant LDH release (Fig. 1A). The lowest dose of ox-LDL tested (50 μg/mL) did not result in significantly elevated LDH release. Similarly, native LDL did not affect the viability of hf-RPE but while 100 μg/mL had no effect on LDH release by hf-RPE, 300 μg/mL caused a modest level of LDH release and 500 μg/mL ox-LDL treatment led to a significant increase in LDH release ($P < 0.001$; Fig. 1B), illustrating the dose-dependent cytotoxic effect of ox-LDL on hf-RPE cells.

To examine the effect of these treatments on hf-RPE cells, cytoskeletal organization was visualized by probing with phalloidin (Fig. 2). The control and LDL-treated hf-RPE appeared as an intact monolayer of hexagonal cells (Figs. 2A, 2B). In contrast, hf-RPE treated with ox-LDL exhibited aberrant cytoskeletal organization and disrupted monolayer integrity (Fig. 2C). Since the altered monolayer suggested disrupted barrier function, TER was measured at the time of treatment (0 hours), 24 hours, and 48 hours after lipoprotein addition. The average TER of the hf-RPE cells at 0 hours was 600 to 700 ohms × cm^2 (Fig. 2D). At 24 hours, there was no difference in the TER of control (682 ± 16.17 ohms × cm^2) and LDL-treated cells (584.3 ± 25.1 ohms × cm^2); however, 24-hour treatment of hf-RPE cells with ox-LDL resulted in a significant decrease in TER values (316.3 ± 20.8 ohms × cm^2; Fig. 2D). After 48 hours, there was further reduction in the TER of the ox-LDL–treated cells (232.7 ± 15.19 ohms × cm^2) compared with control (519 ± 9.07 ohms × cm^2) and LDL-treated cells (491.3 ± 52.29 ohms × cm^2; Fig. 2D). The slight but decrease in TER of control and LDL-treated cells at 48 hours ($P < 0.05$) relative to cells at the 0-hour time point is likely due to their culture in serum-free conditions.

Ox-LDL Is Targeted to the Lysosomes in Human RPE

To determine if ox-LDL is targeted to the lysosomes, hf-RPE and ARPE-19 cells were treated with tracer levels of DiI-labeled–ox-LDL, and its localization was examined by immunostaining for LAMP-1. Fifteen hours after its addition, DiI-labeled–ox-LDL was localized within LAMP-1 positive structures in the hf-RPE (Figs. 3A–C). Similar results were observed with ARPE-19 (Figs. 3D–F). There was also a reduction in the number of lysosomes in ARPE-19 cells treated with 100 μg/mL ox-LDL for 48 hours when compared with LDL-treated cells (Supplementary Fig. S1).

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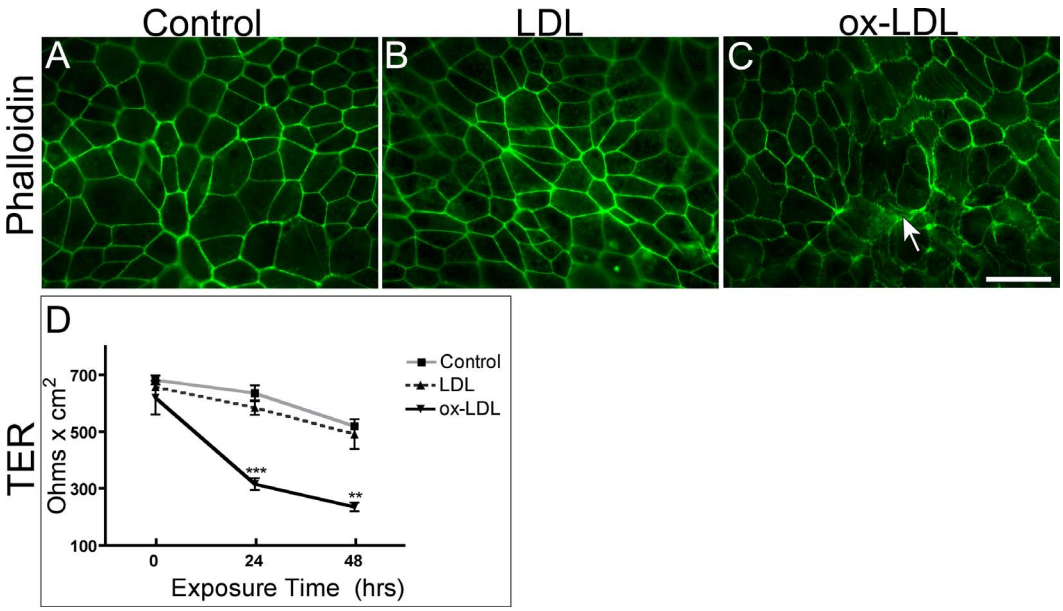

Figure 2. Treatment of Ox-LDL disrupts RPE barrier properties. Human fetal RPE cells grown on 0.4-μm transwell membranes for 2 to 4 weeks were treated with LDL or ox-LDL for 48 hours and then examined for actin cytoskeletal organization using AlexaFluor 488 phalloidin. (**A**) Control hf-RPE cells treated with PBS and (**B**) Human fetal cells treated with LDL (500 μg/mL) exhibited intact hexagonal RPE morphology. (**C**) In comparison with (**A**) and (**B**), treatment of hf-RPE with ox-LDL (500 μg/mL) led to a disrupted cytoskeletal organization. *Scale bar:* 50 μm (**A–C**). (**D**) Measurements of TER in hf-RPE cells treated with 500 μg/mL LDL or ox-LDL were used to assess RPE barrier properties. There was no change in TER following 24 hours of LDL treatment, whereas ox-LDL–treated RPE cells exhibited a dramatic decrease in TER, which continued to decline over the next 24 hours. *** $P < 0.001$, ** $P < 0.01$.

CD36 Receptor Mediates ox-LDL Uptake

Knowing that human RPE cells express the CD36 receptor,[33,34] we examined if this receptor mediates ox-LDL uptake in human RPE cells (Fig. 3). We treated ARPE-19 with 20 μg/mL of control IgA in the presence of DiI-ox-LDL had 510 ± 20 DiI-ox-LDL particles per field of view (Fig. 3I). In contrast, cells treated with 20 μg/mL CD36 antibody showed significantly fewer ($P < 0.05$) DiI-ox-LDL particles per field of view: 299 ± 32 (Fig. 3I). Treatment of ARPE-19 with 40 μg/mL CD36 antibody led to a further reduction of ox-LDL uptake (Figs. 3G–I) compared with 40 μg/mL IgA-treated cells. This result indicates that a significant portion of ox-LDL uptake by human RPE is mediated by the CD36 receptor.

Ox-LDL Increases Expression of the CD36 Receptor in Human RPE Cells

We tested the effect of ox-LDL on the expression of the CD36 receptor by incubating the RPE cells with LDL or ox-LDL. Treatment of hF-RPE cells with 500 μg/mL ox-LDL led to a 2-fold increase in CD36 mRNA level at 24 hours ($P < 0.01$; Fig. 4A) and a significant increase ($P < 0.01$) in CD36 receptor protein level at 72 hours relative to the control and LDL-treated cells (Fig. 4B). As in hf-RPE cells, there was a significant ($P < 0.01$) increase in CD36 mRNA level in ARPE-19 cells treated with 100 μg/mL ox-LDL in comparison with control and LDL-treated cells (Fig. 4C). There was also a significant ($P < 0.01$) increase in CD36 receptor protein level at 48 hours after ox-LDL treatment relative to control and LDL-treated cells (Fig. 4D).

CD36 Mediated ox-LDL Uptake Mediates to Cell Death in Human RPE Cells

We investigated whether blocking of the CD36 receptor would attenuate ox-LDL–mediated RPE cell death. The inclusion of control IgA did not influence ox-LDL induced LDH release (Fig. 5A), whereas the addition of CD36 receptor function blocking IgA significantly reduced ox-LDL–induced RPE cell death by approximately 40% ($P < 0.05$; Fig. 5A).

Uptake of ox-LDL Leads to Inflammasome Activation

To determine the effect of ox-LDL accumulation on NLRP3 inflammasome activation, NLRP3 mRNA levels were measured in RPE cells with LDL or ox-LDL treatment. In both hf-RPE and ARPE-19 cells, ox-LDL–treated cells exhibited a nearly 6-fold increase in NLRP3 mRNA level relative to control and LDL-treated cells (Figs. 5B, 5C).

Inflammasome NLRP3 leads to the activation of caspase-1[2,7]; thus, caspase-1 activation was assessed in RPE cells treated with LDL or ox-LDL (Fig. 6). Caspase-1 activation in hf-RPE cells was quantified by immunoblot analysis, as FLICA assay yielded high-level background due to hf-RPE pigmentation. There was a modest increase in active caspase-1 at 48 hours in cells that were treated with ox-LDL but not in control or LDL-treated cells (data not shown) and a significant increase in procaspase-1 ($P < 0.01$) and active caspase-1 in RPE cells treated with ox-LDL for 72 hours ($P < 0.01$; Fig. 6A) compared with control and LDL-treated cells (Fig. 6A).

Analysis of caspase-1 activation in ARPE-19 cells by FLICA assay showed no caspase-1 activation in control and LDL-treated cells (Fig. 6B), but caspase-1 positive cells (Fig. 6B) were seen in ARPE-19 that had been treated with ox-LDL.

Activation of caspase-1 leads to the processing and secretion of the pro-inflammatory cytokine IL-1β.[2,31] Thus, we analyzed whether treatment of RPE with ox-LDL led to IL-1β activation and secretion. Conditioned media of control ARPE-19 or ARPE-19 cells that were treated with 200 μg/mL LDL for 48 hours did not contain pro–IL-1β or processed IL-1β (Fig. 6C), whereas conditioned media from ARPE-19 cells incubated in

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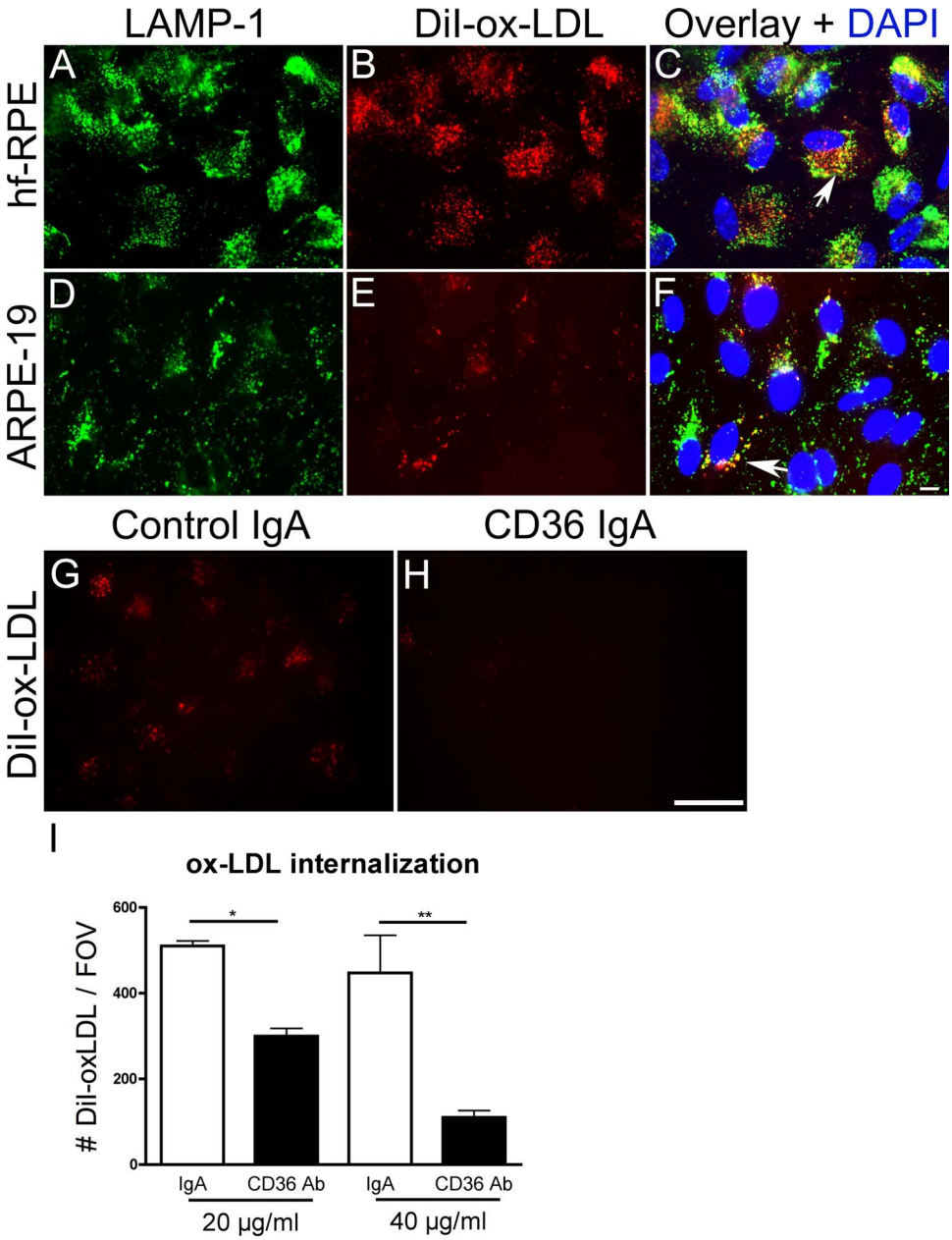

Figure 3. Oxidized LDL taken up by RPE is targeted to the lysosomes through the CD36 receptor. hf-RPE and ARPE-19 cells grown on laminin-coated coverslips for a week were treated with 10 µg/mL DiI-ox-LDL and then visualized at 15 hours by immunofluorescent staining for LAMP-1 to localize the lysosomes. For function blocking assay, ARPE-19 cells were treated with 10 µg/mL DiI-ox-LDL in the presence of 20 or 40 µg/mL of control IgA or anti-CD36 IgA. At 15 hours posttreatment, there was clear localization of DiI-ox-LDL to LAMP-1-staining lysosomes in (**A–C**) hf-RPE and (**D–F**) ARPE-19. Anti-CD36 IgA treatment (40 µg/mL) significantly decreased DiI-ox-LDL uptake in ARPE-19 cells (**H**). (**I**) Quantification of the number of DiI-ox-LDL particles present per field of view. ** $P < 0.01$. *Arrows* in (**C**) and (**F**) show the location of DiI-ox-LDL within LAMP-1–positive lysosomes. *Scale bars*: 10 µm (**A–F**) and 50 µm (**G**, **H**).

200 µg/mL ox-LDL contained both the precursor and mature form of IL-lβ (Fig. 6C). There was a low but detectable level of mature IL-lβ in the conditioned media from cells treated with 100 µg/mL ox-LDL (data not shown).

Our results indicate that ox-LDL-induced NLRP3 inflammasome activation (Figs. 5, 6) leads to RPE cell death. Thus, we investigated whether inhibition of NLRP3 inflammasome activation would block RPE cell death. Treatment with 10 µM isoliquiritigenin, an inhibitor of the NLRP3 inflammasome,[35] significantly ($P < 0.001$) reduced ox-LDL–induced cytotoxicity (Fig. 7A) and preserved RPE cell morphology (Fig. 7B), supporting the conclusion that ox-LDL–induced RPE cell death is mediated through the NLRP3 inflammasome.

Discussion

A number of different molecules, most of which are found in drusen deposits such as lipids, A2E, amyloid β, have been suggested to play a role in AMD pathology.[3,4,19,36] Among the contributing factors, the accumulation of lipids with age is strongly associated with the development of AMD.[12,13] As retinal pigment epithelium cells are key regulators of lipid

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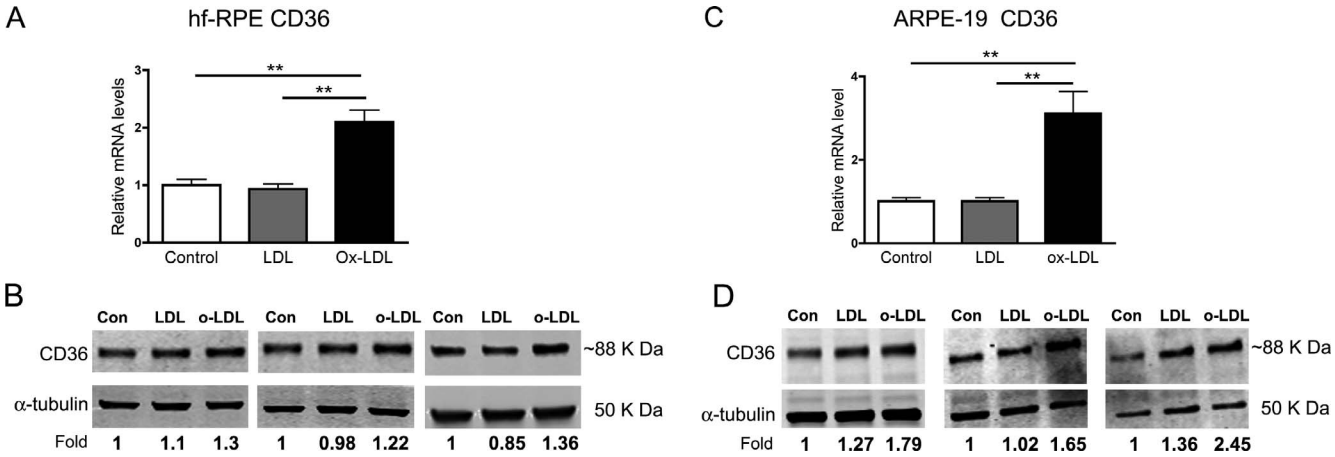

Figure 4. Treatment with Ox-LDL increases CD36 expression level. We treated (**A**) hf-RPE and (**C**) ARPE-19 cells with LDL or ox-LDL for 24 hours and CD36 mRNA levels were measured by RT-PCR. Treatment with LDL did not alter the expression of CD36 mRNA, whereas ox-LDL treatment increased CD36 mRNA levels in both cell types by approximately over 2-fold. We treated (**B**) hf-RPE and (**D**) ARPE-19 cells with LDL or ox-LDL for 72 hours and 48 hours were analyzed for CD36 protein expression levels. Treatment with LDL did not alter CD36 protein level relative to the untreated control. In contrast, ox-LDL treatment significantly increased CD36 protein levels in hf-RPE cells and ARPE-19 cells as compared with LDL and control cells. ** $P < 0.01$.

metabolism in the retina,[37] the accumulation of lipids in and around the RPE cell layer points to a role for impaired lipid handling by RPE cells. The pathogenesis of AMD shares many features with the development of atherosclerosis, including association with oxidized lipid accumulation and inflammation.[13,19,20,38,39] Oxidized lipids have been shown to accumulate preferentially in the macula of human eyes with AMD.[19] Although the levels ox-LDL in the AMD eyes have yet to be quantified, analysis of atherosclerotic lesions has revealed that ox-LDL levels are 70 times higher than the levels of circulating ox-LDL.[40] The increased accumulation of ox-LDL in macrophages leads to foam cell formation and atherogenesis.[41]

Informed by these observations, we investigated the effect of ox-LDL on RPE cell function and integrity. Treatment of RPE

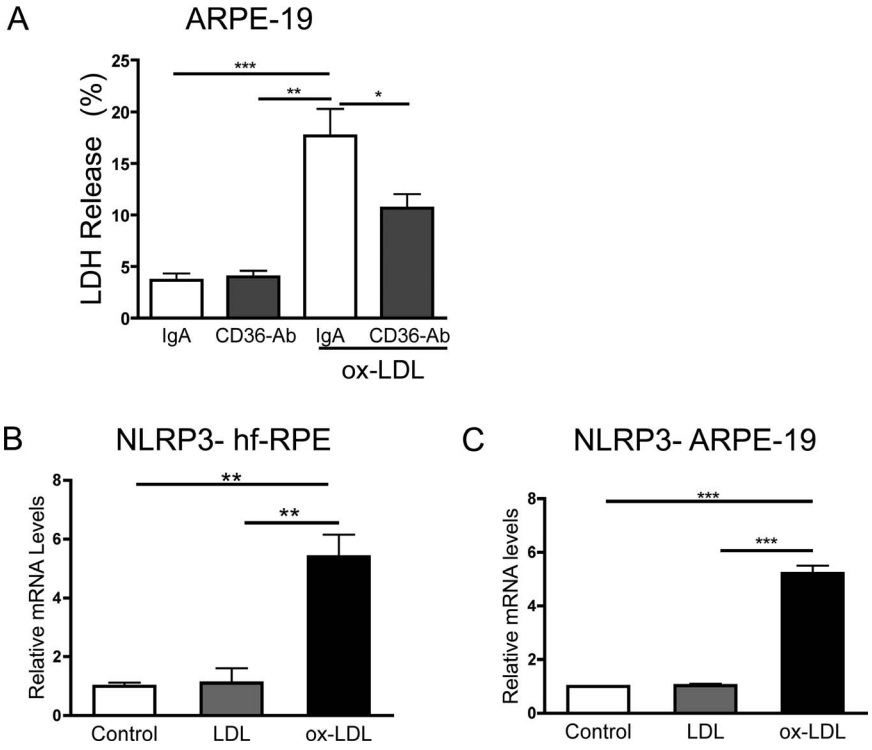

Figure 5. Oxidized LDL increases NLRP3 mRNA expression in RPE cells. We incubated (**A**) ARPE-19 cells grown for 2 to 3 weeks were incubated with 40 μg/mL of control IgA or anti-CD36 IgA in the presence or absence of 100 μg/mL of ox-LDL. After 48 hours of treatment, conditioned media were collected to measure LDH levels. We found that ARPE-19 cells that were grown in the presence ox-LDL showed a significant increase ($P < 0.01$) in LDH level. Treatment of ARPE-19 cells with anti-CD36 IgA significantly ($P < 0.05$) decreased ox-LDL induced cytotoxicity. We treated (**B**) hf-RPE and (**C**) ARPE-19 cells with LDL or ox-LDL for 24 hours and then analyzed for NLRP3 mRNA levels by RT-PCR. Cells treated with Ox-LDL showed nearly a 6-fold increase ($P < 0.01$) in NLRP3 mRNA expression in hf-RPE cells and more than a 5-fold ($P < 0.001$) increase in ARPE-19 cells relative to LDL or untreated control cells. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.01$.

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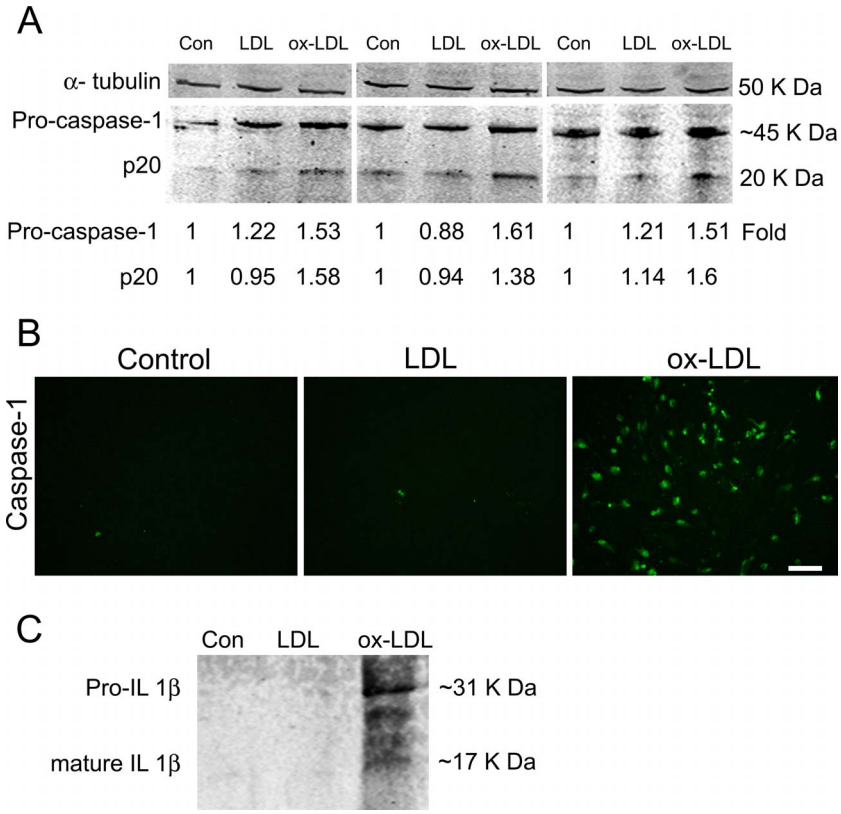

Figure 6. Oxidized LDL leads to caspase-1 activation in RPE cells. (**A**) We treated hf-RPE cells with 500 μg/mL LDL or ox-LDL for 72 hours and then analyzed for pro- and cleaved caspase-1 by protein immunoblot. Human fetal RPE cells treated with ox-LDL exhibited a significant increase in pro-caspase-1 ($P < 0.01$) and cleaved caspase-1 ($P < 0.01$) compared with untreated control and LDL-treated cells. Each sample represents hf-RPE from three different fetal eyes. (**B**) We treated ARPE-19 cells for 48 hours with 100 μg/mL LDL or ox-LDL in the presence of fluorescent-labeled inhibitor of caspase-1 (FLICA, FAM-YVAD-FMK). Untreated and LDL-treated cells displayed little to no caspase-1-positive cells (*top left* and *middle*), whereas cells treated with ox-LDL showed increased number of caspase-1 positive cells (*top right*). *Scale bar*: 100 μm. (**C**) We treated ARPE-19 cells with LDL or ox-LDL for 48 hours, conditioned media were collected and IL-1β release was measured by protein blot. Pro- and mature IL-1β were observed in ox-LDL–treated cells but not in untreated and LDL-treated cells.

cells with ox-LDL–induced cell death in a dose-dependent manner, suggesting that RPE cells can clear lower levels of ox-LDL but that there is a threshold at which they can be overwhelmed by ox-LDL. Our findings are in line with other reports using ARPE-19 cells, which show RPE cell death with increasing concentrations of ox-LDL treatment.[21,42] It is worth noting that our data show that similar results were obtained using both primary hf-RPE and ARPE-19 cells; the effects differ quantitatively (e.g., cell death of primary RPE occurs at 500 μg/mL ox-LDL, whereas 100 μg/mL ox-LDL is cytotoxic for ARPE-19 cells).

Exposure of RPE cells to ox-LDL led to cytoskeletal disorganization and increased stress fiber formation. Observations of the morphology of RPE cells in whole mount preparations of RPE-choroid of human eyes with AMD show a similar phenotype.[43] The layer of RPE is the site of the outer blood–retinal barrier and the extensive junctional complexes along with the cytoskeleton provides structural support for RPE cells to form basolateral interactions to polarize.[44] Accordingly, the significant decrease in TER that we detected in hf-RPE cells treated with ox-LDL suggests that the accumulation of lipids and their subsequent oxidation in the early stages of AMD may underlie early RPE dysfunction, including a decline in barrier function.

To investigate the mechanism of ox-LDL–mediated RPE dysfunction and death, we assessed the role of CD36, a lipid scavenger receptor. Receptor CD36 is an 88 kDa transmembrane glycoprotein expressed by cells such as macrophages, microglia, microvascular endothelium, and RPE cells[26,45] has been shown to bind ox-LDL and oxidized phospholipids in vitro and in vivo.[45,46] Blockade of the CD36 receptor significantly inhibited the uptake of ox-LDL by human RPE cells, and prolonged neutralization of CD36 led to a significant reduction in RPE cell death. Studies conducted in human monocytes, THP-1 cells, and murine macrophages show that ox-LDL treatment activates peroxisome proliferator–activated receptor γ, leading to increased CD36 expression and ox-LDL uptake.[47,48] In a similar manner, we observed that ox-LDL treatment of human RPE cells was associated with a significant increase in CD36 mRNA and protein levels.

Use of DiI-labeled–ox-LDL revealed that, similar to myeloid cells, ox-LDL internalized by the RPE was targeted to the lysosomes.[28] The accumulation of ox-LDL in lysosomes can disrupt the processing and degradation of photoreceptor outer segments (POS),[49] one of the major functions of the RPE. Such alterations may result in intracellular accumulation of toxic photo-oxidized outer segments and contribute to the buildup of lipofuscin.

We have shown that NLRP3 is activated in donor eyes with AMD but not in the eyes of age-matched controls.[2] Other studies also have shown significant upregulation of NLRP3 and IL-1β mRNA levels in the RPE lesion area of human eyes with AMD.[50] In vitro investigations have revealed that destabilization of lysosomes pharmacologically[2] or by A2E results in the NLRP3 inflammasome mediated release of IL-1β[36]; neither of these studies observed IL-18 release. On the other hand, there

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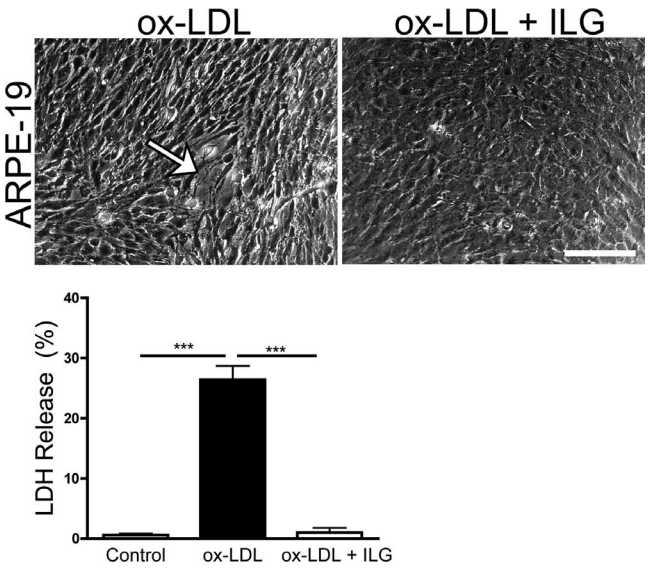

Figure 7. Inhibition of NLRP3 inflammasome activation prevents ox-LDL–induced RPE cell death. We treated ARPE-19 cells for 48 hours with 100 μg/mL ox-LDL in the presence or absence of 10 μM isoliquiritigenin, and conditioned media were collected to measure LDH release. Oxidized LDL induced significant RPE cell death, and inclusion of isoliquiritigenin nearly completely inhibited ox-LDL–induced cell death. Arrow on the *top-left* image denotes cells with abnormal morphology, which are presumably undergoing cell death. *Scale bar*: 100 μm. *** $P < 0.001$.

are studies that suggest that pharmacologic induction of oxidative stress or lysosomal destabilization in primed RPE cells results in release of IL-18.[50,51] Exposure of RPE cells to ox-LDL led to the activation of NLRP3 inflammasome, as evidenced by the upregulation of NLRP3 mRNA, activation of caspase-1, and release of mature IL-1β. Interestingly, studies conducted in atherosclerotic mice models and in macrophages show that exposure to ox-LDL without priming activates NLRP3 inflammasome and induces the production of IL-1β and not IL-18.[6,28] The upregulation of NLRP3 mRNA was seen with ox-LDL, but not native LDL and it is likely mediated by lysosomal destabilization[6,28]; a decrease in RPE lysosomes with ox-LDL treatment as visualized by lysotracker supports this concept. Confocal reflection microscopy of myeloid cells has revealed that CD36-mediated internalization of ox-LDL leads to the formation of crystals within the lysosomes, which causes lysosomal disruption and a release of cathepsins into the cytoplasm.[28] By a mechanism that is not completely understood, cathepsins activate the NLRP3 inflammasome.[6,28]

Isoliquiritigenin has been shown to inhibit ASC oligomerization and activation of the NLRP3 inflammasome in macrophages as well as inflammation in adipose tissue.[35] We tested the effect of isoliquiritigenin on ox-LDL–induced human RPE cell death. Isoliquiritigenin significantly inhibited ox-LDL RPE cell death, suggesting that ox-LDL–induced RPE cell death is mediated primarily through the NLRP3 inflammasome, and that inhibition of this cell death pathway may rescue RPE degeneration.

Our observations point to a mechanism by which the accumulation of oxidized lipids contributes to RPE loss in AMD. The detection of NLRP3 in the RPE cells of human eyes with AMD[2] and the association of myeloid cells with drusen[38] further support the concept that the NLRP3 inflammasome is involved in AMD pathogenesis. Findings from this study suggest several potential mechanisms for protecting RPE cells, including enhancing lipid clearance and inhibition of NLRP3 inflammasome activation.

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