Inflammasome priming increases retinal pigment epithelial cell susceptibility to lipofuscin phototoxicity by changing the cell death mechanism from apoptosis to pyroptosis

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Abstract

Progressive death of retinal pigment epithelium (RPE) cells is a hallmark of age-related macular degeneration (AMD), the leading cause of blindness in all developed countries. Photooxidative damage and activation of the NLRP3 inflammasome have been suggested as contributing factors to this process. We investigated the effects of inflammasome activation on oxidative damage-induced RPE cell death. In primary human RPE cells and ARPE-19 cells, lipofuscin accumulated following incubation with oxidatively modified photoreceptor outer segments. Oxidative stress was induced by blue light irradiation (dominant wavelength 448 nm, irradiance 0.8 mW/cm², duration 3 to 6 hours) of lipofuscin-loaded cells and resulted in cell death by apoptosis. Prior inflammasome priming by IL-1α or complement activation product C5a altered the cell death mechanism to pyroptosis and resulted in a significant increase of the phototoxic effect. Following IL-1α priming, viability 24 hours after irradiation was reduced in primary RPE cells and ARPE-19 cells from 65.3% and 56.7% to 22.6% (p=0.003) and 5.1% (p=0.0002), respectively. Inflammasome-mediated IL-1β release occurred only in association with pyroptotic cell lysis. Inflammasome priming by conditioned media of pyroptotic cells likewise increased cell death. Suppression of inflammasome activation by inhibition of caspase-1 or cathepsins B and L significantly reduced cell death in primed cells. In summary, inflammasome priming by IL-1α, C5a, or conditioned media of pyroptotic cells increases RPE cell susceptibility to photooxidative damage-mediated cell death and changes the mechanism of induced cell death from apoptosis to pyroptosis. This process may contribute to RPE degeneration in AMD and provide new targets for intervention.

Highlights

- RPE cell death by photooxidative damage increases following inflammasome priming.
- Mechanism of cell death changes after priming from apoptosis to pyroptosis.
- Priming can occur by IL-1α, C5a, or conditioned media of pyroptotic cells.
- Inflammasome inhibition decreases photooxidative cell death in primed cells.
1. Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in all industrialized countries [1]. For the late-stage atrophic form of the disease (geographic atrophy), there is currently no effective treatment available. Geographic atrophy secondary to AMD is characterized by progressive degeneration of the retinal pigment epithelium (RPE), resulting in corresponding secondary photoreceptor loss and visual impairment. The mechanism of RPE cell death in AMD has not yet been fully elucidated. Several lines of clinical and experimental evidence indicate that oxidative and lipofuscin-mediated photooxidative damage play an important pathophysiological role [2]. Recent studies suggest that the NLRP3 inflammasome also contributes to RPE cell death secondary to AMD [3,4]. Indeed, NLRP3 inflammasome activation has been demonstrated in RPE cells affected by AMD [3,5], and increased intravitreal and systemic levels of the inflammasome activation products IL-1β and IL-18 have been reported in AMD patients [6,7].

We have identified a mechanism that links oxidative/photooxidative damage and inflammasome activation in RPE cells by demonstrating that lipofuscin phototoxicity results in oxidative damage to lysosomal membranes with subsequent cytosolic leakage of lysosomal enzymes and activation of the NLRP3 inflammasome [8]. Inflammasome activation in RPE cells requires a prior priming signal that can be provided by complement activation product C5a [9]. Inflammasome activation can be accompanied by pyroptosis, a recently described type of programmed cell death that is distinct from other cell death mechanisms including apoptosis and necrosis. Pyroptosis is characterized by a combination of several features including caspase-1 dependence, DNA fragmentation, rapid loss of cell membrane integrity, and inflammatory cytokine release [10].

Against the background of the interrelations between oxidative damage, inflammasome activation, and RPE cell death, we sought to elucidate the effects of inflammasome priming on mechanism and extent of photooxidative damage-induced cytotoxicity in RPE cells.
2. Methods

2.1. Cell culture

Human fetal primary RPE (pRPE) cells (Clonetics H-RPE; Lonza, Cologne, Germany) were cultured in medium provided by the manufacturer (Clonetics RtEGM; Lonza) containing 2% heat-inactivated fetal bovine serum and were used in experiments for a maximum of 6 cell culture passages. The spontaneously immortalized, non-transformed human RPE cell line ARPE-19 (CRL-2302; ATCC, Rockville, MD, USA) was maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 medium (PAN-Biotech, Aidenbach, Germany) containing 2.5 mM L-glutamine, 1.0 mM sodium pyruvate, and 17.5 mM D-glucose, and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Gibco/Thermo Fisher Scientific, Karlsruhe, Germany) at 37°C in a humidified atmosphere of 5% CO2 in air.

2.2. Induction of photooxidative damage

Lipofuscin-mediated photooxidative damage was induced in RPE cells using experimental conditions described previously in detail [8]. Briefly, isolated porcine POS were covalently modified with the lipid peroxidation product 4-hydroxynonenal (HNE) at a concentration of 5 mM at room temperature over night to stabilize them against lysosomal degradation [11]. These treatment parameters result in a modification of 48 nmol/mg protein as previously determined by HNE-specific ELISA [12] and were chosen to correspond to the range of carbonyl modifications detected in aged human cells in vivo [13] as data for human POS in vivo is not yet available. RPE cells were incubated with modified POS (concentration equivalent to 4 mg total POS protein per cm² cell growth area) daily for 7 days, resulting in lipofuscinogenesis [14]. Subsequently, and cells were irradiated with blue light (dominant wave length, 448 nm; irradiance, 0.8 mW/cm²) as described previously [8]. In all experiments, duration of irradiation was 3 hours for pRPE cells and and 6 hours for ARPE-19 cells.

2.3. L-leucyl-leucine-methyl ester (Leu-Leu-OMe) treatment

L-leucyl-leucine-methyl ester (Leu-Leu-OMe) induces lysosomal membrane permeabilisation (LMP) and inflammasome activation in RPE cells similar to lipofuscin phototoxicity [5,15]. We treated cells with 1 mM Leu-Leu-OMe (Bachem, Bubendorf, Switzerland) for 3 hours.
2.4. Inflammasome priming

Cells were primed with 4 ng/ml recombinant human IL-1α (R&D Systems, Wiesbaden, Germany), 50 ng/ml recombinant human C5a (R&D Systems), or 50 pg/ml recombinant human IL-1β (R&D Systems) for 48 hours prior to irradiation or Leu-Leu-OMe treatment.

2.5. Cell death detection assay

To quantify cell death, we analyzed loss of plasma membrane integrity by means of lactate dehydrogenase (LDH) release into the media using a calorimetric assay (Cytotoxicity Detection Kit; Roche, Mannheim, Germany) according to the manufacturer’s instructions. We expressed the assay results as percentage of total cellular viability. For this, LDH release was measured in cells lysed by 1% Triton X-100 and in untreated control cells, and the results were set as 0% and 100% cellular viability, respectively.

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

DNA strand breaks were demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (TdT in Situ Apoptosis Detection Kit – DAB; R&D Systems, Wiesbaden, Germany) following the manufacturer’s recommendations. For this, cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and incubated with TUNEL enzyme and labeling solution for one hour at 37°C in the dark.

2.7. Annexin V-FITC and propidium iodide (PI) labeling

Phosphatidylserine exposure and loss of membrane integrity was assessed simultaneous by labeling with FITC-coupled annexin V and propidium iodide (PI) (FITC Annexin V Apoptosis Detection Kit; Biolegend, Fell, Germany). Labeling was quantified by flow cytometry (FACS Canto II; BD Biosciences, Heidelberg, Germany).

2.8. ELISA detection of IL-1β

Secretion of IL-1β into the media was measured by specific ELISA (BD OptEIA Human IL-1β ELISA Kit II; BD Biosciences, Heidelberg, Germany) according to the manufacturer’s instructions.
2.9. **Priming with conditioned media**

Inflammasome activation was induced by HNE-POS incubation, IL-1α priming, and blue light irradiation as described above. Before start of irradiation, cells were thoroughly washed to remove IL-1α and medium was changed. Immediately after the 6 hours of irradiation, conditioned media were collected. New, treatment-naive cells were primed with the conditioned media for 48 hours. For inhibition of the IL-1 receptor (IL1R), 100 ng/ml anakinra (Kineret; Swedish Orphan Biovitrum, Langen, Germany) was added to conditioned media in the indicated groups.

2.10. **Inflammasome inhibition**

For inhibition of caspase-1, we applied 10 µM of the caspase-1, -4, and -5 inhibitor Z-YVAD-FMK (BioVision, Munich, Germany) 30 min prior to and during irradiation. Cathepsin B inhibitor CA-074 (Merck/Calbiochem, Darmstadt, Germany) and cathepsin B and L inhibitor Z-FF-FMK (Merck/Calbiochem) were used at a concentration of 10 µM each for 1 hour prior to and during irradiation treatment.

2.11. **Statistical analysis**

All LDH release assays were performed in triplets and all IL-1β ELISA experiments in doublets according to the assay manufacturers’ recommendations. Results are presented as mean ± standard deviation. Statistical analyses were performed using two-tailed unpaired Student’s t test. Differences were considered statistically significant at p<0.05. In all figures, significance levels as are indicated using ns for not significant, * for p<0.05, ** for p<0.01, and *** for p<0.001.

3. **Results**

3.1. **Inflammasome priming increase cell death by lipofuscin phototoxicity.**

The cell culture model used in this study was characterized previously in detail. Briefly, we demonstrated in pRPE cells and ARPE-19 cells that incubation with HNE-modified POS induces lipofuscin accumulation [12,14] and that subsequent blue light irradiation results in photooxidative damage to lysosomal membranes and cell death [8]. Substances such as IL-1α or C5a represent priming signals for the inflammasome in RPE cells that induce expression of pro-
IL-1β [5,9]. In primed RPE cells, LMP by photooxidative damage results in activation of the NLRP3 inflammasome with release of IL-1β and IL-18 [8,9].

To investigate the effect of inflammasome priming on the extent of photooxidative damage-induced RPE cell death, we analyzed the time-course of cell death-associated loss of plasma membrane integrity (LDH release) over 48 hours in ARPE-19 cells treated by 6 hours of irradiation (Figure 1A) and pRPE cells irradiated for 3 hours (Figure 1B). At the beginning of the time-course, all treatment groups exhibited full viability, including the HNE-POS group that had received 7 days of HNE-POS treatment prior to the start of the experiment and the C5a and IL-1α groups that had been exposed to the respective substance for 48 hours preceding the experiment. This indicates that none of these treatments alone had an effect on cell viability without irradiation. Likewise, irradiation alone did not result in cell death in lipofuscin-free unprimed control cells (viability of 96% after 48 hours, p=0.3).

Irradiation of lipofuscin-loaded unprimed cells, however, resulted in a significant reduction of cell viability. The measured loss of plasma membrane integrity occurred delayed with a peak between 12 and 24 hours after the start of irradiation. Priming with either IL-1α or C5a prior to irradiation significantly altered the time course of induced cell death. Primed cells responded to irradiation with rapid cell lysis that was most pronounced within the first 6 hours after the start of irradiation. More importantly, the extent of cell death was also affected by inflammasome priming with priming resulting in a significant amplification of the observed phototoxic effect. E.g. viability measured in lipofuscin-loaded ARPE-19 cells 24 hours after the start of the 6 hours irradiation treatment was reduced from 56.7% in unprimed cells to 28.3% in C5a-primed cells (p=0.008) and 5.1% in IL-1α-primed cells (p=0.0002; Figure 1A). Similarly, priming by C5a and IL-1α reduced viability in lipofuscin-loaded pRPE cells 24 hours after the start of the 3 hours irradiation from 65.3% to 33.1% (p=0.004) and 22.6% (p=0.003), respectively (Figure 1B).

We have previously demonstrated that inflammasome-induced IL-1β release is stronger following IL-1α priming compared to C5a priming [9], possibly due to a stronger effect of IL-1α on the induction of inflammasome components such as pro-IL-1β. Our current observation that inflammasome-mediated cytotoxicity is more pronounced following IL-1α priming compared to C5a priming is consistent with these previous results. The finding that priming increased the cytotoxic effect of irradiation even in cells without lipofuscin-loading by HNE-POS incubation
may be due to the accumulation of autophagy-derived lipofuscin as previously described [8,14].

In summary, our results suggest that inflammasome priming by substances such as C5a or IL-1α increase the susceptibility of RPE cells to photooxidative damage-mediated cell death.

3.2. **Inflammasome priming changes the mechanism of lipofuscin phototoxicity-induced cell death from apoptosis to pyroptosis**

The differences between unprimed and primed cells in the time course of cell lysis suggest different underlying mechanisms of cell death. To delineate the predominant mechanism of cell death in each group, we analyzed additional features of cell death following lipofuscin loading and light irradiation (Figure 2). Morphological investigations by light microscopy demonstrated the occurrence of plasma membrane blebbing and cell shrinkage in unprimed irradiated cells whereas primed cells exhibited early cell swelling after irradiation (Figure 2A). Analysis of DNA fragmentation by TUNEL assay revealed a progressive increase in TUNEL staining over 24 hours after irradiation in unprimed cells (Figure 2B). Primed cells likewise exhibited positive TUNEL labelling that, however, was already detectable immediately after irradiation. In flow cytometric analysis of cells double-labelled with annexin V-FITC and PI to differentiate healthy (FITC-/PI-), apoptotic (FITC+/PI-) and necrotic/pyroptotic (FITC+/PI+) cells, the majority of unprimed irradiated cells were apoptotic whereas primed irradiated cells were predominantly necrotic/pyroptotic (Figure 2C). In addition to these findings, we previously demonstrated that HNE-POS/blue light treatment of IL-1α primed RPE cells as employed in this study results in caspase-1 activation [8] and that the treatment induces release of IL-1β and IL-18 in both IL-1α primed and C5a primed but not unprimed RPE cells [9].

In summary, unprimed cells exhibited delayed cell lysis, plasma membrane blebbing and cell shrinkage, TUNEL-positive DNA fragmentation, predominantly annexin V-positive/PI-negative cell staining, and lack of IL-1β and IL-18 release. In contrast, primed cells demonstrated cell swelling and early cell lysis, TUNEL-positive DNA degradation, predominantly PI-positive cell staining, caspase-1 activation, and release of IL-1β and IL-18. These combinations of features identify the cell death in unprimed and primed cells as apoptosis and pyroptosis, respectively [16]. Thus, our results reveal that inflammasome priming changes the predominant cell death mechanism induced by photooxidative damage in RPE cells from apoptosis to pyroptosis.
3.3. Interleukin-1β release secondary to LMP is closely associated with pyroptotic cell lysis

IL-1β can be released passively during pyroptotic cell lysis (terminal release) as well as by active processes not requiring cell death, with the predominant mechanism of release depending on cell type and stimulus type and strength [17,18]. Elucidating the association of inflammasome-mediated IL-1β release and cell death in RPE cells may help to understand the role of RPE inflammasome activation in different disease stages of AMD. To determine whether IL-1β release by RPE cells secondary to LMP-induced inflammasome activation can occur independent of pyroptosis, we simultaneously measured release of IL-1β and LDH in cells exposed to increasing durations of lipofuscin/light treatment, increasing durations of the chemical LMP-inducer Leu-Leu-OMe, or increasing concentrations of Leu-Leu-OMe (Figure 3). In all experimental conditions tested, significantly increased IL-1β release was detectable only in association with significant LDH release but never without it. This finding suggests that at least in our experimental setting, IL-1β release by RPE cells secondary to LMP occurs in association with pyroptotic cell lysis, suggesting terminal release of IL-1β as the predominant mechanism.

3.4. Priming by conditioned media of inflammasome-activated RPE cells increases cell death by lipofuscin phototoxicity

Similar to IL-1α, the inflammasome-regulated cytokine IL-1β can itself induce inflammasome priming via the IL-1 receptor and thus initiate a paracrine amplification loop of inflammasome activation [9,19,20]. We therefore sought to investigate whether cytokine release of RPE cells following inflammasome activation increases the susceptibility of neighboring RPE cells to photooxidative damage-mediated cell death. For this, we assessed the cytotoxic effect of lipofuscin/light treatment in RPE cells after priming with conditioned media of inflammasome-activated RPE cells (Figure 4).

Incubation with conditioned media for 48 hours did not itself affect cell viability as indicated by the full viability of all treatment groups measured at the beginning of irradiation. After irradiation, cells primed with conditioned media of non-irradiated control cells demonstrated a delayed time course of cell death. In contrast, cells primed with conditioned media of irradiated cells exhibited rapid cell lysis, suggestive of pyroptosis. More importantly, the extend of cell death 48 hours after irradiation was significantly increased in cells primed with conditioned
media of irradiated cells as compared with cells primed with conditioned media of non-irradiated control cells (viability 45% vs. 16%, p=0.0006). Inhibition of IL-1 receptor (IL1R) by the drug anakinra during priming completely prevented the effect of conditioned media on cytotoxicity whereas recombinant IL-1β had an priming effect similar to conditioned media of irradiated cells.

These results suggest that cytokines such as IL-1β released by RPE cells after inflammasome activation increase the susceptibility of bystander RPE cells to photooxidative damage-induced cell death, possibly via their previously demonstrated IL1R-mediated priming effect [9].

3.5. Inhibition of Inflammasome activation reduces cell death by lipofuscin phototoxicity

Inflammasome activation in RPE cells by photooxidative damage is dependent on lysosomal leakage of cathepsins and activation of caspase-1 [8]. To further delineate the mechanism by which inflammasome priming increased susceptibility to phototoxic cell death, we subjected ARPE-19 cells and pRPE cells to inhibition of cathepsin B/L and caspase-1 during irradiation (Figure 5). As expected, apoptotic cell death of unprimed ARPE-19 cells was unaffected by cathepsin B or caspase-1 inhibition (Figure 5A). In contrast, pyroptosis of primed ARPE-19 cells was partially prevented by inhibition of cathepsin B/L or caspase-1 (Figure 5B). Moreover, inhibitor treatment changed the time course of cell death in primed cells back towards a delayed type similar to that observed in unprimed cells. In pRPE cells, cathepsin B/L and caspase-1 inhibition experiments yielded similar results (Figure 5C and Figure 1B). These findings demonstrate that the increased susceptibility of primed RPE cells to photooxidative cell death is dependent on activity of cathepsin B/L and caspase-1.

4. Discussion

Blue light irradiation of RPE cells in vitro results in lipofuscin-dependent generation of reactive oxygen species [21], LMP by oxidative damage [22,23], and cell death [24]. We have demonstrated that LMP by lipofuscin-mediated photooxidative damage not only results in oxidative-damage dependent cell death but also in activation of the NLRP3 inflammasome in primed RPE cells [8,9]. This mechanism may underlie the inflammasome activation observed in the RPE of AMD patients [3,5] and may contribute to the progressive RPE damage in AMD.
Inflammasome activation by photooxidative damage-mediated LMP requires prior inflammasome priming that can be induced by substances such as IL-1α or the complement activation product C5a [5,9]. In the absence of a priming signal, the mechanism of cell death secondary to oxidative or photooxidative damage-induced LMP has been identified as apoptosis in various cell types other than RPE [25,26]. Our combined results from this and previous studies demonstrate that LMP-induced cell death in RPE cells likewise occurs by apoptosis as characterized by cell membrane blebbing, late loss of cell membrane integrity, lack of IL-1β and IL-18 release [9], annexin V-positive/PI-negative staining, and positive TUNEL labelling. In contrast, cells exposed to the priming signals IL-1α or C5a prior to photooxidative damage-mediated LMP exhibit a distinctly different mechanism of cell death that we identified as pyroptosis by means of cell swelling, early loss of cell membrane integrity, caspase-1 activation [8], IL-1β and IL-18 release [9], lack of annexin V-positive/PI-negative staining, and positive TUNEL labelling. Importantly, inflammasome priming resulted not only in a different mechanism but also in a significantly higher extent of photooxidative cell death. This increased susceptibility to phototoxicity was reversible by treatment of RPE cells with inflammasome inhibitors such as caspase-1 inhibitor Z-YVAD-FMK.

Evidence from genetic studies indicates an important role for complement activation in the pathogenesis of complex, multifactorial AMD. Activated complement components such as C5a are abundant both in the sub-RPE space and in plasma of AMD patients [27,28]. Several inhibitors of C5 or complement components upstream of C5 activation such as CFD and C3 are currently under clinical investigation for their potential to prevent progressive RPE cell death in patients with GA secondary to AMD. We have previously shown that the C5 activation product C5a primes RPE cells for inflammasome activation [9]. Here, we demonstrate that inflammasome priming agents such as IL-1α and C5a increase the susceptibility of RPE cells to oxidative damage-mediated cell death. Thus, our results support the rational for therapeutic complement inhibition in atrophic AMD. In addition, agents with direct inhibitory effects on inflammasome activation such as small molecule NLRP3 inhibitors have been shown to be effective in vivo and, thus, represent interesting candidates for future interventional approaches in atrophic AMD [29].

IL-1β induces expression of its own precursor pro-IL-1β via IL1R [19,20]. We previously reported that conditioned media from RPE cells collected following inflammasome-mediated IL-1β release induces IL1R-dependent inflammasome priming in other RPE cells, thus providing an
paracrine amplification loop of inflammasome activation [9]. Consistently, our current results indicate that conditioned media of pyroptotic ARPE-19 cells increase cell death by photooxidative damage in other RPE cells in an IL1R-dependent fashion. It is conceivable that in situations of localized RPE cell death such as in atrophic AMD, this mechanism could result in increased susceptibility of bystander RPE cells to inflammasome-mediated cell death and thus contribute to the progressive RPE cell loss in AMD.

IL-1β is a major inflammatory effector cytokine of inflammasome activation. As IL-1β lacks a secretory signal sequence it is not secreted by conventional routes. Rather, its release occurs either actively from the living cell via secretory lysosomes, microvesicles, or exosomes or passively by cell lysis during cell death (terminal release) [17,18]. The prevailing mechanism can differ with cell type and stimulus type and strength. To elucidate the predominant mechanism of IL-1β release in RPE cells, we subjected ARPE-19 cells to increasing durations of lipofuscin/light treatment or increasing concentrations and incubation times of Leu-Leu-OMe, a substance that induces inflammasome activation by LMP similar to lipofuscin phototoxicity [5,15]. In these experiments, IL-1β release occurred only in association with loss of cell membrane integrity as measured by LDH release. This suggests that in our experimental setting, IL-1β is released from the cell predominantly during cell death-associated loss of plasma membrane integrity rather than by active mechanisms of the living cells. As RPE cell death in AMD occurs mainly in late-stage disease, further elucidation of the mechanisms of IL-1β release in RPE cells in vivo will help to delineate the AMD stages in which inflammasome activation in the RPE may play a pathogenic role.

In summary, we examined the mechanisms of photooxidative damage-induced cell death in pRPE cells and ARPE-19 cells and demonstrated that inflammasome priming by substances such as IL-1α and C5a not only changes the cell death mechanism from apoptosis to pyroptosis but also increases the cells’ susceptibility to photooxidative damage-mediated cytotoxicity. These results provide new insights into the complex interplay of complement system, inflammasome activation, oxidative damage, and RPE cell pathology in diseases such as AMD and suggest inhibition of inflammasome priming or activation as potential treatment strategies for atrophic AMD.
Conflict of interest


Acknowledgment

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References


Figure legends

Figure 1. Inflammasome priming increases cell death by lipofuscin phototoxicity. (A) ARPE-10 cells and (B) pRPE cells were incubated with HNE-POS to induce lipofuscin accumulation or were incubated without POS. In addition, cells were primed for inflammasome activation by C5a or IL-1α or left unprimed. Subsequently, all treatment groups were irradiated with blue light for 6 hours (ARPE-19) or 3 hours (pRPE). The time-course of cellular viability was assessed by means of LDH release over 48 hours after the start of irradiation treatment. Significance levels for primed cells as compared to the unprimed HNE-POS group are indicated $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***)
Figure 2. Inflammasome priming changes the mechanism of lipofuscin phototoxicity-induced cell death from apoptosis to pyroptosis. (A) Differences in cell death morphology between unprimed and IL-1α-primed ARPE-19 cells following HNE-POS/light treatment were assessed by light microscopy. Images of unprimed cells demonstrate plasma membrane blebbing (arrowheads) suggestive of apoptosis. In contrast, IL-1α-primed cells exhibited early cell swelling (arrowheads). Scale bars represent 100 μm (upper row) and 50 μm (lower row). (B) DNA degradation/fragmentation was analyzed by TUNEL labelling in unprimed and IL-1α-primed ARPE-19 cells at different time points after start of irradiation. DNA fragmentation was detectable in both unprimed and IL-1α-primed cells but occurred earlier in primed cells. Scale bars represent 100 μm. (C) Flow cytometry was employed to assess annexin V-FITC and PI staining in unprimed and IL-1α-primed ARPE-19 cells immediately following irradiation.
Figure 3. Interleukin-1β release secondary to LMP is closely associated with pyroptotic cell lysis. (A) HNE-POS-treated ARPE-19 cells were primed with IL-1α and irradiated with blue light for increasing durations to induce LMP. Alternatively, LMP was induced by treatment of IL-1α-primed ARPE-19 cells with (B) increasing incubation times Leu-Leu-OMe at a concentration of 1 mM or with (C) increasing concentrations of Leu-Leu-OMe for an incubation time of 3 hours. In all experiments, simultaneous analysis of IL-1β release and cell lysis assessed by LDH release was performed. Significance levels compared to the respective untreated control groups are indicated as not significant (ns), p<0.05 (*), p<0.01 (**), and p<0.001 (***)

Figure 4. Priming by conditioned media of inflammasome-activated RPE cells increases cell death by lipofuscin phototoxicity. ARPE-19 cells were incubated with HNE-POS and primed
with conditioned media that had been collected from IL-1α-primed ARPE-19 cells treated by HNE-POS without irradiation (HNE-POS / 0h light) and by HNE-POS and 6 hours irradiation (HNE-POS / 6h light). In control experiments, the IL1R inhibitor anakinra was added during priming with conditioned media from HNE-POS/light-treated cells (HNE-POS / 6h light + IL1R inhibitor), or cells were primed with recombinant human IL-1β instead of conditioned media (IL-1β). Following priming, all treatment groups as well as an untreated, unprimed control group (Control) were subjected to irradiation for 6 hours, and the time-course of cellular viability was analyzed by LDH release assay over 48 hours after the start of irradiation. Significance levels of the HNE-POS / 6h light group as compared to the HNE-POS / 0h light group are indicated as p<0.05 (*), p<0.01 (**), and p<0.001 (***).

**Figure 5.** Inhibition of inflammasome activation reduces cell death by lipofuscin phototoxicity. (A) The time-course of cell viability was measured in unprimed ARPE-19 cells over 48 hours after the start of irradiation treatment without prior incubation with POS (Control) or after incubation with HNE-POS. For inhibition of caspase-1 and cathepsin B, cells were co-incubated with Z-YVAD-FMK and CA-074, respectively, during irradiation. In addition, we analyzed the
time-course of viability of (B) IL-1α-primed ARPE-19 cells and (C) IL-1α-primed pRPE cells following HNE-POS/light treatment and additional inhibition of caspase-1 and cathepsin B/L by Z-YVAD-FMK and Z-FF-FMK, respectively. Significance levels of inhibitor-treated cells as compared to the respective HNE-POS groups without inhibitor treatment are indicated as not significant (ns), p<0.05 (*), p<0.01 (**), and p<0.001 (***).