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Decline in cellular clearance systems induces inflammasome signaling in human ARPE-19 cells

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ABSTRACT

Retinal pigment epithelium (RPE) plays a major role in the maintenance of photoreceptors, and degeneration of RPE results in the development of age-related macular degeneration (AMD). Accumulation of intracellular protein aggregates, increased oxidative stress, and chronic inflammation are all factors damaging the functionality of aged RPE cells. Here, we report that inhibition of proteasomal degradation with MG-132 and autophagy with bafilomycin A1 resulted in the release of IL-1β but not that of IL-18 in human ARPE-19 cells. NLRP3 receptor became upregulated, and caspase-1, the functional component of an inflammasome complex, was activated. In addition to accumulating intracellular protein aggregates, inhibition of degradation systems induced oxidative stress which was demonstrated by elevated amounts of intracellular 4-hydroxynonenal (HNE)-protein adducts. Along with IL-1β, exposure to MG-132 and bafilomycin A1 resulted in the secretion of IL-8. A low concentration (1 pg/ml) of IL-1β was capable of triggering significant IL-8 production which also became attenuated by treatment with a specific caspase-1 inhibitor. These results suggest that decline in intracellular degradation systems results not only in increased amounts of intracellular protein aggregates and oxidative stress but also in the activation of NLRP3 inflammasomes, arisen as a result of elevated production of biologically active IL-1β.

1. Introduction

Retinal pigment epithelium (RPE) is a single-cell layer of postmitotic cells, which performs many important functions in the human retina. RPE cells play a crucial role in maintaining the function of photoreceptor cells by ingesting and degrading the spent tips of photoreceptor outer segments (POS) and by providing photopigments to rods and cones [1–3]. RPE cells also transport nutrients and waste products between the sensory retina and choroid layers, protect the outer retina from light-generated oxygen reactive species (ROS), and take responsibility for the immune defense of macula [2,4]. Due to their extremely active metabolism, high oxygen consumption, and prolonged exposure to light RPE cells are subjected to high levels of oxidative stress [5]. Oxidative stress, concurrently with the age-related decrease in antioxidant production, disturbs homeostatic regulation by RPE, and contributes to the accumulation of aggregates by oxidizing cellular macromolecules [3]. Moreover, age-related accumulation of nondegradable lipofuscin in the lysosomes of RPE cells disturbs the degradation of waste material and aged mitochondria resulting in the increased production of reactive oxygen species (ROS) [6]. Since lipofuscin is a photoinducible material, it can accelerate oxidative stress also by itself [6]. Lipofuscin contains lipid peroxidation- or glucoxidation-induced end products 4-hydroxynonenal (HNE), malondialdehyde (MDA), and advanced glycation end products (AGEs) which have been associated with decreased proteolysis, inflammation, and RPE degeneration [7]. A dysfunction of RPE cells is the primary event leading to the photoreceptor damage, loss of vision, and the clinically observed changes encountered in AMD [8].

Autophagy and the ubiquitin-proteasome pathway (UPP) are two central degradation systems in mammalian cells [9]. UPP plays a key role in eliminating short-lived and misfolded proteins that cannot be repaired by chaperones. Autophagy is a specialized process for eliminating long-lived proteins, protein aggregates and cell organelles [9]. In the final phase of autophagy, the cargo of autophagosomes is digested by lysosomal hydrolases [10]. When degradation systems are functioning

Abbreviations: AMD, Age-related macular degeneration; BafA, Bafilomycin A; HNE, 4-Hydroxy-2-nonenal; LDH, Lactate dehydrogenase; RPE, Retinal pigment epithelium

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well, waste material does not accumulate and thus it does not induce adverse effects, such as increased oxidative stress, and tightly controlled cellular functions, e.g. proliferation, cell survival and signal transduction progress without disturbance. However, both proteasomal and autophagosomal degradation are known to decline in aged RPE cells [11,12].

It has been shown recently that autophagy is capable of regulating inflammasomes, and it probably inhibits the intracellular signaling by removing dysfunctional mitochondria that would otherwise produce increased amounts of ROS [13,14]. Furthermore, cytosolic mtDNA can contribute to inflammasome activation [13–15]. Inflammasomes are intracellular protein complexes that play a major role in the induction of inflammation. Currently, the best-known inflammasomes are those having NLRP3 (Nucleotide-binding domain and Leucine rich repeat Receptor containing a Pyrin domain 3) as their receptor component. The activation of NLRP3 inflammasomes is a two-step process. The priming signal can come either through TLR (Toll-like receptor) or cytokine receptors, and this results in the production of NLRP3 protein and the inactive pro-forms of proinflammatory cytokines IL-1β and IL-18 [16,17]. Oligomerization of NLRP3 after sensing its activator enables the complex to recruit the adaptor protein ASC (apoptosis-associated speck-like protein containing caspase recruitment domain [CARD]) and pro-caspase-1 to the complex, which results into autoactivation of the enzyme. Thereafter, activated caspase-1 is able to process pro-forms of IL-1β and IL-18 into their mature and secreted forms. Lately, several research groups have linked the activation of NLRP3 inflammasomes to RPE degeneration and AMD pathogenesis [18–20].

In the present study, we have explored the effect of dysfunctional degradation machinery in RPE cells on inflammasome signaling. We induced intracellular protein aggregates in human ARPE-19 cells by inhibiting proteasomal degradation with MG-132, and prevented the autophagy-mediated clearance of aggregates by neutralizing lysosomal enzymes with the antibiotic, bafilomycin A1. Thereafter, we determined inflammasome activity. Our data suggest that decline in intracellular degradation systems is capable of inducing inflammasome signaling and increasing the secretion of IL-1β without elevating the production of IL-18. Particularly low instead of high intracellular amounts of HNE-protein adducts seem to favor IL-1β release over IL-18. Inflammasome activation with IL-1β liberation also shows biological relevance in its capability to induce the production of IL-8.

2. Materials and methods

2.1. Cells and stimulations

ARPE-19 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultivated in a humidified 10% CO_2 atmosphere at 37 °C in Dulbecco's MEM/Nut MIX F-12 (1:1) medium (Life Technologies, Paisley, UK) containing 10% inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin (Cambrex, Charles City, IA, USA), 100 μg/ml streptomycin (Cambrex), and 2 mM L-glutamine (Life Technologies). Confluent cell cultures were washed with serum-free DMEM/F12 medium and stimulated with MG-132 (5 μM; Calbiochem, San Diego, CA) in the serum-free medium for 24 h in a humidified 10% CO_2 atmosphere at 37 °C. After incubation, the cells were further stimulated with bafilomycin A1 (50 nM; Sigma-Aldrich, Steinheim, Germany) in fresh serum-free medium. The samples were collected after 24 h incubation in a humidified 10% CO_2 atmosphere at 37 °C. Cell cultures were also treated with irreversible caspase-1 inhibitor (20 μM; caspase-1 inhibitor II, Ac-YVAD-CMK, Calbiochem, San Diego, CA) prior to both MG-132 and bafilomycin A1 stimulations where indicated. Both MG-132 and bafilomycin A1 were solubilized in DMSO, which alone did not induce any significant release of cytokines nor did treatment with a caspase-1 inhibitor alone (data not shown). In addition, we tested boosting of inflammasome priming by stimulating cells with lipopolysaccharide (LPS, 10 μg/ml; Sigma) for 24 h in a humidified 10% CO_2 atmosphere at 37 °C before the exposure to MG-132. Where indicated, cells were also stimulated with recombinant human (rh)IL-1β (1 pg/ml; Life Technologies, Paisley, UK) or by different concentrations of 4-hydroxynonenal (HNE) for 24 h in a humidified 10% CO_2 atmosphere at 37 °C.

2.2. Sample preparation

Medium samples and cell lysates were collected 24 h after the bafilomycin A1 treatment. Cells were rinsed with 1× DPBS (Dulbecco's phosphate buffered saline, Lonza Group Ltd., Switzerland) before lysis with M-PER solution (Thermo Scientific, Waltham, MA, USA) for Western blot analyses and with the cell lysis buffer provided by Cell Signaling Technologies (Danvers, MA, USA) for ELISA measurements. Cell lysates were centrifuged, and the supernatants were transferred to clean tubes. In the caspase-1 activity assays, lysis buffer was provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). The lysates for ELISA assays were sonicated 3 × 10 s. Both medium samples and cell lysates were stored at −70 °C until analyzed.

2.3. ELISA measurements

Concentrations of pro-inflammatory cytokines IL-1β, IL-6, and IL-8 were determined using commercial enzyme-linked immunosorbent assay (ELISA) reagents (BD Pharmingen, San Jose, CA, USA). IL-1β was also measured using a high-sensitive ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's protocol. Since IL-1β ELISA assays do not commonly specify, whether they measure only the mature cytokine or also the inactive pro-form, an ELISA kit designed specifically to detect pro-IL-1β (R&D Systems, United Kingdom) was also used. Additionally, concentrations of IL-18 and NLRP3 were measured using commercial ELISA kits (eBioscience, San Diego, CA, USA and Cusabio, Wuhan, China, respectively). Since IL-18 was not detected, the result was verified using an ELISA kit from another manufacturer (MBL International).

2.4. Caspase-1 activity assay

Caspase-1 activity was measured from cell lysates using a commercial kit from R&D Systems according to the manufacturer's protocol. Results were normalized to protein concentrations measured by the Bradford method [21] from cell lysates.

2.5. Cell viability assay

In order to determine the integrity of the cellular membrane, the amount of lactate dehydrogenase (LDH) was measured from medium samples with a commercial kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

2.6. Western blot assays

For detecting pro-caspase 1, whole 20 μg of protein in cell lysates were run in 15% SDS-PAGE gels and then wet-blotted onto nitrocellulose membranes (Amersham, Pittsburgh, PA, USA). Transfer of proteins from gels to membranes was ensured by staining with Ponceau S (Sigma-Aldrich, St. Louis, MO, USA). The membranes were blocked overnight in 5% fat-free milk in 0.1% Tween-20/Tris buffered saline (TBS; 50 mM Tris, 150 mM NaCl) at +4 °C. The blocking buffer was also used as a washing buffer and a diluent of primary and secondary antibodies. Thereafter, the membranes were incubated for 2 h at RT with a rabbit monoclonal caspase-1 antibody (1:1000; Cell Signaling Technologies). After washing four times for 10 min, the membranes were incubated for 1 h at RT with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:10,000; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The membranes were washed before detection

as mentioned above. Protein–antibody complexes were detected with an enhanced chemiluminescent assay for horseradish peroxidase (Millipore, Billerica, MA, USA), and normalized to α-tubulin values using mouse monoclonal α-tubulin (Sigma-Aldrich, St. Louis, MO, USA) and polyclonal HRP-conjugated sheep anti-mouse IgG (GE Healthcare Life Sciences) antibodies. The results were quantified using the ImageJ program (http://rsb.info.nih.gov/ij/index.html).

HNE was detected by loading 10–20 μg of protein in cell lysates to 12.5% SDS-PAGE gels. After electrophoresis, proteins were wet-blotted to nitrocellulose membranes (Millipore, Bedford, MA, USA). Equal transfer was checked as described above. After blocking in 5% fat-free milk solution for 60 min at 37 °C, the membranes were treated with a rabbit monoclonal HNE (Alpha Diagnostics, San Antonio, TX, USA) and DyLight 800-conjugated goat anti-rabbit IgG antibodies (Thermo Scientific, Rockford, IL, USA) as described previously by Oksala et al. [22]. The results were normalized to β-actin (Sigma) values using mouse monoclonal β-actin (Sigma) and DyLight 800-conjugated goat anti-mouse IgG antibodies. Immunoblots were visualized with an Odyssey imaging system (Li-Cor Biosciences Inc., Lincoln, NB, USA) and quantified using the Odyssey Software.

2.7. NLRP3 siRNA treatments

ARPE-19 cells at 60–80% confluency were washed with fresh DMEM/F12 medium and treated with Silencer Select siRNA (ID: s41556; Ambion by Life Technologies, Europe BV) targeting the NLRP3 gene using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen by Life Technologies) according to the manufacturer's instructions. Silencer Select Negative Control siRNA and Silencer Select GAPDH siRNA served as negative and positive controls, respectively. Following the 24 h transfection, cells were treated with MG-132 and bafilomycin A1 as previously described. Total RNA was extracted from cells using the Illustra RNAspin Mini Kit (GE Healthcare Life Sciences).

2.8. Real-time PCR

Total RNA was reverse-transcribed to cDNA using SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen by Life Technologies Europe BV). The expression of human GAPDH and human NLRP3 RNAs was studied with the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems by Life Technologies Europe BV) using SYBR® Green chemistry (Applied Biosystems). The primers purchased from TAG Copenhagen (Copenhagen, Denmark) were forward 5′-CGAGAT CCCTCCAAAATCAA-3′ and reverse 5′-GGTGCTAAGCAGTTGGTGGT-3′ for GAPDH, and forward 5′-GGCATATCACAGTGGGATTC-3′ and reverse 5′-GATCTTCGCTGCGATCAAC-3′ for NLRP3. The thermo-cycling program consisted of 40 cycles at 95 °C for 15 s and at 60 °C for 60 s with an initial cycle at 95 °C for 10 min. The melting curve analysis was performed over the range of 60–95 °C. cDNA was semi-quantified and analyzed using the ΔΔCt (change in cycle threshold, Ct) method.

2.9. Statistical analysis

Statistical analyses were conducted using GraphPad Prism (Graphpad Software, San Diego, CA). Pairwise comparisons between independent groups were assessed using the Mann–Whitney *U*-test. Results are presented as mean ± standard error of means (SEM), and *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Intracellular protein aggregates induce IL-1β but not IL-18 secretion in ARPE-19 cells

In order to study the ability of dysfunctional intracellular degradation to activate the inflammasome signaling, ARPE-19 cells were exposed to the proteasome inhibitor, MG-132, for 24 h. Viiri et al. have recently shown that MG-132 treatment induces the accumulation of intracellular aggregates in those cells and under normal conditions, autophagy will clean the aggregates away [23]. In addition to proteasomal inhibition, we therefore exposed our cells to bafilomycin A1 for another 24 h in order to block autophagy. As shown in Fig. 1, MG-132 induced a slight but detectable increase in the release of inflammasome-related cytokine IL-1β. The increase was not statistically significant until the subsequent treatment with bafilomycin A1, which blocked autophagy (Fig. 1). Meanwhile, no increase was observed in the production of IL-18. Due to the low concentrations, the IL-1β production was confirmed from three independent experiments also using a high-sensitivity ELISA kit from another manufacturer (e-Bioscience), and the results were similar as those obtained with the ELISA kit (Becton Dickinson) routinely used in our laboratory (data not shown). Moreover, in order to boost the priming of inflammasome signaling, cells were pretreated with LPS (10 μg/ml) before the exposure to MG-132 and bafilomycin A1. The profile of IL-1β production remained similar, LPS priming only resulted in slightly higher IL-1β concentrations. The highest IL-1β concentrations measured using LPS + MG-132 and LPS + MG-132 + bafilomycin A1 were 1.31 ± 0.16 and 2.6 ± 0.14 pg/ml (mean ± SEM), respectively, whereas control cells and those treated with LPS alone did not produce any measurable levels of IL-1β. Statistical significances were $P < 0.01^{**}$ between both control vs. LPS + MG-132, and control vs. LPS + MG-132 + bafilomycin A1 (Mann–Whitney *U*-test).

3.2. MG-132 + bafilomycin A1 treatment releases mature IL-1β instead of inactive pro-form of the cytokine

Proteasome inhibitor MG-132 with bafilomycin A1 is a robust treatment compromising cell membrane integrity. This can be observed as an increased release of LDH enzyme to the culture medium. In these experiments, ARPE-19 cells tolerated bafilomycin A1 much better than MG-132 but when bafilomycin A1 was added after exposure to MG-132, the combined effect was even more pronounced than that induced by MG-132 alone (Fig. 2).

In order to ensure that the IL-1β being measured from medium samples was not inactive pro-IL-1β which had leaked out of the cells due to ruptured cell membranes, we measured the concentration of pro-IL-1β from both cell lysates and the medium samples. As shown in Fig. 3 (left panel), there was no significant difference in the amount of pro-IL-1β between groups in the medium or lysate samples. This is important since when the mature cytokine is concerned, MG-132 + bafilomycin A1 greatly and significantly increased the amount of secreted IL-1β when compared to control cell mediums (Fig. 3, right panel). In cell lysates, MG-132 + bafilomycin A1 did not significantly increase the concentration of mature IL-1β (Fig. 3, right panel).

3.3. IL-1β secretion is associated with the inflammasome activation

In order to study the association of inflammasome activation with increased IL-1β secretion in ARPE-19 cells, we measured the caspase-1 activity from cell lysates. As shown in Fig. 4A, MG-132 + bafilomycin A1 treatment significantly increased the caspase-1 activity. Treatment with the inhibitor alone or DMSO did not have any significant effect on caspase-1 activity (data not shown). When the function of caspase-1 was inhibited with a specific inhibitor, MG-132 and bafilomycin A1-mediated IL-1β secretion was significantly reduced (Fig. 4B). Concurrently with the caspase-1 activation, there was also a significant decrease in the intracellular concentrations of pro-caspase-1 (Fig. 4C).

It has been convincingly shown that NLRP3 inflammasome activation can take place in ARPE-19 cells [18–20,24,25], and therefore, we studied the expression of inducible NLRP3 protein in our current samples. These results show that there was a significant increase in the

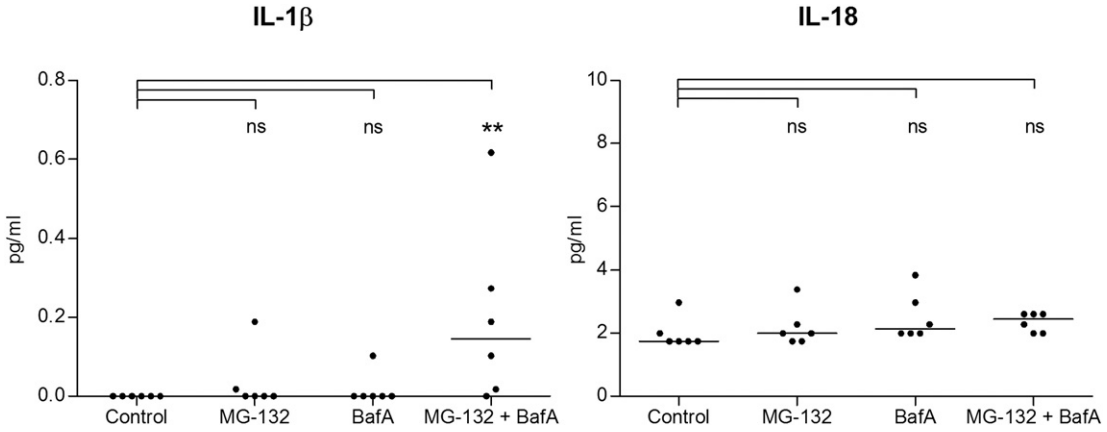

Fig. 1. Production of inflammasome-related cytokines by MG-132 and bafilomycin A1 (BafA). ARPE-19 cells were treated with 5 μM MG-132 for 24 h and exposed to 50 nM bafilomycin A1 for another 24 h. Untreated cells plated at same numbers and grown under similar conditions served as controls. Identical volumes of culture medium samples were taken and IL-1β and IL-18 cytokines were measured with an ELISA technique. Results are presented as mean ± SEM of six parallel samples/group, and they are representative of nine (IL-1β) and five (IL-18) independent experiments. $^{**}P < 0.01$, ns = not significant, Mann–Whitney *U*-test.

amount of NLRP3 occurring after the treatments with MG-132 and bafilomycin A1 when compared to controls (Fig. 5A). Expression of MG-132 + bafilomycin A1-induced NLRP3 was inhibited by specific siRNAs (Fig. 5B).

3.4. MG-132 induces the production of HNE in ARPE-19 cells

We have previously shown that HNE is able to induce NLRP3 inflammasome signaling in ARPE-19 cells, and therefore we measured intracellular amounts of HNE-protein adducts. As shown in Fig. 6A, MG-132 significantly increased the amount of HNE-protein adducts in ARPE-19 cells. The effect of bafilomycin A1 was also statistically significant but much weaker than that of MG-132. When administered together, however, an additive effect was apparent (Fig. 6A). In comparison, our previous treatment of ARPE-19 cells with 30 μM HNE, induced three to four times higher amounts of intracellular HNE-protein adducts than could be obtained with MG-132 + bafilomycin A1 (Fig. 6B). LPS used for the priming of inflammasome signaling did not markedly change the effect of HNE (Fig. 6B). When the cells were treated with different concentrations of HNE, there was a clear trend in the release of higher amounts of IL-1β by the lower HNE concentrations 1 and 5 μM when compared to higher concentrations 15 and 30 μM (Fig. 7).

3.5. IL-8 production induced by MG-132 can be secondary to IL-1β release

In addition to inflammasome-related cytokines, we measured the production of two other pro-inflammatory mediators, i.e. IL-6 and IL-8. We observed that MG-132 + bafilomycin A1 increased the production of IL-8 but not that of IL-6 (Fig. 8). Moreover, the production of IL-8 by MG-132 + bafilomycin A1 declined when the cells were treated with the specific caspase-1 inhibitor prior to stimulation (Fig. 8). Caspase-1 inhibitor had no influence on the production of IL-6 (Fig. 8). As presented in Fig. 9, IL-1β at the concentration of 1 pg/ml significantly increased the IL-8 production. The whole pathway from a decline in degradation systems up to IL-8 production is shown in a

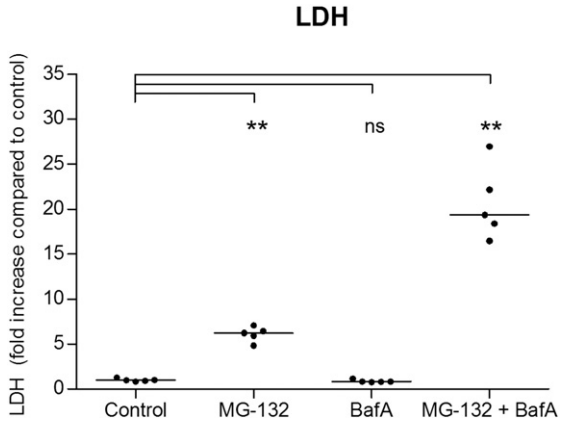

Fig. 2. Effect of MG-132 and bafilomycin A1 (BafA) treatments on cellular viability as measured by the release of LDH. Enzyme levels were measured from medium samples using a commercial kit after exposing cells consecutively to MG-132 for 24 h and then to bafilomycin A1 for 24 h where indicated. Data were normalized to protein concentrations and compared to control values. Results are shown as mean ± SEM of six parallel samples/group, and they are representative of four (MG-132 and bafilomycin A1 alone) and six (MG-132 + bafilomycin A1) independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, ns = not significant, Mann–Whitney *U*-test.

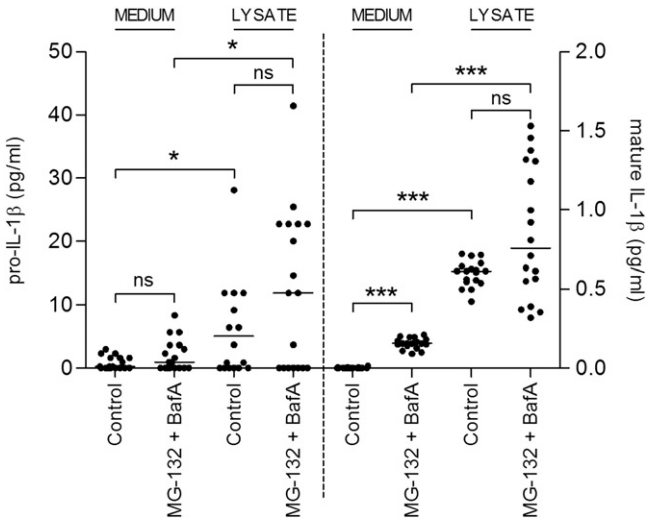

Fig. 3. Concentrations of pro (left panel) and mature (right panel) IL-1β inside and outside of the cell. ARPE-19 cells were treated with MG-132 (24 h) and bafilomycin A1 (BafA, 24 h), and both medium and cell lysate samples were collected. pro and mature forms of IL-1β were measured using ELISA. Data are combined from three independent experiments with six parallel samples in each group/experiment. IL-1β has been measured using a highly sensitive IL-1β kit. The results are presented as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$, ns = not significant, Mann–Whitney *U*-test.

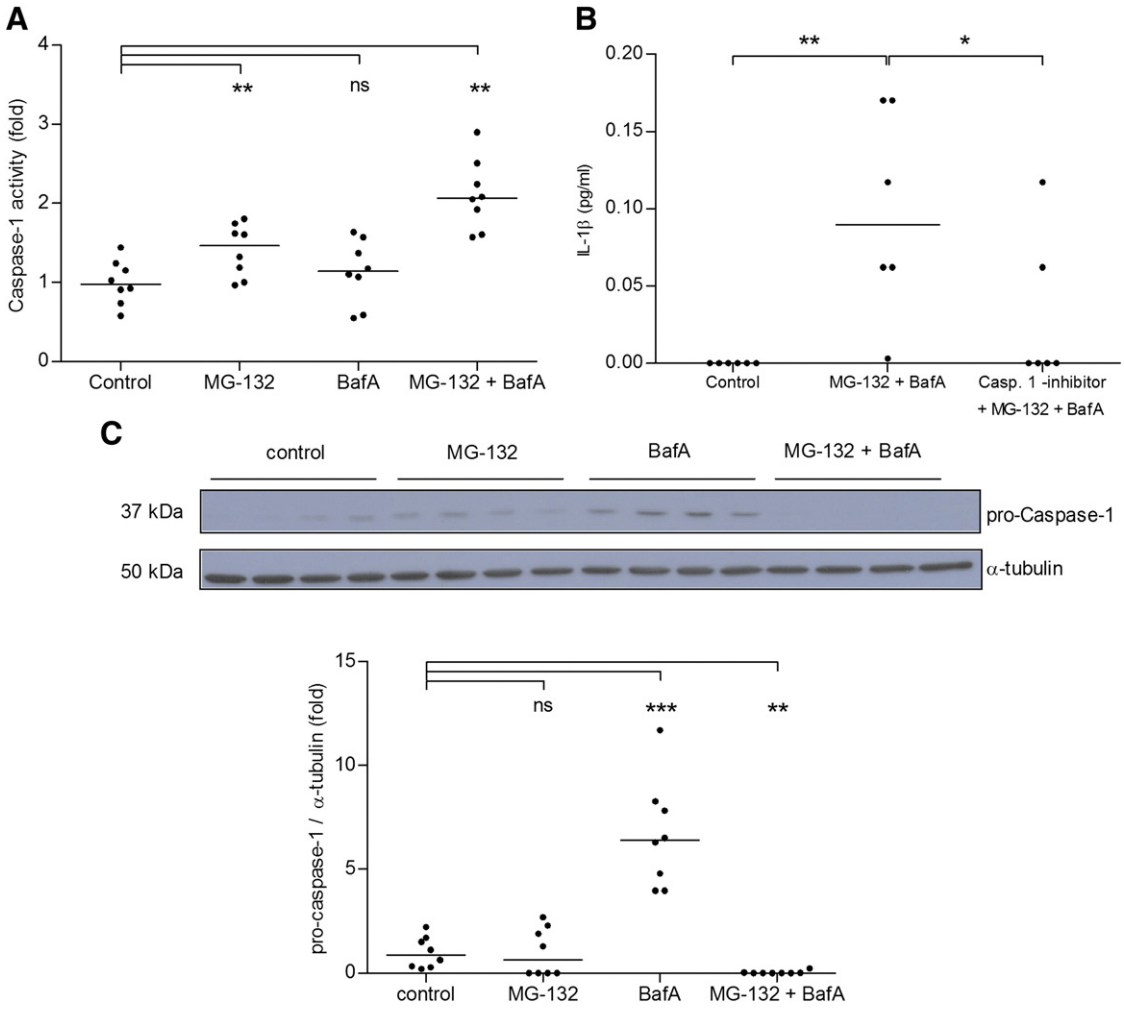

Fig. 4. Activity of caspase-1 following the MG-132 and bafilomycin A1 (BafA) treatments. Cells were treated first with MG-132 for 24 h and then with bafilomycin A1 for another 24 h. The caspase-1 inhibitor (20 μM) was added prior to each treatment where indicated. Medium samples and cell lysates were collected for analyses. (**A**) Caspase-1 activity measured from the cell lysates using a commercial kit. Data are combined from four independent experiments with two parallel samples in each group/experiment. (**B**) Effect of caspase-1 inhibitor on the production of IL-1β by MG-132 + bafilomycin A1. Results measured from medium samples by ELISA are representative of three independent experiments with six parallel samples/group. (**C**) Amount of pro-caspase-1 in cell lysates. The pro-enzyme concentration was measured using the Western blot technique, and a representative blot picture is shown. Densitometric data are combined from two independent analyses with four parallel samples/group. All data in bar graphs are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns = not significant, Mann–Whitney *U*-test.

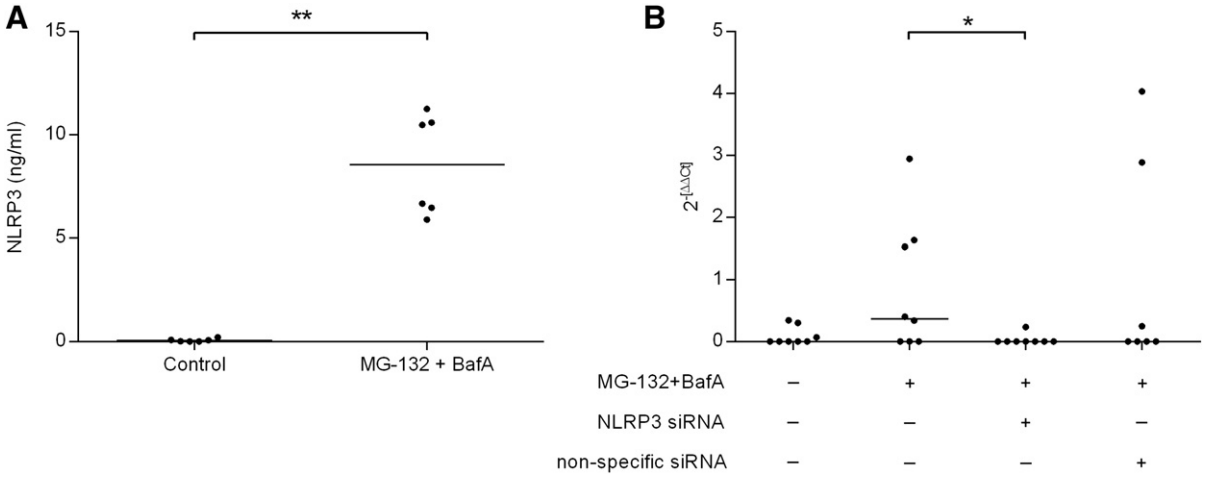

Fig. 5. Effect of MG-132 and bafilomycin A1 (BafA) on the NLRP3 expression. (**A**) Cells were treated with MG-132 and bafilomycin A1 for 24 h each, and culture medium samples were analyzed for the level of NLRP3 protein using ELISA. Results are representative of three independent experiments with six parallel samples/group. (**B**) siRNA treatment (24 h) preceded the exposures to MG-132 and bafilomycin A and thereafter, the expression of NLRP3 was determined using qPCR. Data are combined from two independent experiments with four parallel samples in each group/experiment. Results are presented as mean ± SEM. $^{*}P < 0.05$, Mann–Whitney *U*-test.

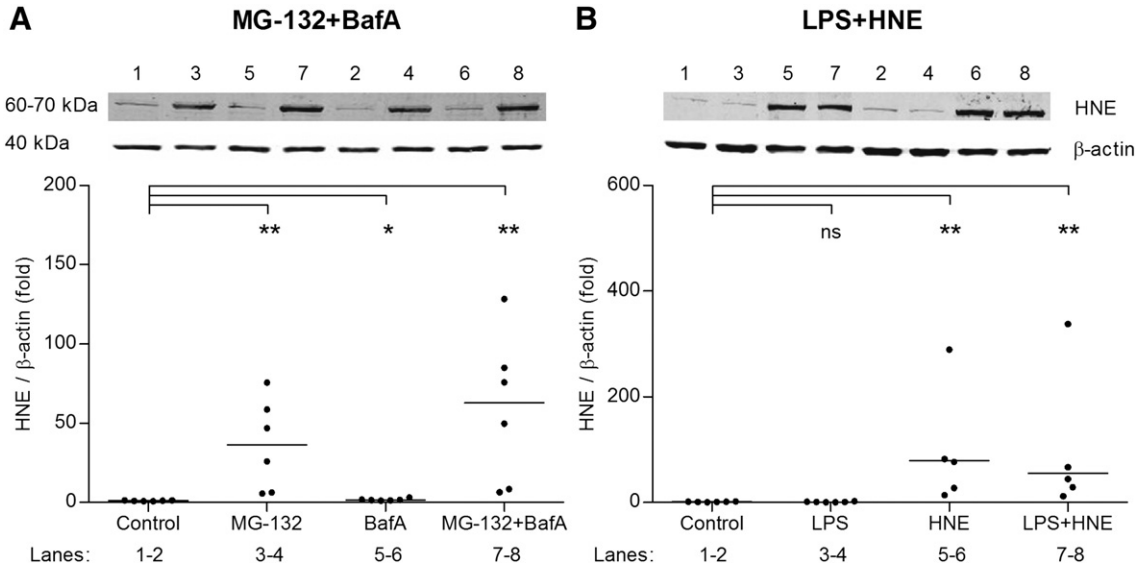

Fig. 6. Effects of **(A)** MG-132 + bafilomycin A1 (BafA), and **(B)** LPS + HNE treatments on the formation of HNE-protein adducts in ARPE-19 cells. Each of the MG-132, bafilomycin A1, LPS, and HNE treatments on ARPE-19 cells lasted 24 h. Intracellular HNE-protein adducts were measured from cell lysates by the Western blot method, and a representative blot picture is shown. The antibody used in this study detects 4-HNE histidine adducts and therefore, it quite specifically labels the protein most sensitive to histidine-bound HNE. Densitometric data are combined from three independent experiments with two parallel samples in each group/experiment. The results are presented as mean ± SEM. $^{**}P < 0.01$, Mann–Whitney *U*-test.

schematic summary of the effects of MG-132 + bafilomycin A1 on human ARPE-19 cells (Fig. 10).

4. Discussion

There are many distinct age-related events that can disrupt the function of RPE cells contributing to the vision loss diagnosed as AMD. Both proteasomal degradation and autophagy activity become reduced which results in the accumulation of intracellular waste material, oxidative stress, and inflammation [11,12,23]. Lysosomal rupture is known to be a potent player in the activation of inflammasome signaling [26]. With respect to autophagy, lysosomal damage could be a possible mediator but in the present study, neutralization of the lysosomal enzymes by bafilomycin A1 blockade did not prevent the activation of inflammasomes even though it removed the possibility of cathepsin B to contribute to the activation process. The increased amounts of protein aggregates and the presence of dysfunctional mitochondria evoke excessive oxidative stress that is one of the most prominent inducers of inflammasome signaling [12]. It has even been suggested that all of the various NLRP3 activators induce some kind of oxidative stress, which could act as a common mediator in the inflammasome activation [27–30]. We have previously shown that oxidative stress is capable of inducing NLRP3-mediated inflammasome signaling in human ARPE-19 cells [18]. In the present study, we examined the effect of degenerated intracellular clearance on the induction of inflammasome signaling.

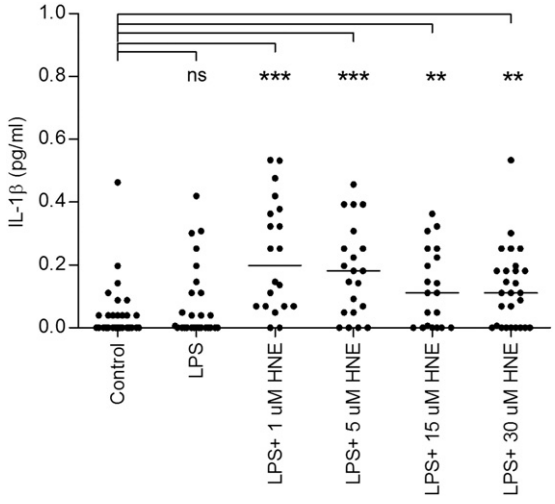

Fig. 7. IL-1β production in response to different HNE concentrations. Cells were primed with 10 μM LPS, and exposed to different concentrations of HNE. Medium samples were collected and concentrations of IL-1β measured with an ELISA method. The data are combined from four independent experiments with 4–17 parallel samples in each group. The results are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, ns = not significant, Mann–Whitney *U*-test.

After inducing intracellular protein aggregation by inhibiting proteasomes using MG-132, we observed a small but detectable increase in the release of IL-1β. It has recently been shown that autophagy eliminates the intracellular protein aggregates induced by MG-132 in ARPE-19 cells [23]. Since our cells were not aged or derived from AMD patients, it is probable that their fully functional autophagy degraded the aggregates as rapidly as they were being produced by MG-132, and thus reduced the ability of protein aggregates to induce severe inflammasome activation. When inhibiting also autophagy by bafilomycin A1, the release of IL-1β was significantly increased. The low levels of IL-1β were not surprising since it is known to be a very powerful cytokine under very strict regulation [31]. The cytokine secreted into extracellular space may rapidly bind to cell surface receptors, soluble receptors, or receptor antagonists, which increases challenge in its measurement from medium samples. Moreover, our protocol to perform experiments in serum-free medium most probably contributes to the low IL-1β levels detected. Due to its resilience, ARPE-19 cell line is a good study model to be used without bovine-derived material, which could contribute to cell responses or provide non-specific background in measurements. According to our experience, ARPE-19 cells could manage up to 1 week with nutrients included in the serum-free basal medium. Despite the fact that the specific increase of IL-1β by MG-132 + bafilomycin A1 was repeated several times in our experiments, nonetheless we confirmed the increased IL-1β production by using a highly-sensitive ELISA kit from another manufacturer. In addition, we tested the effect of LPS priming prior to exposure to MG-132 and bafilomycin A1. LPS priming enhanced the IL-1β production but did not change the secretion profile, which further proved that the

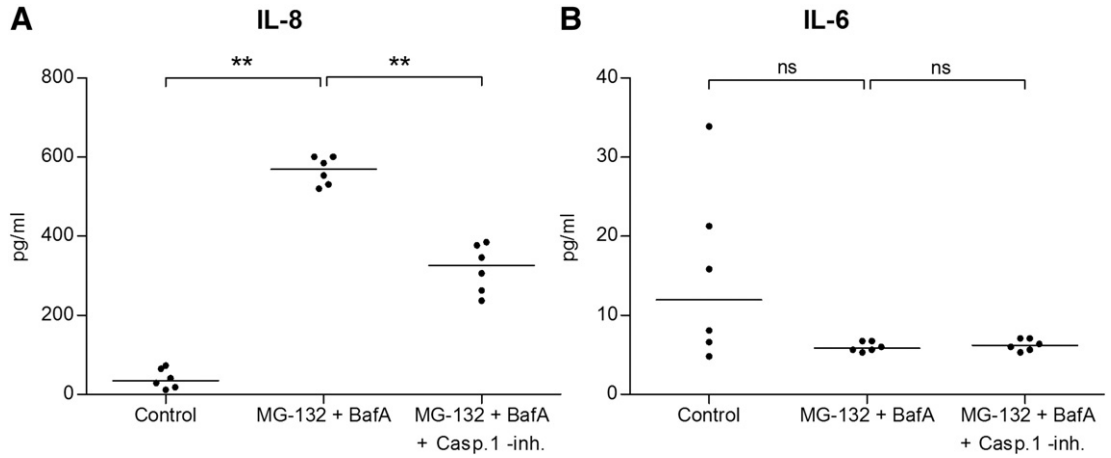

Fig. 8. Productions of **(A)** IL-8 and **(B)** IL-6 by MG-132 and bafilomycin A1 (BafA), and the effect of caspase-1 inhibitor on them. Cells were treated with MG-132 for 24 h and bafilomycin A1 for 24 h, and a specific caspase-1 inhibitor was added prior to treatments where indicated. Results are presented as mean ± SEM of six parallel samples/group, and they are representative of three independent experiments. $^{**}P < 0.01$, ns = not significant, Mann–Whitney *U*-test.

IL-1β production was real. Since it seemed that MG-132 was sufficient to prime the inflammasome signaling, we did not want to involve LPS in our later experiments with MG-132 and bafilomycin A1.

MG-132 + bafilomycin A1 treatment significantly increased the amount of LDH in the culture medium which indicates that the treatment compromised the integrity of cell membranes. Our results do not exclude pyroptosis, a caspase-1-mediated cell death, but only shows that cell membranes become ruptured. In this study, the mechanism was purposely left secondary since we were more interested in the form of IL-1β measured from our medium samples. Therefore, in addition to IL-1β measured with common ELISA kits which do not necessarily rule out the inactive pro-form, we analyzed the amount of pro-IL-1β from both cell lysate and medium samples with a specific ELISA kit. The concentrations of both pro and mature IL-1β were higher in lysates than in medium samples, and the finding was applicable to both control and treatment groups. There may be several reasons for this phenomenon. It is probable that more pro-IL-1β is found in lysates since it should not be actively released outside of the cell. Instead, the mature IL-1β in lysate samples may result from binding of the cytokine to its specific receptors on the cell surface. In the preliminary experiments conducted for our previous article [18], we found that the exposure to the IL-1 receptor antagonist before inflammasome activation by HNE evoked a more consistently increased secretion of IL-1β. Since there was no significant difference in the amount of pro-IL-1β between groups in the medium or lysate samples, and MG-132 + bafilomycin A1 greatly and significantly increased the amount of secreted IL-1β when compared to control cell mediums, we can infer that IL-1β measured from our present medium samples, was a mature form of the cytokine. Moreover, MG-132 + bafilomycin A1 did not significantly increase the concentration of mature IL-1β in cell lysates when compared to controls.

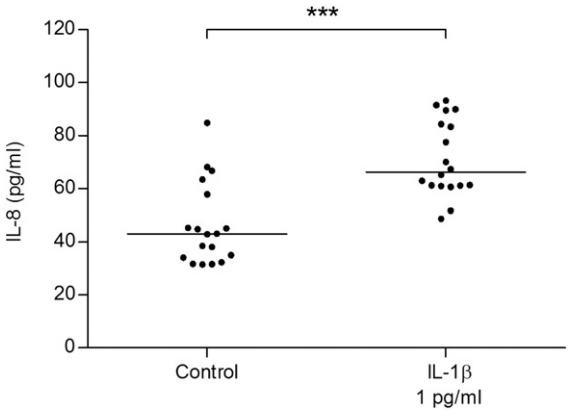

Fig. 9. Ability of IL-1β to induce the production of IL-8. Cells were exposed to recombinant human IL-1β (1 pg/ml) for 24 h, and concentrations of IL-8 were measured from medium samples. The data are combined from three independent experiments with six parallel samples in each group/experiment. The results are shown as mean ± SEM. $^{***}P < 0.001$, Mann–Whitney *U*-test.

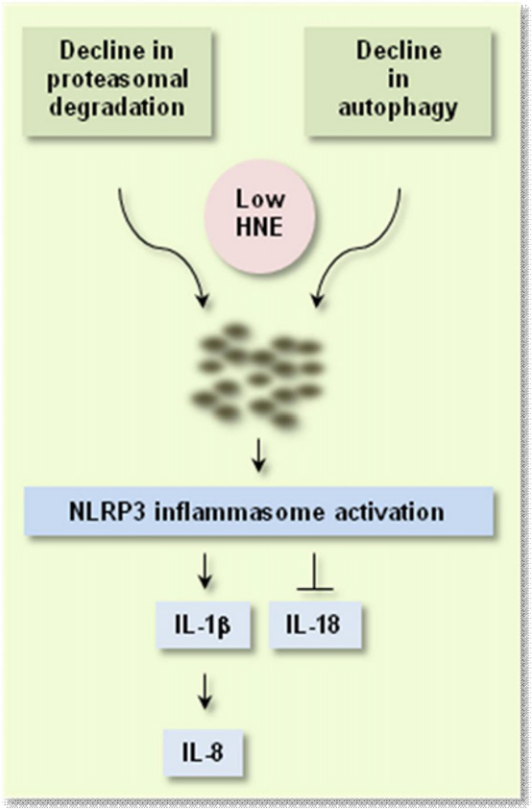

Fig. 10. Summary of the signaling induced by MG-132 + bafilomycin A1 in ARPE-19 cells. Inhibition of intracellular degradation induces a slight production of HNE and an accumulation of protein aggregates. The NLRP3 inflammasomes become activated and start to produce IL-1β but not IL-18. IL-1β already at a concentration as low as 1 pg/ml is able to upregulate significantly the production of IL-8.

We have previously shown that the lipid peroxidation end product 4-hydroxynonenal (HNE) is capable of inducing inflammasome signaling in ARPE-19 cells [18]. After exposure to 30 μM HNE, the cells also produced over 100 pg/ml IL-18, whereas in the present study, MG-132 + bafilomycin A1 treatment stimulated the release of IL-1β but not that of IL-18. As an interesting detail, we observed that MG-132 exposure was able to induce intracellular HNE-protein adduct formation in ARPE-19 cells. Since the levels of HNE-protein adducts induced by MG-132 were about four-fold lower than those induced by our previous HNE exposure (30 μM), we hypothesized that lower concentrations of endogenous HNE could specifically prefer the production of IL-1β. When we exposed our cells to different HNE concentrations, the results indeed supported the proposal that low amounts of HNE tend to release preferentially IL-1β.

HNE is a major marker of oxidative stress in retina and also an intriguing molecule. At low concentrations, it is known to promote proliferation and cell survival, whereas at high concentrations it acts in the opposite way [32–34]. Especially when one considers the situation in AMD, it is very interesting that HNE also regulates the VEGF production in a concentration-dependent manner. It has been shown that low HNE concentrations (<5 μM) trigger VEGF release in human ARPE-19 and RPE-28 cells, whereas concentrations higher than 5 μM reduce its release [32,34]. VEGF secreted by RPE cells also exhibits true angiogenic potential as demonstrated by HUVEC cells that displayed increased migration and tube formation in matrigel when grown with a culture medium from RPE cells treated with 1 μM HNE [34]. Therefore, our present results showing that low HNE concentrations lead to the production of IL-1β are in line with the previous studies mentioned above since IL-1β is known to promote VEGF production [35]. Interestingly, it was recently shown that blocking the NLRP3 or IL-1β signaling resulted in reduced RPE barrier breakdown and neovascularization in mice [36]. The finding suggests that NLRP3 inflammasome activation with IL-1β secretion can contribute to the pathogenesis of wet AMD. Tarallo et al., in turn, have recently demonstrated that there is an association between IL-18 release and geographic atrophy [19]. The detailed role of this polarization in the pathogenesis of AMD needs to be elucidated in further studies.

Our present data demonstrate that degenerated intracellular clearance is associated with the induction of inflammation through an intracellular danger sensor system. Concurrent treatment with MG-132 and bafilomycin A1 also induced intracellular production of HNE providing evidence that declined degradation, oxidative stress, and inflammation play a role in disturbing the functionality of RPE cells. Our data also showed that already low concentrations (1 pg/ml) of IL-1β are able to induce a secondary response by promoting the production of IL-8, which is a major chemotactic mediator of human RPE attracting neutrophils and monocytes [37]. Although IL-1β levels produced by the MG-132 + bafilomycin A treatment remained lower than 1 pg/ml, a pretreatment of cells with LPS increased the production to 1–3 pg/ml and therefore, IL-1β at 1 pg/ml was chosen for a stimulatory concentration. LPS activates nuclear factor kappa B (NF-κB) signaling, which can become activated *in vivo* in RPE cells e.g. by fragments of extracellular matrix or stress-related alarmins, such as HSP60 and HMGB1 [38,39]. NF-κB signaling is a major pathway in mediating the inflammasome priming but in our experimental settings, it becomes inhibited by MG-132 [40,41]. It is known that IL-1β is also able to stimulate the production of IL-6 in ARPE-19 cells [42]. However, since the production of IL-6 could not be detected in the cell cultures directly releasing IL-1β due to the inhibitory effect of MG-132 on its major regulator NF-κB [43], the contribution of IL-6 has been omitted from the present analysis. Further studies are naturally needed for verifying the link between the inflammasome activation and leukocyte chemotaxis but it seems possible that age-related decline in intracellular cleaning systems with the subsequent production of IL-1β has even broader biological significance than shown in this study. However, our data indicate that malfunctional clearance can result in the inflammasome activation in human RPE cells.

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