NLRP3 inflammasome activation by photooxidative damage provides a novel link between hallmark pathogenic features of age-dependent macular degeneration

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I. Summary

Summary

In the developed world, age-related macular degeneration (AMD) is the most common cause for severe visual loss and legal blindness in the elderly. In this progressive disease, degeneration of the retinal pigment epithelium (RPE) results in the death of photoreceptors and secondary loss of central vision. Two late manifestations of AMD can be distinguished, neovascular AMD and atrophic AMD. Neovascular AMD is characterized by rapid visual loss secondary to VEGF-mediated ingrowth of choroidal neovascularizations (CNV). In contrast, atrophic AMD causes slowly progressive central visual decline by RPE cell degeneration known as geographic atrophy. Anti-VEGF treatment has proven highly effective in neovascular AMD and is now widely used clinically. In contrast, there is still no treatment available for atrophic AMD. Several lines of evidence indicate that oxidative and lipofuscin-mediated photooxidative damage play an important role in AMD pathology. Further characteristics of the disease include the formation of extracellular deposits called drusen, and chronic low-grade immune processes including complement activation in the sub-RPE space. Inflammation is recognized as a major driving force in atrophic AMD. However, the mechanisms triggering and maintaining the retinal inflammation remain incompletely understood. Recent studies have shown that the NLRP3 inflammasome, a key mediator of the innate immune system, is activated in the RPE of patients with atrophic and neovascular AMD. The NLRP3 inflammasome is a multiprotein complex, which induces caspase-1 activation resulting in secretion of the pro-inflammatory cytokines Interleukin-1β (IL-1β) and Interleukin-18 (IL-18). An initial priming signal in combination with a subsequent activation signal, such as reactive oxygen species or lysosomal membrane permeabilization (LMP), leads to assembly of the NLRP3 inflammasome. Studies demonstrated that phototoxic damage mediated by intralysosomal accumulation of photoreactive lipofuscin leads to LMP in RPE cells. The present thesis investigated whether lipofuscin-mediated phototoxicity activates the NLRP3 inflammasome in RPE cells in vitro and aimed to identify a novel mechanism of inflammasome activation by light damage that may provide new treatment targets for blinding diseases such as AMD. For this purpose, lipofuscin-loaded primary human RPE cells and human RPE cell line ARPE-19 cells were irradiated with blue light (dominant wavelength 448 nm, irradiance 0.8 mW/cm^2 , duration 6 h) to induce photooxidative stress. The obtained results, presented in chapter IV demonstrate that accumulation of lipofuscin rendered RPE cells in vitro susceptible to phototoxic destabilization of lysosomes and cytosolic leakage of lysosomal enzymes. This resulted in NLRP3 inflammasome activation as evidenced by caspase-1 activation, processing and release of IL-1β and IL-18. Investigating the secretion profile of inflammatory cytokines after

inflammasome activation revealed predominantly apical secretion of inflammatory cytokines, that corresponds to the neuoretinal side in vivo. In addition, secreted cytokines exerted a chemotactic effects on microglia cells as well as reduced constitutive secretion of VEGF (chapter V). In contrast to inflammasome activation, the mechanism of inflammasome priming in AMD has been little investigated so far. A recent study in patients with early or intermediate AMD demonstrated the complement factor H risk genotype to be associated with significantly increased plasma levels of the inflammasome-regulated cytokine IL-18, suggesting a role for activated complement components in inflammasome activation in AMD. Inflammasome priming by complement activation products has also been proposed in the context of other diseases such as atherosclerosis and gout. The thesis further reveales the capacity of activated complement components to prime human RPE cells for inflammasome activation. Obtained results, presented in chapter VI demonstrate that incubation of ARPE-19 cells with complement-competent normal human serum (NHS) induced expression of pro-IL-1β and enabled secretion of IL-1β in response to lipofuscin phototoxicity, indicating inflammasome priming by NHS. For further delineation of the relevant priming competent component in NHS, complement factors were heat inactivated or blocked by complement inhibitors which enabled the identification of complement activation product C5a as the priming signal for RPE cells. Likewise, conditioned media of inflammasome-activated RPE cells provided a priming effect that was mediated by the IL-1 receptor, thus suggesting a paracrine amplification loop of inflammasome activation. Further investigation demonstrated that cell priming by IL-1α or C5a increased susceptibility of RPE cells to oxidative/photooxidative damage-mediated cell death (chapter VII). Morphological observations and investigation of the underlying cell death mechanism revealed a change in cell death mode from apoptosis to pyroptosis. Moreover, conditioned media of pyroptotic ARPE-19 cells increased cell death by photooxidative damage in other cells in an interleukin-1 receptor (IL-1R) dependent fashion. These results make it conceivable that in situations of localized RPE cell death such as in atrophic AMD, this mechanism could result in increased susceptibility of immediate bystander RPE cells to inflammasome-mediated cell death, thus contributing to the centrifugal progression pattern of RPE cell loss in AMD. In summary, this study identified a novel mechanism of inflammasome activation by light damage and provides a functional link between key factors of AMD pathogenesis including lipofuscin accumulation, photooxidative damage, complement activation, and RPE degeneration. Thereby, this study provide new potential treatment targets for blinding diseases such as AMD.

II. List of Publications

List of Publications

The data presented in this thesis were published previously in the following publications:

I.

Brandstetter, C., Mohr, L. K. M., Latz, E., Holz, F. G. & Krohne, T. U. Light induces NLRP3 inflammasome activation in retinal pigment epithelial cells via lipofuscin-mediated photooxidative damage. J. Mol. Med. 93, 905–916 (2015).

Key findings: In this study, blue light photooxidative stress to human RPE cells, intensified by accumulated lipofuscin, causes lysosomal membrane permeabilization and subsequent activation of the NLRP3 inflammasome by leaking lysosomal enzymes, resulting in secretion of inflammatory cytokines IL-1β and IL-18. These results identify blue light damage as a new mechanism of inflammasome activation and thus contribute to our understanding of light damage to the RPE.

Documentation of authorship: The author participated in the study design, performed all experiments and analyzed the results. The author participated in writing the manuscript.

II.

Mohr, L. K. M., Hoffmann, A. V., **Brandstetter, C.,** Holz, F. G. & Krohne, T. U. Effects of Inflammasome Activation on Secretion of Inflammatory Cytokines and Vascular Endothelial Growth Factor by Retinal Pigment Epithelial Cells. Invest. Ophthalmol. Vis. Sci. 56, 6404–6413 (2015).

Key findings: Lysosomal membrane permeabilization–induced activation of the NLRP3 inflammasome in RPE cells results in apical secretion of inflammatory cytokines with chemotactic effects on microglia cells and reduced constitutive secretion of VEGF. Via these mechanisms, lipofuscin phototoxicity may contribute to local immune processes in the outer retina as observed in AMD.

Documentation of authorship: Author designed, performed and analyzed the microglia chemotaxis assay. Furthermore, Carolina Brandstetter was significantly involved in the supervision of the project as well as planning and execution of the experiments.

III.

Brandstetter, C., Holz, F. G. & Krohne, T. U. Complement component C5a primes retinal pigment epithelial cells for inflammasome activation by lipofuscin-mediated photooxidative damage. J. Biol. Chem. 290, 31189–31198 (2015).

Key findings: By investigating the capacity of activated complement components to prime human RPE cells for inflammasome activation by lipofuscin-mediated photooxidative, complement activation product C5a was identified as a priming signal for RPE cells that allows for subsequent inflammasome activation by stimuli such as lipofuscin-mediated photooxidative damage.

Documentation of authorship: The author participated in the study design, performed all experiments and analyzed the results. The author participated in writing the manuscript.

IV.

Brandstetter, C., Patt, J., Holz, F. G. & Krohne, T. U. Inflammasome priming increases retinal pigment epithelial cell susceptibility to lipofuscin phototoxicity by changing the cell death mechanism from apoptosis to pyroptosis. J. Photochem. Photobiol. B, Biol. 161, 177–183 (2016).

Key findings: Examination of the mechanisms of photooxidative damage-induced cell death in cultured human RPE cells demonstrated that inflammasome priming with IL-1α or C5a not only changes the cell death mechanism from apoptosis to pyroptosis but also increases the susceptibility of the cells 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.

Documentation of authorship: The author designed, performed and analyzed all experiments, with the exception of the experiment to determine IL-1β release association with pyroptotic cell lysis. The author participated in writing the manuscript.

III. General Introduction

3.1 Age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause blindness in all industrialized countries, affecting primarily persons aged 50 years and above. The disease affects roughly four and a half million people in Germany and vaguely 30 million in the rest of the world.[1] A further significant increase in incidence and prevalence of the disease is predicted as a result of demographic development.[2] AMD is a progressive and degenerative condition that affects cells and structures of the outer retina resulting in damage to the *macula lutea*.[3] The *macula lutea*, further referred as "macula" is described as a small oval area that is characterized by the rich and close packing with cone photoreceptors. The macula accounts for fine spatial resolution and spatially differentiated perception, - the vision that is critical for activities such as reading, driving, and facial recognition.[4] Characteristic for the commonly asymptomatic initial stage of AMD is the occurrence of drusen, deposits of extracellular debris and inflammatory material at the interface of the retinal pigment epithelium (RPE) and Bruch´s membrane that comprise lipid- and protein-rich debris.[5] Drusen deposition is also part of a normal phenomena during natural aging,[6] and usually do not affect visual function. But due to lipoidal character, drusen may act as a diffusion barrier that affects the transport of fluids and nutrients from the choroid to the retina. By this, the presence of drusen may contribute to an age-related degeneration of the RPE.[7] Presumably, degenerative dysfunctions of the RPE and oxidative stress play a crucial role in drusen formation.[8] However, an increase in number, size, and confluence of drusen, that is accompanied by retinal hyperpigmentation, is a significant risk factor for the sight-threatening advanced forms of AMD. A representative funduscopic image with drusen is presented in figure III.1a. Their presence may be accompanied by RPE cell death,[9] but the connection between these AMD related events is not fully understood yet.

Due to their clinical manifestation, AMD is characterized by two types, the slowly developing atrophic AMD and the fast developing neovascular AMD. The atrophic form is an advanced form of AMD that culminates in geographic atrophy (GA). GA is characterized by the loss of the RPE and the formation of atrophic patches within the macula. Adjacent photoreceptors also degenerate due to their metabolic dependence on RPE cell function. This causes a slow but continuous progressive loss of central vision.[10,11] As atrophic patches increase in size and may become confluent, they eventually progress into central scotomas and areas of central vision loss with relative sparing of the surrounding peripheral retina.[12] Despite extensive efforts, there are currently no available treatment options for atrophic AMD, although randomized clinical trials have reported that oral supplementation of various

antioxidants (i.e., vitamins C and E, beta carotene, zinc, lutein and zeaxanthin) may reduce the odds of disease progression from early to late stage AMD.[3,13] A representative eye with GA is shown in figure III.1b. The neovascular form is an advanced form of AMD that culminates in the formation of choroidal neovascularizations (CNV) and fibrovascular scarring primarily affecting the macula.[12] CNV is characterized by newly formed vessels that penetrate the Bruch's membrane and sprout into the sub-retinal space.[14] Unlike physiological blood vessels, neovascular vessels are leaky and blood components drain into the sub-retinal space. Therefore, this form of AMD may include lipid deposition, hemorrhage, sub-retinal fluid accumulation, RPE detachment and fibrotic scarring, as presented in figure III.1c. The neovascularizations are initiated by an increased secretion of vascular endothelial growth factor (VEGF).[15] If left untreated, they can lead to severe blindness with scarring within several months. Progression of neovascular AMD may be halted by VEGF inhibiting drugs, which represent an important CNV treatment option.[16] However, these advanced stages of AMD should not be seen as stationary, since neovascular lesions may be present in the periphery of eyes with geographic atrophy and vice versa.[17]

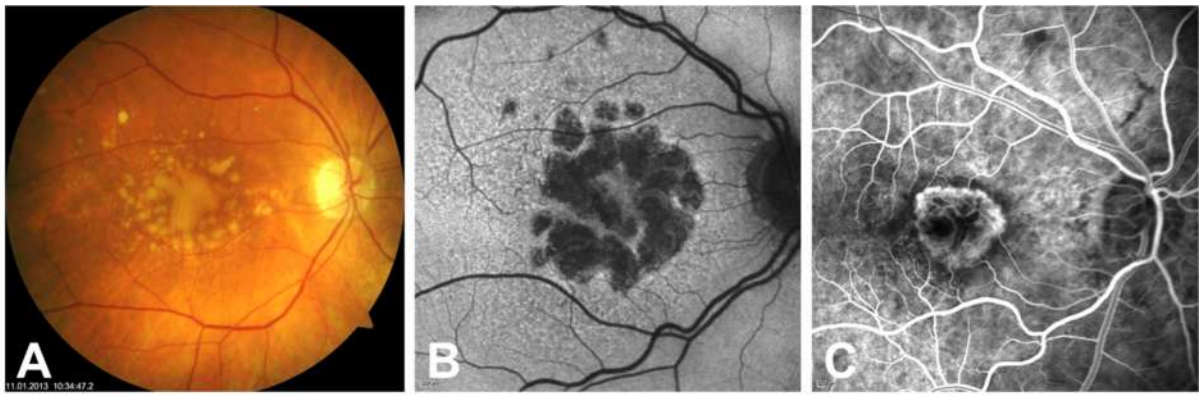

Figure III.1. Funduscopic images from AMD patients demonstrating hallmarks of disease. (A) Fundus photography in intermediate AMD showing drusen. (B) Fundus autofluorescence in atrophic AMD showing geographic atrophy. (C) Fluorescein angiography in neovascular AMD showing choroidal neovascularisation.

While aging represents its primary determinant, environmental factors such as cigarette smoking,[18,19] dietary habits,[20–22] and light exposure,[23–25] together with genetic predispositions[26–32] contribute to the risk of AMD occurrence. In addition, oxidative stress, by releasing reactive oxygen species, represents another trigger in AMD pathogenesis.[3,33] Numerous findings suggest RPE dysfunction and degeneration as a critical event in AMD pathogenesis.[34]

3.2 Retinal pigment epithelium cells as primary disease target in AMD

The RPE and the neuroretina constitute the retina, the neural portion of the eye. The neuroretina comprises five types of layered neurons: (1) photoreceptor cells, (2) bipolar cells, and (3) ganglion cells, which together provide the most direct route of transmitting visual information to the brain via the optic nerve, as well as (4) horizontal cells and (5) amacrine cells. Other cells present in the retina are glia cells: astrocytes, Müller cells, and microglia (figure III.2). The latter are located in the inner retina but transmigrate into and out of the subretinal space in diseases such as AMD.[35] The RPE is built of a single layer of melanin-pigmented cells of cuboidal form located on the Bruch's membrane between the neural retina and the choriocapillaries. The RPE together with the Bruch´s membrane form the outer blood-retinal barrier (BRB) which is highly indispensable for sustaining vision. Tight junctions are strengthening the RPE laterally. Therefore, the BRB prevents the migration of immune cells and macromolecules from the underlying choroid into the photoreceptor layer.[36] The RPE features a variety of metabolic functions that are of crucial importance for the healthy retina, such as exchange of nutrients, metabolites and fluids between the neuroretina and choriocapillaris,[37] synthesis of vitamin-A metabolites (retinoid recycling) and protection against oxidative stress. Another function is the phagocytosis of shed photoreceptor outer

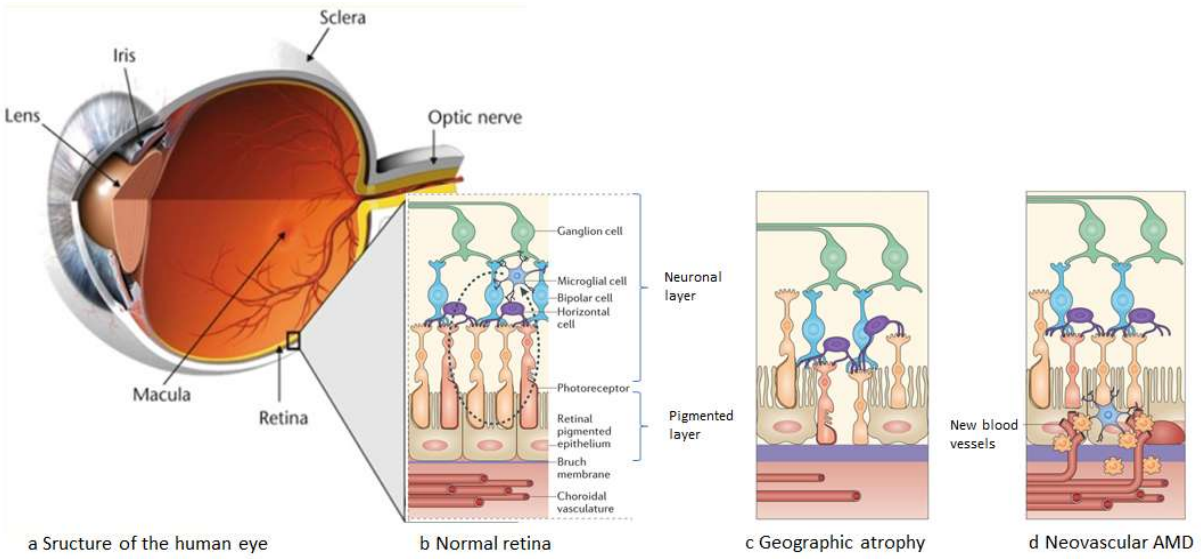

Figure III.2: Structure of the human eye and organization of the retina. a) Profile of the human eye. b) Normal retinal architecture is comprised of the neuroretina that consists of photoreceptor cells, neuronal cells and glia cells (Müller cells, astrocytes, and microglia); the retinal pigment epithelium (RPE), the Bruch´s membrane and a choroidal vascular network. Microglia transmigrate into and out of the subretinal space (as shown by the dashed arrow). c) Geographic atrophy and d) Neovascular AMD demonstrate the advanced forms of AMD. Further information will be given in the text. Images modified from Ambati et al., 2013.[38]

segments (POS). These POS are digested and thereby essential substances such as the aldehyde all-trans-retinal get regenerated. After transformation into the photoactivated isomer 11-cis-retinal the POS is returned to the photoreceptors to rebuild light-sensitive POS from the basic constituents of the photoreceptors.[39–42] Therefore, as a consequence of its strategic location and vital roles, the RPE is the primary site associated with AMD pathology.[7,43,44] RPE degeneration results in loss of sensory retina, leading to vision loss in advanced AMD. The lysosomal compartment of the RPE is responsible for breaking down POS into their basic components for recycling. However, as aging is characterized by a gradual functional decline of all organ systems,[45] likewise lysosomes are affected by age-dependent decline in degradative capacity, resulting in incompletely degraded material.[46] In addition, oxidative stress has been shown to be implicated in decreased lysosomal function, again promoting the accumulation of incompletely degraded material.[47] Incomplete degradation of oxidized material has been proposed to be the beginning bud for aggregate formation that further cross-link to ultimately emerge in lipofuscin, one of the main hallmark features in AMD pathology. The accumulation of lipofuscin has been demonstrated to precede RPE dysfunction and degeneration with concurrent severe functional loss.[48] This RPE degeneration is thought to be mediated, at least in part, by photoreactive properties of lipofuscin that accumulate in RPE over a lifetime. Therefore, as a consequence of its strategic location and vital roles, the RPE is the primary site associated with AMD pathology.[7,43,44]

3.3 Oxidative stress in AMD

As mentioned above, oxidative processes are a powerful feature in formation of lipofuscin.[8,49] Oxidative stress is related to a number of age-dependent diseases such as atherosclerosis,[50] and Alzheimer's disease,[51] as well as several ocular diseases, such as diabetic retinopathy,[52,53] cataract,[54] and age-dependent macular degeneration.[55] Oxidative stress is characterized as an imbalance in reactive oxygen species (ROS) and antioxidants.[56,57] ROS can arise endogenously by the activity of NADPH oxidases in inflammatory process,[58] in small amounts by normal mitochondrial activity[59] or due to ionizing radiation, ultraviolet rays, tobacco smoke or photosensitizers.[60,61] Antioxidant systems have evolved to protect biological systems against the deleterious effects of a wide array of ROS. Antioxidant enzymes such as peroxidase, catalase, and superoxide dismutase,[62] as well as the antioxidant protection mechanisms of the vitamins E, C and glutathione reduce the harmful

effects of ROS.[63] In addition, the RPE contains various pigments that are specified for different wavelengths[64,64] such as the carotenoids lutein and zeaxanthin that possess antioxidant properties by filtering short-wavelength light.[65] The outer retina is particularly vulnerable to photooxidative damage due to the age-dependent decline in RPE antioxidant defense[66,67] as well as their high oxygen concentration, the intense light exposure combined with the presence of photosensitizers and the high content of polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid,[64,68,69] permitting oxidative stress to ultimately overwhelm RPE cell defenses, leading to oxidative damage and retinal aging.[64,70–72] Oxidative stress is an important mediator of damage to cell structures (termed oxidative damage), including single- and double desoxyribonucleic acid (DNA)-strand breaks, DNA–protein and protein–protein cross-linking, protein fragmentation and oxidation of lipids, reffered to as lipid peroxidation.[73–75]

3.3.1 Lipid peroxidation

Lipid peroxidation is the oxidative degradation of lipids and proceeds by a complex free radical chain reaction by utilizing the interaction of ROS with PUFAs. PUFA are particularly prone to oxidation due to their multiple double bonds, that are more vulnerable to oxidation than saturated ones. Membrane and organelle surroundings are constituted by phospholipid layers that are rich in PUFAs, making them a vulnerable target for lipid peroxidation. Therefore, this process is particularly detrimental to cellular functions as it disturbs membrane fluidity, integrity and function.[68] Free radicals exhibit an unstable and reactive status, due to an unpaired electron in the outer orbital shell.[76] Therefore, free radicals aim to achieve a stable state. This can be achieved by abstracting an electron from other molecules. By this, unsaturated fatty acids are susceptible to free radical attack, due to their unsaturated carbon double bonds. Carbon double bonds constitute electrons bonds, in which one of those (allylic) bonds has loosely held electrons. Initially, lipid peroxidation occurs when ROS is generated in close proximity to a PUFA containing structure, such as the lysosomal membrane or phagocytosed POS. The radical immediately abstracts the allylic hydrogen forming water and a peroxyl radical. This lipid radical itself abstracts a hydrogen from another lipid molecule, generating a new lipid hydroperoxide. This leads to an autocatalytic chain reaction of oxidants. The peroxyl radical turns into a lipid hydro-peroxidase, while simultaneously a new peroxyl radical is generated.[77] As a part of these reactions, the valuable unsaturated fatty acids are cleaved in cytotoxic effective lipid peroxidation products. Termination of the chain reaction can only occur when either an antioxidant scavenger (e.g. vitamin E) donates a hydrogen atom to a radical, or if two

radicals react with each other to produce a non-radical species. The vitamin E radical reacts with another forming non-radical products.[78] If not terminated fast enough, these reactions lead to cleavage of unsaturated fatty acids in cytotoxic effective lipid peroxidation products and irreparable damage to membranes under attack, with ensuing leakage of enclosed components (e.g. ions, proteins and lysosomal enzymes). The POS membranes are highly enriched by PUFAs. Docosahexaenoic acid, a precursor of α-linolenic acid with six double bonds, dominates among the fatty acids of the phospholipids.[79] Therefore, the POS membranes are a sensitive site for oxidative damage. Phagocytosis of spent POS by RPE cells would be expected to introduce pre-formed lipid peroxidation products-modified POS proteins, and PUFAs prone to subsequent peroxidation into the RPE lysosomal compartment.

Two prevalent lipid peroxidation products are malondialdehyde (MDA) and 4-hydroxynonenal (HNE). MDA predominantly arises from oxidation of the polyunsaturated fatty acids docosahexaenoic acid and arachidonic acid.[68] HNE usually emerge by oxidation of arachidonic acid and linoleic acid.[68] MDA and HNE attach covalently to proteins by forming adducts with lysine, histidine or cyteine residues. In this way, MDA and HNE stabilize POS proteins against degradation, resulting in accumulation of undigested POS within the lysosomes.[80] Cross-linkage of oxidized proteins by HNE or MDA as well as decreased proteolysis of modified proteins leads to the formation of aggregates that may be the underlying mechanisms of lipofuscinogenesis. In addition, detection of HNE- and MDA-modified proteins in a proteomic analysis of human RPE lipofuscin confirmed the intralysosomal occurrence of these aldehydes and brought attention to the pivotal role of lipid peroxidation products in the induction of lysosomal dysfunction and lipofuscinogenesis in RPE.[47,81]

3.3.2 Lipofuscin-mediated photooxidative stress

Lipofuscin accumulates in non-dividing metabolically active cells like neurons, cardiomyocytes and RPE cells[82,83] and is associated with a variety of pathological conditions such as ceroid-lipofuscinosis, cirrosis of the liver, cardiac hypertrophy, as well as with ocular diseases such as AMD, Stargardt disease and Best's disease.[84–86] Unlike most cell types cells, in which lipofuscin is produced through the lysosomal degradation of intracellular organelles,[39] the major substrate for RPE lipofuscin is the undegradable end product resulting from POS phagocytosis.[87] Due to several fluorophores, RPE lipofuscin has a characteristic broad-band fluorescence.[88] RPE lipofuscin autofluorescence facilitates the

detection of lipofuscin accumulation and distribution in the living eye[89,90] by means of fundus spectrophotometry, a noninvasive measurement of intrinsic fluorescence and reflectance of the ocular fundus.[91] The signal for lipofuscin accumulation in the RPE is augmented in the macula, maximally in the parafoveal ring where rods are densest.[92–95] Additionally, autofluorescence increases linearly with age in the healthy retina,[95] demonstrating age-dependent increase in lipofuscin accumulation. It still needs to be proven whether lipofuscin is the causal factor in retinal degeneration. However, a growing number of evidence propose lipofuscin as detrimental factor, influencing RPE function and viability. The observation of lipofuscin fluorescence and progression of atrophic areas in AMD patients revealed that excessive lipofuscin accumulation preceeds RPE atrophy with concurrent damage to photoreceptors and choriocapillaris, leading to GA.[48] A feasible approach is provided by studies that revealed increased RPE susceptibility to light-induced cell damage by accumulation of lipofuscin.[96–98] In a consequence, a role of light-induced RPE cell damage has been postulated for AMD pathogenesis. However, several clinical studies that investigated the association of daylight exposure and the formation of AMD reported ambiguous results. Some studies demonstrated a relationship, whereas others failed to do so. Among the positive results, the Beaver Dam Eye Study reported that early signs of AMD positively correlate with excessive exposure to sunlight (> 5 hours per day) during the teenage years and beyond.[99] Similarly, the Chesapeake Bay Watermen Study revealed that late AMD positively correlate to cumulative sunlight exposure.[100] Ultraviolet radiation (UV) turns out not to be the causative agent for retinal damage, as UV is almost completely absorbed by the crystalline lens. However, shorter wavelengths of the visible spectrum (i.e. blue-light, 400 to 480 nm) demonstrate strong effects, possibly due to photooxidative damage to the RPE.[100–102]

Lipofuscin represent the main photosensitizer in the retina, beside the visual pigments in photoreceptor cells.[60] As soon as a photosensitizing molecule (e.g. lipofuscin, riboflavin, retinal) absorbs light in its particular wavelength, it gets converted into an excited state. In order to return to its ground state, the photosensitizer transfers the increase in energy to an adjacent oxygen molecule, creating a singlet oxygen. The singlet oxygen is highly aggressive and immediately seeks to attack and damage its surroundings when formed.[103] Lipofuscin accumulation can be a result of photooxidative damage to the retina. Furthermore, once present in the RPE, lipofuscin can itself propagate photooxidative stress.[104] Especially when excited by blue light, lipofuscin exerts photoreactive properties that induce oxidative stress to the lysosomal membrane via massive peroxidation of lysosomal membrane lipids.[104]

3.3.3 Lysosomal membrane permeabilization

Lysosomes are membrane-bound acidic organelles, present in virtually all eucaryotic cells.[105] The endolysosomal system comprise early endosomes, with an slight acidic intra-lumenal pH of 6.0-6.6, late endosomes, with a more acidic pH (~5), and lysosomes with the most acidic compartment (pH ~4.5). The size of the lysosomes is approximately between 0.5 and 2 micrometers depending on the content and stage of the degradation process.[106,107] Lysosomes are considered the major site for degradation of exo- and endogenously derived material.[108] For this purpose, lysosomes comprise a variety of hydrolases including cathepsins, nucleases, glycosidases, sulfatases, and lipases. Of the lysosomal hydrolases, the cathepsin family of proteases is the best characterized. The cathepsins are subdivided, according to their active site amino acids, into cysteine (cathepsins B, C, F, H, K, L, O, S, V, W, and X), serine (cathepsins A and G), and aspartic cathepsins (cathepsins D and E).[109] Lysosomal enzymes are regulated by the pH of their surrounding environment that dictates enzymatic activity.[105] The material to be digested is delivered to the endolysosomal compartment via endocytosis, autophagocytosis, and in specialized cells, through phagocytosis.[110–112]. The degraded material pass out of the lysosome via membrane transporter proteins and is reused by the cell. Lysosomes are limited by a single 7–10 nm phospholipid-bilayer.[113] The lysosomal membrane provides an essential physical barrier from the acidic hydrolases to protect the interior of the cell from degradation. This is achieved by lysosome-specific expression of the highly glycosylated lysosome-associated membrane proteins (LAMPs) and lysosomal integral membrane proteins (LIMPs), that are considered responsible for lysosomal resistance to self-degradation from the inside.[114,115] The lysosomal membrane harbors a proton pump that uses energy from ATP hydrolysis to maintain intralysosomal acidic conditions. [116–118]

Photosensitizers that are located in lysosomes and generate singlet oxygen upon radiation have been shown to cause LMP via massive peroxidation of lysosomal membrane lipids.[119–121] Another mechanisms leading to LMP may be induced by application of lysosomotrophic agents such as L-Leucyl-L-leucine methyl ester (Leu-Leu-OMe),[122] or ciprofloxacin[123]. These agents accumulate within the lysosomes and permeabilize the membrane via a detergent mechanism. In addition, lipofuscin fluorophore A2E was also proposed to exert lysosomotrophic features.[124] Lysosome membrane permeabilization leads to subsequent relocation of lysosomal constituents into the cytosol[125] that triggers various cellular responses, such as inflammasome activation, autophagy, and apoptotic cell death.[108,126] The idea of lysosomal involvement in cell death was first suggested by de Duve and Wattiaux in

1966.[108] The importance of the cytosolic location of cathepsins for their pro-apoptotic function is highlighted by studies in which microinjection of cathepsins into the cytosol was sufficient to induce apoptosis. In each of these cases, a specific inhibitor of cathepsin B or D attenuated cell damage.[127–129] Cathepsins have the ability to participate in both the initiation and execution phases of apoptosis but may in some cases also directly activate caspases.[130]

3.4. Complement system in AMD

Over the past decade, studies revealed that inflammation plays a crucial role in the pathogenesis of AMD. The most convincing evidence for the contribution of inflammation to AMD comes from studies that have strongly implicated the complement system. As an integral part of the innate immune system, the complement system plays a pivotal role in the innate defense against common pathogens as well as in the clearance of cellular debris from host tissues and other roles related to tissue homeostasis.[131] The complement system is comprised of over 50 blood-circulating, mostly liver-derived, and membrane-bound proteins, regulators and effector molecules (figure III.3). The latter largely exist in a precursor state that is activated rapidly in a cascade-like fashion following activation of the system via any of three pathways: the classical pathway, the lectin pathway and the alternative pathway (AP) of the complement system.[132] The classical pathway can be initiated by the binding of the pattern recognition molecule C1q to immunglobulin (IgG or IgM) in antibody:antigen complexes. It can also bind to C-reactive protein (CRP) on self or microbial surfaces or directly to molecules expressed on microbial membranes. The lectin pathway is independent of antibody binding and is triggered by interaction of mannose-binding lectin (MBL) with mannose residues or ficolin with N-acetyl glucosamine residues both present in bacterial cell walls.[133] C1q and MBL are structurally similar molecules, and both the classical and lectin pathways require C2 and C4 complement components for the generation of the C3 convertase. While the classical or lectin pathway are largely dependent on protein:carbohydrate or protein:protein interaction, the AP is constantly autoactivated in the fluid phase, a process referred to as „tickover“.[134] AP activation by tickover appears at low rates by hydrolysis of complement component C3, leading to the formation of C3(H2O) and the binding of small amounts of C3b to cell surface carbohydrates or proteins of target cells such as bacterial cells, followed by the binding of factor B, which is then cleaved by Factor D to form the C3 convertase complex C3bBb. Once deposited on the surface of cells or pathogens, C3b binds more factor B, and this binding gradually amplifies the activation

cascade. The binding of properdin (P) stabilizes the C3 convertase and enhances the process.[135] Although triggered by different signals, all activated pathways cumulate into the generation of a C3 convertase, a protein complex that cleaves the key complement effector molecule C3 into the anaphylatoxin C3a and the bioactive opsonin C3b. The latter contributes to the formation of the C5 convertase, which in turn cleaves C5 to C5a and C5b. The complement system not only helps the cell to defend against pathogens, but also has the potential to harm self-tissues. Therefore, it is particularly important for the cell to balance complement activation and inhibition.[136]

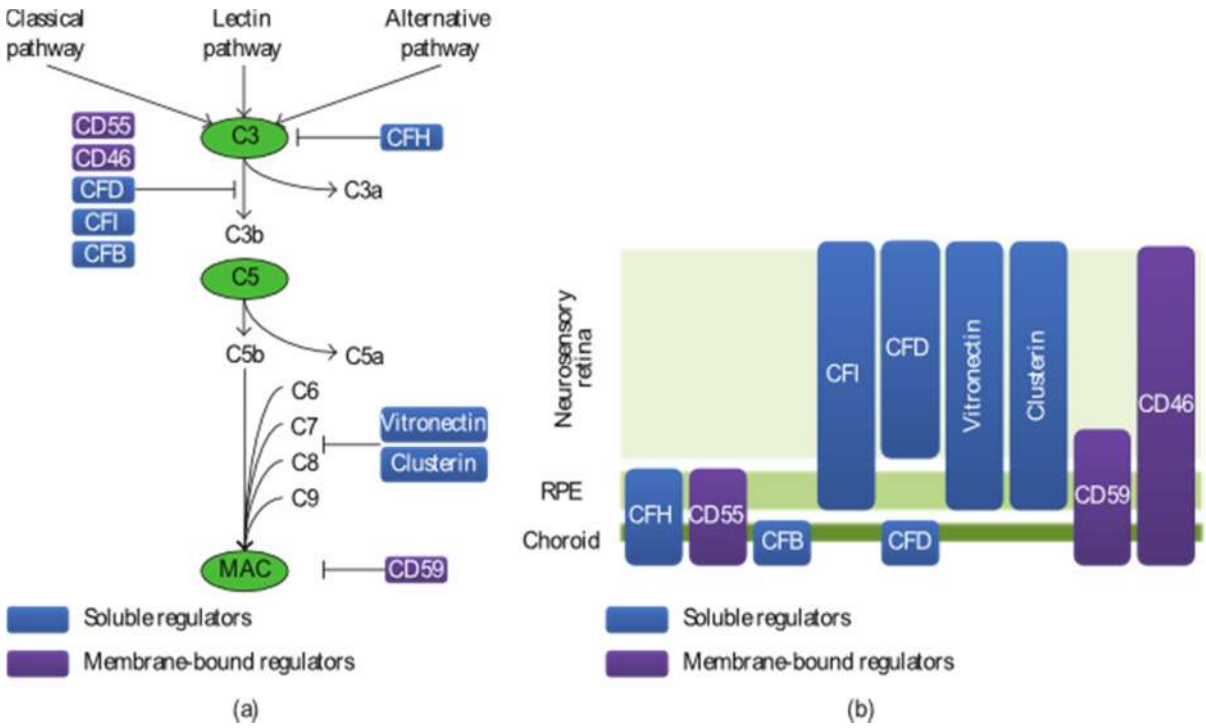

Figure III.3: Complement system activation. (a) Complement system activation. Local effects of the inhibitors on complement components. (b) Complement system in the eye. Local production of the complement inhibitors by retinal cells. Image from Kawa et al., 2014.[137]

Complement factor H is the major soluble regulator of the alternative complement pathway, and has a pivotal role because of the constant activation of that pathway.[138] A single nucleotide polymorphism (SNP) in CFH that changes amino acid residue 402 from a tyrosine (Y402) to a histidine (402H) leads to functional consequences. The 402H variant binds with reduced affinity to several of its ligands which reduces the effectiveness by restricting activation of the alternative pathway. Therefore, there is a greater AP activation level in the retina of an individual carrying the negatively effecting risk variant 402H than in individuals

with Y402.[139–147] This results in constant induction of the inflammatory cascade resulting in tissue damage and ultimately drusen formation in AMD.[148] Evidence for complement activation in AMD were provided by the identification of complement proteins including C3, and the products of its activation and degradation, such as the anaphylatoxins C3a and C5a in drusen from eyes of AMD patients.[149,150] The anaphylatoxins C3a and in particular C5a, are potent inflammatory factors, stimulating secretion of cytokines, proteolytic enzymes, reactive oxygen species, and other pro-inflammatory molecules that lead to amplification of inflammatory responses[151,152] and aplenty other physiological responses that range from chemoattraction to apoptosis.[153,154] The anaphylatoxins operate through their respective receptors, namely C3aR and C5aR. These receptors belong to the seven membrane-spanning G-protein-coupled receptors.[152] A recent study demonstrated activation of the NF-κB pathway in RPE cells after stimulation with C5a.[155] Due to the sub-retinal deposition of drusen, RPE cells may be constantly stimulated by these inflammatory factors.[38,150]

3.5. The Inflammasome

The inflammasome is a key pro-inflammatory signaling pathway primarily discovered in cells of the innate immune system. The name “inflammasome” refers to structural and functional similarities with an apoptotic signaling pathway, the “apoptosome”.[156,157] Both pathways involve cytoplasmic sensor molecules that upon activation assemble large, cytosolic, multiprotein complexes. The activation and subsequent assembly of inflammasomes is induced by divers stimuli which are often microbial products or subjects associated with cellular damage.[158,159] Beside cells of the innate immune system such as dendritic cells and neutrophils,[160,161] varying levels of inflammasome activity have also been demonstrated in certain nonimmune cells, such as keratinocytes and RPE cells.[162] Although NLRP3 inflammasome activity frequently plays a protective role, such as mediating host defense against intracellular microbes and viruses as well as tissue and wound repair,[163–166] it contributes to pathology in some instances. Impaired inflammasome pathway function was first associated with a group of rare autoinflammatory diseases called cryopyrin-associated periodic syndromes (CAPS), and are caused by mutations in one of the inflammasome sensor molecules, the NLRP3 (also known as cryopyrin).[167,168] Evidence indicates that the NLRP3 inflammasome is also implicated in a growing number of common metabolic diseases involving chronic inflammation[169] such as atherosclerosis, and type 2 diabetes.[170–172]

3.5.1 Inflammasomes activate caspase-1 resulting in IL-1β and IL-18 processing

Caspase-1 is a cysteine protease that use cysteine as the nucleophilic group for substrate cleavage and cleaves the peptide bond carboxy-terminal to aspartic acid residues.[173] The best known substrates of caspase-1 are the cytosolic zymogens pro-IL-1β and pro-IL-18.[174]. Cleavage by caspase-1 generates the mature and biologically active pro-inflammatory cytokines IL-1β and IL-18. Therefore, caspase-1 is characterized as an inflammatory caspase in contrast to apoptotic caspases. Furthermore, caspase-1 does not play a role in apoptosis; it is responsible for induction of a distinct form of programmed cell death, referred to as pyroptosis. However, caspase-1 resemble the apoptotic cell death initiating caspases 8,9 and 10, as caspase-1 has large prodomains, the "death domain" that enable homotypic interaction with other proteins.[175,176] This interaction domain typically consist of six or seven antiparallel α-helices, the relative orientation determines their classification as a caspase activation and recruitment domain (CARD) or pyrin domain (PYD). Caspase-1 is expressed as an inactive zymogen that needs to be activated before it can exert its function as a protease. An essential step in caspase-1 activaion is the assembly of the inflammasome.[177]

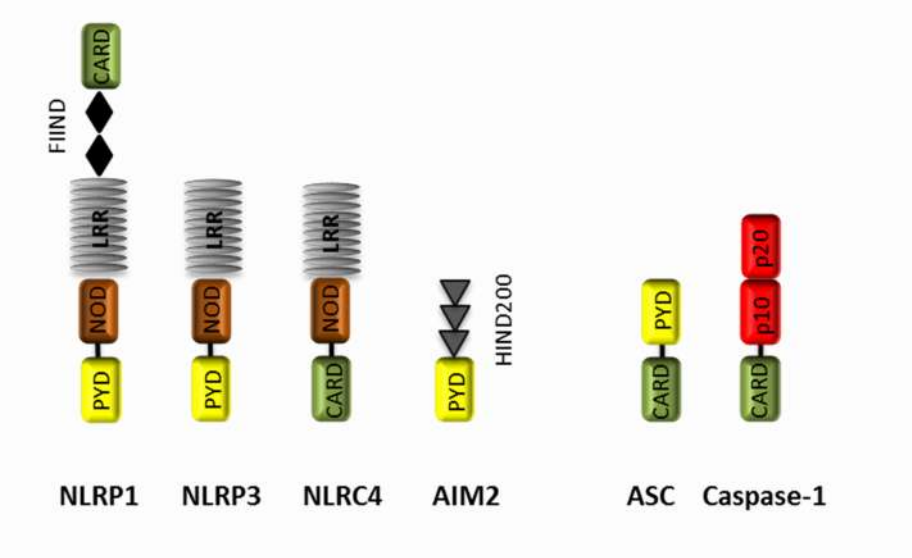

Figure III.4: Domain structure of inflammasome proteins. The image presents the best known inflammasome forming PRRs that belong to two families, the NLR family and the PYHIN family. The NLR familiy (NLRP1, NLRP3 and NLRC4) contain a nucleotide-binding oligomerization domain (NOD), carboxy-terminal leucine-rich repeat (LRR), and contain either a pyrin domain (PYD) or a caspase activation and recruitment domain (CARD) or both. NLRP1 has C-terminal an additional function-to-find domain (FIIND domain) and a CARD. The PYHIN familiy (AIM2) contain an N-terminal PYD that is followed by a C-terminal PYHIN family signature domain, the hematopoietic interferon-inducible nuclear protein domain (HIN200). The image was adapted from Walsh et al., 2014.[178]

The inflammasome is an intracellular multiprotein complex that consists of pro-caspase-1, the adapter ASC (apoptosis-associated speck-like protein containing a CARD), and a pattern recognition receptors (PRR). The best known PRRs that are capable of forming an inflammasome complex belong either to the cytosolic nucleotide-binding oligomerization domain receptors, in short NOD-like receptors (NLRs), such as NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP3 or NLRC4 (NOD-, LRR- and CARD-containing 4) or to the AIM2 like receptor (ALR) family, namely AIM2 (absent in melanoma 2).[175,177,179] With the exception of NLRP1, all NLR proteins have a tripartite domain structure.[180] This structure is composed of a central nucleotide-binding oligomerization domain (NOD), C-terminal series of leucine-rich repeats (LRRs), and a N-terminal protein-protein interaction domain, either a PYD or a CARD. The human NLRP1 has C-terminal an additional function-to-find domain (FIIND) and a CARD. Therefore, NLRP1 possess two interaction domains for homotypic protein-protein interaction. AIM2 harbours a N-terminal PYD that is followed by a C-terminal PYHIN family signature domain, the hematopoietic interferon-inducible nuclear protein domain (HIN200).[181] Figure III.4 demonstrates the domain structure of inflammasome proteins.

3.5.2 Inflammasome activation

Inflammasome activation is regulated by a “two-signal” mechanism. Classically, the first signal is referred as inflammasome priming and involves the transcription of pro-IL-1β that is induced by activation of the transcription factor nuclear factor-κB (NF-κB). Therefore, priming can be accomplished by NF-κB agonists such as the TLR4 ligand lipopolysaccharide (LPS), tumor necrosis factor α (TNF-α), and the cytokines IL-1α and IL-1β.[182–185] Although, in many cells pro-IL-18 is constitutively expressed and does not need priming,[186] its expression is increased by cellular activation.[187] In contrast, the NLRP3 protein demonstrates a basal expression that is not sufficient for inflammasome activation in a resting cell.[183,188] Thus, similarly to IL-1β generation, a priming step is required to drive the NLRP3 transcription. Interestingly, various immune cells regulate IL-1 production differently. Macrophages require a two signal process (TLR ligand and NLR stimuli), whereas monocytes possess a constitutively activated form of caspase-1 through the release of endogenous ATP, and that is why monocytes only need cell priming by stimulation with TLR2 or TLR4 ligands.[189] NLRP3 transcription can be activated by diverse immune signalling or cytokine receptors, such as the tumor necrosis factor receptor (TNFR) or interleukin-1 receptor (IL-1R). By this, immune cells are influenced by their susceptibility to NLRP3 inflammasome triggers.[183,188] Once primed, the “second signal” triggers inflammasome activation that results in oligomerization

of the inflammasome constituents, recruitment of the adapter protein ASC and pro-caspase-1 as well as subsequent cleavage of pro-IL-1β and pro-IL-18 into its mature forms.[190]

Each inflammasome scaffold is activated by particular PAMPs or DAMPS. The sensor molecules of inflammasomes can directly detect distinct signals such as the bacterial cell wall component muramyl dipeptide (MDP) by NLRP1[191] and double stranded DNA by AIM-2.[188,192–194] NLRC4 is activated by flagellin and certain components of bacterial secretion systems.[195] However, NLRP3 responds to a diverse array of PAMPs and DAMPs.[170,196] Therefore, it is unlikely that all bind directly to NLRP3. For this reason, a common molecule or pathway that is responsible for NLRP3 inflammasome activation has been proposed. Studies indicate three possible pathways. The first model for NLRP3 inflammasome activation suggest ROS as a proximal trigger since increased ROS level have been demonstrated upon treatment with NLRP3 activators.[197–199] The second model proposes a drop in intracellular potassium (K^+) concentration due to pore-forming bacterial toxins or the formation of endogenous ion channels that cause inflammasome activaiton.[200] A third model suggests disruption of the lysosomal membrane as NLRP3 inflammasome activation signal. Phagocytosis of particles or live pathogens may result in lysosomal membrane rupture that leads to release of lysosomal components and the putative NLRP3-activating molecule into the cytosol. The lysosomal protease cathepsin B or a cytosolic substrate of cathepsin proteolytic activity are most likely to activate the NLRP3 inflammasome.[126,170,201]

The inflammasome-forming NLR´s, and the HIN domain in the case of AIM2, are stabilized in an inactive but signaling-competent state in the absence of an activating stimulus. With ligand binding, the auto-inhibitory state undergoes a conformational change, leading to exposure of the N-terminal PYD or CARD domain[202,203] that enables homotypic interaction (CARD-CARD or PYD-PYD) with other CARD or PYD possessing proteins. Pro-caspase-1 possess a CARD, thus scaffolding proteins that also have a CARD, such as NLRP1 and NLRC4, can directly bind pro-caspase-1.[204] Inflammasome scaffolds that lack a CARD, such as NLRP3, AIM2 and NLRP1 at its C-terminal side, have a PYD domain instead. This domain allows interaction with the adapter molecule ASC, which has both a PYD domain and a CARD, and provides the connection to the CARD domain of caspase-1.[205] Although NLRP1 and NLRC4 can each recruit pro-caspase-1 independently of ASC via their CARDs, ASC might enhance inflammasome activation.[191,206] The association of caspase-1 with the inflammasome components allows its processing and activation. Pro-caspase-1 is a 44 kDa protein that consist of one large subunit (20 kDa) and one small subunit (10 kDa).

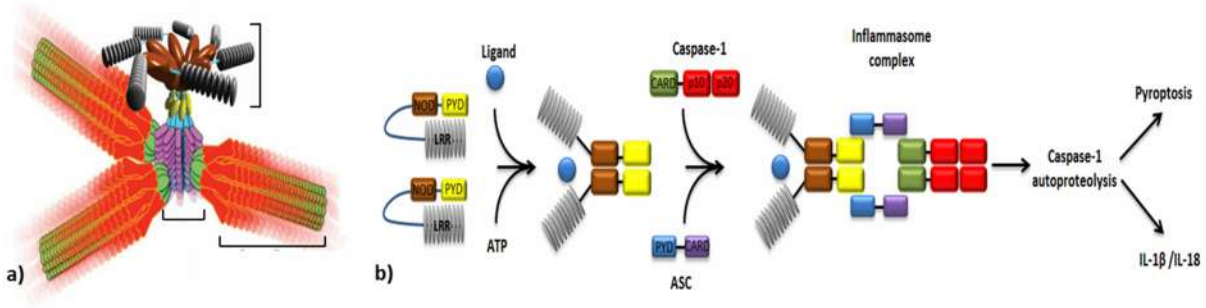

Figure III.5: Generic model of inflammasome activation. Cytosolic nucleotide-binding domain, leucine-rich repeat (NOD-LRR) proteins are maintained in an autoinhibited state until ligand recognition and ATP binding drive oligomerization via the NOD domain. Recruitment of caspas-1 to form the inflammasome complex occurs directly via CARD-CARD interactions or indirectly via the adaptor ASC (a, modified from Lu et al., 2014;[207] b, modified from Moltke et al., 2013.[208]

Recruitment of pro-caspase-1 to the inflammasome results in a high local concentration of the zymogen. This pro-caspase-1 clustering triggers proximity-induced oligomerization of caspase-1,[209] resulting in autoproteolysis, release of the CARD domain and tetramerization of two large and two small subunits, leading to the formation of the active enzyme.[210,211] Figure III.5 gives an generic model of the NLRP3 inflammasome and its polymerization mechanism. Upon activation, caspase-1 cleaves pro-IL-1β and pro-IL-18, generating the corresponding mature, biologically active cytokines IL-1β and IL-18. Both, pro-IL-1β and pro-IL-18 lack a signal peptide for protein secretion by the ER-Golgi secretory pathway. Therefore, these cytokine precursors reside in the cytosol of the cell. Nevertheless, following processing of the mature forms (IL-1β and IL-18), the cytokines are secreted from the cells by a poorly understood pathway termed unconventional protein secretion, which occurs independently of the classical Golgi pathway.[212]

3.5.3 Pyroptosis versus Apoptosis

Cell death is generally described dichotomously as either passive or programmed cell death. Necrosis (from the Greek ‘necro’, meaning death, and ‘sis’, meaning a condition of) is a passive form of cell death that is induced by accidental damage of tissue. During necrosis no activation of caspases or any cell death specific program occurs.

Apoptosis (from the Greek ‘apo’, meaning away from, and ‘ptosis’, meaning falling) represents the best-studied form of programmed cell death that needs activation of caspases. Apoptosis represents an active and programmed cell death mechanism that is observed during homeostatic cell processes such as normal cell turnover in healthy adult tissues as well as during embryonic development.[213] Meanwile, it has been appreciated that

various non-apoptotic forms of programmed cell death also exist, such as pyroptosis. Apoptosis and pyroptosis differ in terms of molecular and morphological changes of the affected cells as presented in table III.1. Morphologically, apoptotic cell death is characterized by cytoplasmic shrinkage and chromatin condensation (pyknosis) that is followed by nuclear fragmentation (karyorrhexis). During apoptosis, caspase-mediated proteolysis of inhibitor of caspase-activated DNase (ICAD) releases caspase-activated DNase (CAD). The activated DNase CAD cleaves DNA between nucleosomes. As a result, characteristic oligonucleosomal DNA fragments of about 180 bp occure.[214] In addition, during apoptosis the plasma membrane forms cytoplasmic blebs and cell membrane enclosed cell fragments (apoptotic bodies). In healthy cells, phosphatidylserine (PS) is actively restricted to the inner leaflet of the plasma membrane by aminophospholipid translocase activity. In apoptosis, a combined effect of decreased phospholipid translocase activity and activation of a phospholipid scramblase[215] leads to PS externalization. PS exposure on the cell surface can be recognized by phagocytes as a signal for engulfment.[216,217] Therefore, apoptosis is considered to be immunologically silent.[218] Importantly, apoptotic cells retain their membrane integrity and therefore do not evoke acute inflammation.

	Characteristics	Apoptosis	Pyroptosis
Morphology	Cell lysis	–	+
	Cell swelling	–	+
	Pore formation	–	+
	Membrane blebbing	+	–
	DNA fragmentation	+	+
Mechanism	Caspase-1 activation	–	+
	ICAD cleavage	+	–
Outcome	inflammation	–	+

Table III.I: Differences between pyroptosis and apoptosis. Despite sharing characteristics with apoptosis, the morphological, mechanistic and physioloical features of pyroptosis make it a distinct form of programmed cell death. The table was adapted from Labbé and Saleh, 2011. [219]

Pyroptosis (from the Greek 'pyro', relating to fire or fever, and 'ptosis', meaning falling) was first described as an atypical cell death mode of macrophages infected with *Salmonella typhimurium* by Brennan and Cookson in 2000.[220] However, pyroptosis is not restricted to macrophages or bacterial infections.[221] Pyroptosis is a caspase-1 dependent form of

programmed cell death. An important function of caspase-1 is its key role in the processing of inactive IL-β and IL-18 into mature inflammatory cytokines, demonstrating pyroptosis as an inherently pro-inflammatory cell death mode.[222] Perhaps the most striking difference between pyroptosis and apoptosis is at the plasma membrane. Microscopically, pyroptotic cell death is characterized by an increase in cell size due to cytosolic swelling generated by the caspase-1 dependent formation of plasma membrane pores followed by rapid mebrane rupture and release of cytosolic contents. Some of these components, such as ATP, pro-IL-1α, and HMGB1 become pro-inflammatory effectors which are able to recruit neutrophils and induce inflammation.[223] Although cleavage of chromosomal DNA was assumed to indicate apoptotic cell death,[224] DNA damage also occures during pyroptosis. [220,225–228] However, DNA cleavage during pyroptosis is carried out by an unidentified nuclease that does not induce ICAD degradation and does not display the oligonucleosomal pattern characteristic of apoptosis.[214,225,229]

3.5 Aims of the study

Rationale

Various studies demonstrated that oxidative and lipofuscin-mediated photooxidative damage play an important pathophysiological role in AMD. Further characteristics of the disease include a chronic low-grade immune process including complement activation in the sub-RPE space. Yet the identity of a molecular link between hallmark features of AMD pathogenesis including lipofuscin accumulation, photooxidative damage, innate immune response in sub-RPE space, and RPE cell degeneration remains unclear. Recent studies have shown that the NLRP3 inflammasome, a key mediator of the innate immune system, is activated in the RPE of patients with atrophic and neovascular AMD. However, the triggers for NLRP3 inflammasome activation in AMD pathology remain unknown.

The purpose of this study was to examine NLRP3 inflammasome activation by oxidative/ photooxidative damage in human RPE cells as a potential link between hallmark pathogenic features in age-dependent macular degeneration.

The specific aims of this study were:

- to test whether lipofuscin-mediated photooxidative stress activates the inflammasome pathway and induces IL-1β and IL-18 secretion in human cultured RPE cells via lysosomal membrane permeabilization **(chaper IV)**,

- to investigated the effects of LMP-induced inflammasome activation on the secretion profile of inflammation-related cytokines and VEGF as well as their secondary effects on microglial and vascular endothelial cells in vitro **(chapter V)**,

- to identify a potential priming effect for activated complement in human serum and to further delineate the responsible complement component **(chapter VI)**,

- to elucidate the effects of inflammasome priming on mechanism and extent of photooxidative damage-induced cytotoxicity in RPE cells **(chapter VII)**.

IV. Light induces NLRP3 inflammasome activation in retinal pigment epithelial cells via lipofuscin-mediated photooxidative damage

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4.1 Abstract

Photooxidative damage and chronic innate immune activation have been implicated in retinal pigment epithelium (RPE) dysfunction, a process that underlies blinding diseases such as age-related macular degeneration (AMD). To identify a potential molecular link between these mechanisms, we investigated whether lipofuscin-mediated phototoxicity activates the NLRP3 inflammasome in RPE cells in vitro. We found that blue light irradiation (dominant wavelength 448 nm, irradiance 0.8 mW/cm^2, duration 6 h) of lipofuscin-loaded primary human RPE cells and ARPE-19 cells induced photooxidative damage, lysosomal membrane permeabilization (79.5 % of cells vs. 3.8 % in nonirradiated controls), and cytosolic leakage of lysosomal enzymes. This resulted in activation of the inflammasome with activation of caspase-1 and secretion of interleukin-1β (14.6 vs. 0.9 pg/ml in nonirradiated controls) and interleukin-18 (87.7 vs. 0.2 pg/ml in nonirradiated controls). Interleukin secretion was dependent on the activity of NLRP3, caspase-1, and lysosomal proteases cathepsin B and L. These results demonstrate that accumulation of lipofuscin-like material in vitro renders RPE cells susceptible to phototoxic destabilization of lysosomes, resulting in NLRP3 inflammasome activation and secretion of inflammatory cytokines. This new mechanism of inflammasome activation links photooxidative damage and innate immune activation in RPE pathology and may provide novel targets for therapeutic intervention in retinal diseases such as AMD.

4.2 Introduction

Age-related macular degeneration (AMD) is a neurodegenerative disease of the retina that represents the most common cause of blindness in all industrialized countries.[230] The molecular pathogenesis of AMD is still not completely resolved which hinders the development of effective therapies, in particular for the atrophic subtype of the disease. In AMD, the retinal pigment epithelium (RPE) becomes progressively dysfunctional and eventually degenerates, resulting in photoreceptor death and visual function loss. Experimental and clinical studies identified oxidative and photooxidative damage to the RPE as a contributing factor.[3,33] This damage is thought to be mediated, at least in part, by the phototoxic properties of lipofuscin that accumulate in RPE cells over a lifetime as a result of constant photoreceptor outer segment phagocytosis and degradation. Moreover, genetic and histochemical evidence supports a role of chronic innate immune activation at the level of the RPE in AMD pathology.[149,231] A candidate innate immune signaling receptor for RPE cell

pathology in AMD pathogenesis is the nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing protein 3 (NLRP3) inflammasome.[185,232] Two steps are required for activation of the NLRP3 inflammasome.[186] First, a priming signal results in the transcriptional induction of NLRP3 and pro-interleukin (IL)-1β. Second, an activation signal triggers the assembly of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and pro-caspase-1 into the inflammasome protein complex. The activated inflammasome mediates caspase-1 activation, which in turn proteolytically activates pro-IL-1β and pro-IL-18 into their mature forms. These highly pro-inflammatory cytokines have pleiotropic autocrine and paracrine effects on a variety of cell types. NLRP3 inflammasome activation in the RPE has been reported in both atrophic and neovascular AMD.[185,232] Several substances have been suggested to provide the signal for inflammasome activation in AMD including drusen components such as C1q[233] and amyloid-beta,[234] Alu RNA accumulation secondary to DICER1 deficiency,[232] the lipofuscin component Nretinylidene-N-retinyl-ethanolamine (A2E),[235] and the lipid peroxidation product 4-hydroxynonenal (HNE).[236] A key pathway upstream of the NLRP3 inflammasome is lysosomal membrane permeabilization (LMP) and subsequent cytosolic leakage of lysosomal components.[126,186] Indeed, this mechanism has been reported to activate the inflammasome in RPE cells following chemical induction of LMP.[185] Another mechanism resulting in LMP in RPE cells is phototoxic damage mediated by intralysosomal accumulation of photoreactive lipofuscin.[96,97] In this study, we investigated whether blue light-induced photooxidative stress in human RPE cells, intensified by accumulated lipofuscin, and subsequent lysosomal membrane permeabilization result in activation of the NLRP3 inflammasome by leaking lysosomal enzymes. Thereby, this study aimed to identify a novel mechanism of inflammasome activation by light damage that may provide new treatment targets for blinding diseases such as AMD.

4.3 Material and methods

4.3.1 Cell culture and treatments

Human fetal primary RPE cells (pRPE) were obtained from Lonza (Cologne, Germany), cultured as recommended by the manufacturer, and used in experiments for a maximum of six passages. The human nontransformed RPE cell line ARPE-19 (CRL-2302; ATCC, Rockville, MD, USA) was cultured as previously reported.[237] The immortalized murine

wildtype and NLRP3 knockout macrophage cell lines used in our study have been characterized previously[126] and were cultured as described.[80] For inhibition of photooxidative damage, the singlet oxygen scavenger 1,4-diazabicyclooctane (DABCO; SigmaAldrich, Munich, Germany) was added to the culture media at a concentration of 30 mM during blue light irradiation. Lysosomal membrane permeabilization was induced by incubation of cells with 1 mM ciprofloxacin (Sigma-Aldrich, Munich, Germany) for 3 h or 1 mM L-leucyl-L-leucine methylester (Leu-Leu-OMe; Bachem, Bubendorf, Switzerland) for 3 h. Cathepsin B inhibitor CA-074 (Calbiochem, Darmstadt, Germany) and cathepsin L inhibitor Z-Phe-Phe-fluoromethylketone (Z-FF-FMK; Calbiochem) were used for cell treatment at a concentration of 10µM each for 1 h prior to and during irradiation treatment. For inhibition of caspase-1, we applied 10µM of Z-Tyr-Val-Ala-Asp-fluoromethylketone (Z-YVAD-FMK; BioVision, Munich, Germany) 30 min prior to and during irradiation.

4.3.2 Induction of lipofuscin accumulation

Isolated porcine POS were modified with lipid peroxidation products malondialdehyde (MDA) or 4-hydroxynonenal (HNE) as described.[80] Lipofuscin accumulation was induced by incubation of cells with modified POS for 7 days or, alternatively, by incubation with native POS and concomitant lysosomal inhibition by ammonium chloride (NH_4Cl; Sigma-Aldrich, Munich, Germany) as reported previously.[237] Intracellular lipofuscin granules were documented by fluorescence microscopy (IX71; Olympus, Hamburg, Germany) using a fluorescein filter set (excitation filter wavelength 480/40 nm, emission filter wavelength 535/50 nm). For flow cytometric quantification (FACS Canto II; BD Biosciences, Heidelberg, Germany), we employed the FITC channel (excitation laser wavelength 488 nm, detection filter wavelength 530/30 nm). Data was acquired and analyzed by appropriate software (DIVA; BD Bioscience, Heidelberg, Germany; FlowJo; Tree Star, Ashland, OR, USA).

4.3.3 Blue light irradiation

For blue light irradiation of RPE cells, we employed a 3×3 array of blue LEDs (XLamp XP-E royal blue; Cree, Durham, NC, USA). The LED spectrum as measured by a spectroradiometer (PR-655 SpectraScan; Photo Research, Chatsworth, CA, USA) exhibited a peak wavelength of 448 nm with a full width at half maximum (FWHM) of 24 nm. Cells were irradiated from a distance of 35 cm within a cell culture incubator for the indicated times. In this setup, cells were exposed to an irradiance of 0.8 mW/cm2 as measured using a power meter (FieldMax-TOP with PM10V1 sensor; Coherent, Santa Clara, CA, USA). Measurements confirmed that irradiation did not affect the temperature of the cell culture

medium. A dose–response curve of the phototoxic effect of irradiation treatment on RPE cells is provided in Fig. IV.2b (control group). Photooxidative damage in blue light-irradiated cells was assessed by immunostaining of protein carbonyls using a commercially available kit (OxyICC Oxidized Protein Detection Kit; Merck Millipore, Darmstadt, Germany) according to manufacturer's instructions, followed by quantification by flow cytometry (FACS Canto II; BD Bioscience, Heidelberg, Germany).

4.3.4 Cell death detection assays

Blue light-induced cell death was documented by light microscopy. Cell death-associated plasma membrane disintegration was assessed by means of release of cytoplasmic lactate dehydrogenase (LDH). Measurements of LDH release were performed in cell supernatants 48 h after the start of irradiation using a calorimetric assay (Cytotoxicity Detection Kit; Roche, Mannheim, Germany) according to the manufacturer's instructions. As an additional cell death assay, we quantified loss of cell attachment 48 h after the start of irradiation by crystal violet assay as previously described.[238]

4.3.5 Analysis of lysosomal membrane permeabilization

Cells were incubated with 1µg/ml acridine orange (Sigma-Aldrich, Munich, Germany) for 15 min and washed two times with PBS. Lysosomal staining (red) was documented by fluorescence microscopy. Lysosomal permeabilization was quantified by flow cytometry (FACS Canto II; BD Bioscience, Heidelberg, Germany) as percentage of cells with loss of lysosomal staining. For analysis of leakage of lysosomeal enzymes, lysosomal and cytosolic cell fractions wereseparated following plasma membrane permeabilization using digitonin (SigmaAldrich, Munich, Germany) as reported.[239] Subsequently, cytosolic activity of lysosomal acid phosphatase was assessed by measuring cleavage of a specific substrate using a commercially available assay (Sigma-Aldrich, Munich, Germany).

4.3.6 Analysis of inflammasome activation

RPE cells were primed with 4 ng/ml human recombinant IL-1α (R&D Systems, Wiesbaden, Germany) for 48 h[185] and macrophages with 200 ng/ml lipopolysaccharide (LPS, from Escherichia coli0127:B8; Sigma-Aldrich, Munich, Germany) for 6 h. For analysis of caspase-1 activity, a fluorochrome-labeled inhibitor of caspase (FLICA) detection assay specific for caspase-1 [carboxyfluorescein-Tyr-Val-Ala-Asp-fluoromethylketone (FAM-YVAD-FMK); Immunochemistry Technologies, Bloomington, MN] was used according to the manufacturer's instructions. Caspase-1 activation was documented by fluorescence microscopy and quantified by flow cytometry (FACS Canto II; BD Bioscience, Heidelberg,

Germany). Interleukin secretion following inflammasome activation in RPE cells was measured by specific ELISAs for human IL-1β (BD Biosciences, Heidelberg, Germany) and human IL-18 (BD Bioscience). Inflammasome activation in murine macrophages was assessed by an ELISA against murine IL-1β(R&D Systems, Wiesbaden, Germany).

4.3.7 NLRP3 siRNA knockdown

Lipofuscin accumulation was induced in ARPE-19 cells by incubation with HNE-modified POS as described above. Then, cells were transfected with 100 nM small interfering RNA (siRNA) targeting human NLRP3 (Ambion Silencer Select siRNA, ID s41556; Life Technologies, Darmstadt, Germany) or 100 nM nonspecific siRNA (Ambion Silencer Select Negative Control siRNA; Life Technologies) for 24 h using a transfection reagent (Invitrogen Lipofectamin RNAiMax; Life Technologies) according to the manufacturer's instructions.[240] Subsequently, cells were primed with IL-1α and irradiated with blue light as described above.

4.3.8 Statistical analysis

Experiments were performed in doublets (Figs. IV.5 and IV.6) or triplets (all other experiments). Results are presented as mean ± standard deviation. Statistical analysis was performed using one-tailed (Fig. IV.7c) or two-tailed (all other experiments) unpaired Student'st tests. Differences were considered statistically significant at $p<0.05$. In all figures, significance levels as compared to controls are indicated using * for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$.

4.4 Results

4.4.1 Blue light irradiation of lipofuscin-loaded RPE cells induces photooxidative damage

Lipofuscinogenesis was induced in pRPE cells and ARPE-19 cells either by incubation with isolated photoreceptor outer segments (POS) and simultaneous inhibition of lysosomal degradation by ammonium chloride (NH_4Cl) or by incubation with POS stabilized against lysosomal degradation by covalent modification with lipid peroxidation products malondialdehyde (MDA) or 4-hydroxynonenal (HNE) as described.[80,237,241] Lipofuscinogenesis was evaluated by assessing the accumulation of granular material with lipofuscin-like autofluorescence using fluorescence microscopy and flow cytometry (Fig. IV.1a,b). Consistent with our previous reports, lipofuscin accumulation was significantly increased in all three treatment groups compared to control groups of cells treated with either

native POS alone, with the lysosomal inhibitor alone, or left untreated. The finding that cells treated with the lysosomal inhibitor generate some amount of lipofuscin even in the absence of POS is attributable to impaired autophagy as described before.[237] The spectral profiles of lipofuscin generated in this in vitro model have been reported.[237] Following lipofuscin loading, RPE cells were irradiated with blue light to induce photooxidative stress. Immuno-cytochemistry confirmed that blue light irradiation resulted in oxidative damage by lipid peroxidation-mediated formation of protein carbonyls (Fig. IV.1c,d). Even control cells exhibited some photooxidative damage secondary to blue light irradiation.

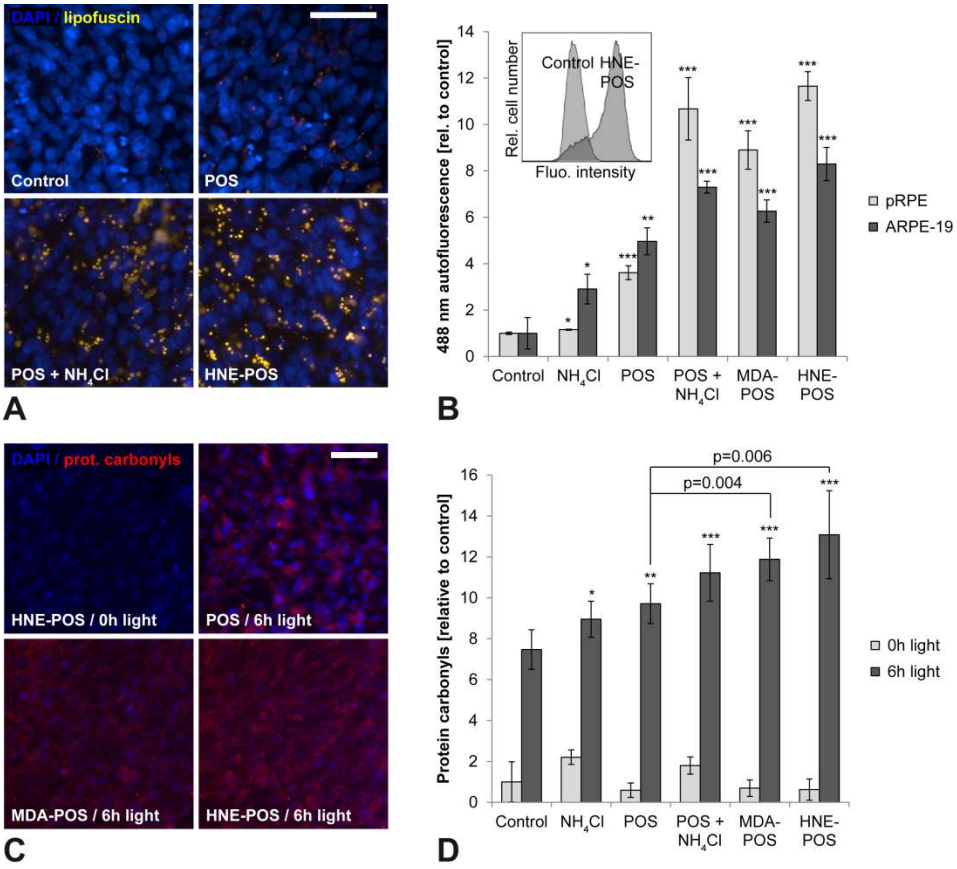

Figure IV.1: Blue light irradiation of lipofuscin-loaded RPE cells induces photooxidative damage. (A) Intracellular lipofuscin accumulation (yellow) was documented in ARPE-19 cells by fluorescence microscopy. Nuclei were visualized by DAPI staining (blue). (B) Lipofuscinogenesis was quantified in pRPE cells and ARPE-19 cells by flow cytometry measurements of mean cellular autofluorescence intensity. An example result is shown as an insert. Cells were left untreated (control) or incubated with the lysosomal inhibitor ammonium chloride (NH4Cl), unmodified photoreceptor outer segments (POS), POS and ammonium chloride (POS+NH4Cl), malondialdehyde-modified POS (MDA-POS), and 4-hydroxynonenal-modified POS (HNE-POS). (C) Photo-oxidative damage was assessed in ARPE-19 cells by immunocytochemical detection of protein carbonyls (red). Nuclei were labeled by staining with DAPI (blue). (D) Mean cellular fluorescence of immunocyto-chemically labeled protein carbonyls was quantified by flow cytometry. Scale bars, 50 µm.

However, photooxidative damage was significantly increased in lipofuscin-loaded cells as compared to controls, consistent with the known photoreactive properties of lipofuscin. Moreover, cells incubated with MDA- or HNEmodified POS exhibited significantly more photooxidative damage compared to cells treated with unmodified POS. We thus verified that the lipofuscin-like material generated by RPE cells in our in vitro model possesses photoreactive properties that result in photooxidative cell damage, similar to lipofuscin generated in human RPE in vivo.[96]

4.4.2 Blue light irradiation of lipofuscin-loaded RPE cells causes phototoxic cell death

As excessive photooxidative damage may result in cell death, we aimed to determine the phototoxic threshold in our cell culture model by measuring cytotoxicity following increasing durations of irradiation of up to 24 h (Fig. IV.2a,b). Cytotoxicity was assessed in parallel by means of loss of plasma membrane integrity (LDH release assay) and loss of cell adhesion (crystal violet assay). Consistently in both assays, cytotoxicity in creased with blue light dose up to an irradiation time of 24 h when cytotoxicity was nearly complete (90–99 %) in all three lipofuscin-loaded treatment groups. Reduction of photooxidative damage by cell incubation with the singlet oxygen scavenger DABCO during irradiation treatment suppressed the cytotoxic effect (Fig. IV.2c). These results confirm that in our RPE cell culture model, lipofuscin generation from phagocytosed POS renders the cells susceptible to photooxidative damage. Based on the results, we selected an irradiation duration of 3 and 6 h for our further experiments as for these irradiation times the detected cytotoxic effect of irradiation is limited.

4.4.3 Lipofuscin-mediated photooxidative damage results in lysosomal membrane permeabilization with cytosolic leakage of lysosomal enzymes

Photoreactivity of intralysosomal lipofuscin can induce lysosomal destabilization.[96,97] We therefore investigated the effects of lipofuscin photoreactivity on lysosomal membrane stability in our model. Lysosomal staining by acridine orange demonstrated intact lysosomes in lipofuscin-loaded cells without irradiation treatment as well as in irradiated cells without lipofuscin loading (Fig. IV.3a). In contrast, cell treatment with both lipofuscin loading and blue light irradiation resulted in a significant loss of lysosomal staining, indicating lysosomal membrane permeabilization. Quantification of lysosomal staining by flow cytometry demonstrated that loss of lysosomal membrane integrity increased with both light dose and lipofuscin load as measured by means of lipofuscin-like autofluorescence (Fig. IV.3b). Cytosolic activity of leaked lysosomal enzymes has been proposed as a mechanism of inflammasome activation.[126]

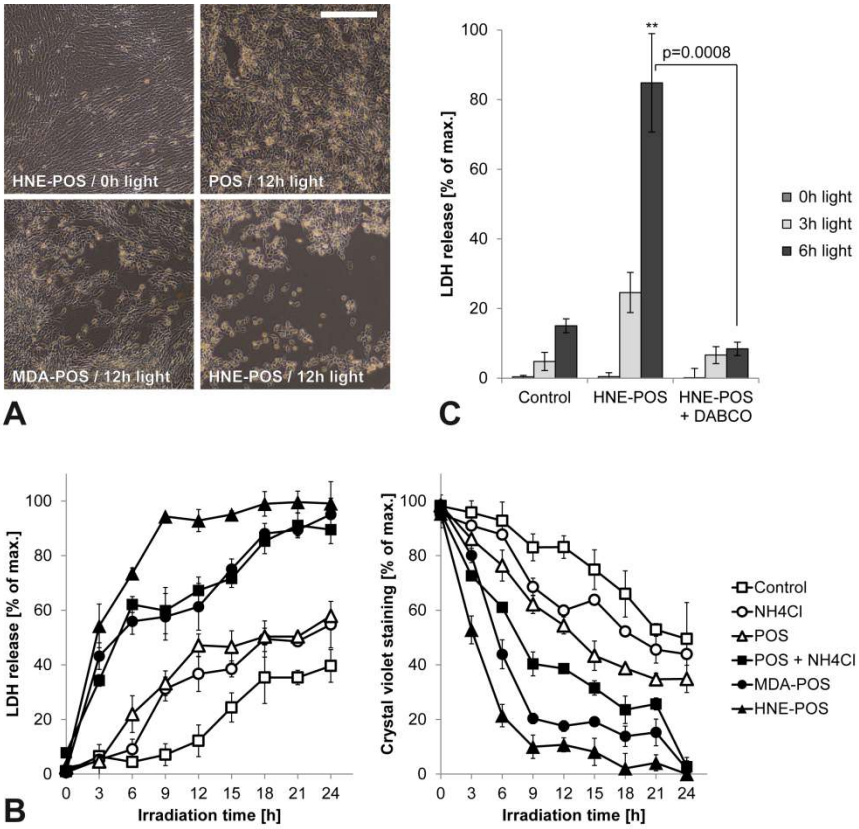

Figure IV.2: Blue light irradiation of lipofuscin-loaded RPE cells causes phototoxic cell death. (A) Photooxidative damage-induced cytotoxicity was documented in ARPE-19 cells by light microscopy. (B) To quantify the phototoxic effect, both loss of plasma membrane integrity and loss of cellular adhesion were analyzed by measuring LDH release and crystal violet staining, respectively. (C) Photooxidative damage secondary to irradiation was reduced by incubation with the singlet oxygen scavenger DABCO.Scale bar,250µm.

To assess whether lysosomal enzyme leakage occurs in light-exposed RPE cells, we separated cytosolic and lysosomal cellular fractions by plasma membrane permeabilization using digitonin. We employed a digitonin concentration of 20µg/ml as for this concentration the isolated cytosolic fraction of untreated control cells exhibited a near maximum concentration of the cytosolic marker enzyme LDH while at the same time contamination by the lysosomal marker enzyme acid phosphatase was low (Fig. IV.3c). Consistent with the results of acridine orange staining, acid phosphatase activity in the isolated cytosolic cell fractions increased with light dose and lipofuscin load (Fig. IV.3d). The observed background cytosolic acid phosphatase activity that was detectable even in nonirradiated control cells may be explained by partial lysosomal membrane permeabilization by the digitonin treatment as indicated by the digitonin titration curve. Our results demonstrate that lipofuscin-mediated photooxidative damage in RPE cells is associated with lysosomal membrane permeabilization and cytosolic leakage of lysosomal enzymes.

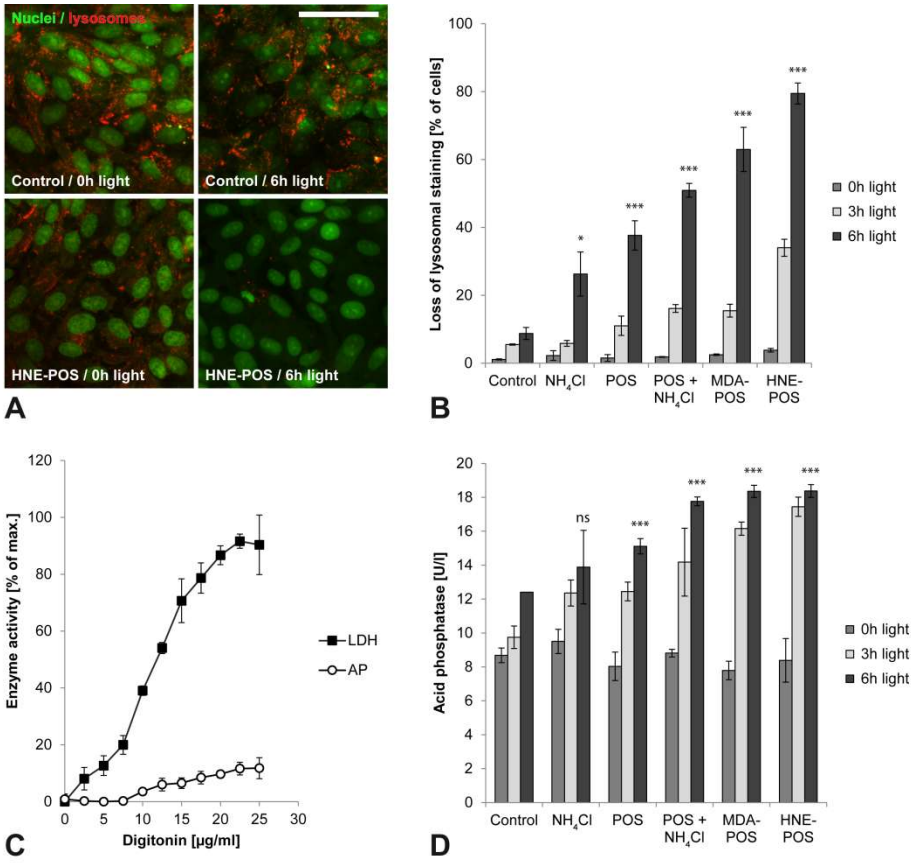

Figure IV.3: Lipofuscin-mediated photooxidative damage results in lysosomal membrane permeabilization with cytosolic leakage of lysosomal enzymes. (A) Intact lysosomes (red) and nuclei (green) were visualized in ARPE- 19 cells by means on acridine orange staining. (B) Lysosomal membrane permeabilization resulted in a loss of lysosomal staining that was quantified by flow cytometry. (C) Digitonin effect on ARPE-19 cells was titrated for maximum plasma membrane permeabilization (release of cytosolic LDH) and at the same time minimal lysosomal membrane permeabilization (release of lysosomal acid phosphatase, AP). (D) A digitonin concentration of 20 µg/ml was selected for separation of cytosolic and lysosomal cellular fractions, and cytosolic leakage of lysosomal enzymes was assessed by analyzing the activity of lysosomal marker enzyme acid phosphatase in the cytosolic fractions. Scale bar, 50 µm.

4.4.4 Lysosomal membrane permeabilization by lipofuscin phototoxicity induces inflammasome activation with activation of caspase-1 and secretion of IL-1β and IL-18

We then investigated inflammasome activation in RPE cells in response to light treatment. As inflammasome activation requires a priming signal, cells were treated with IL-1α that has been shown to induce NRLP3 inflammasome priming in RPE cells.[185] IL-1α-primed cells were irradiated with blue light, and inflammasome activation was assessed subsequently by measuring caspase-1 activation using the FLICA probe FAMYVAD-FMK (Fig. IV.4).

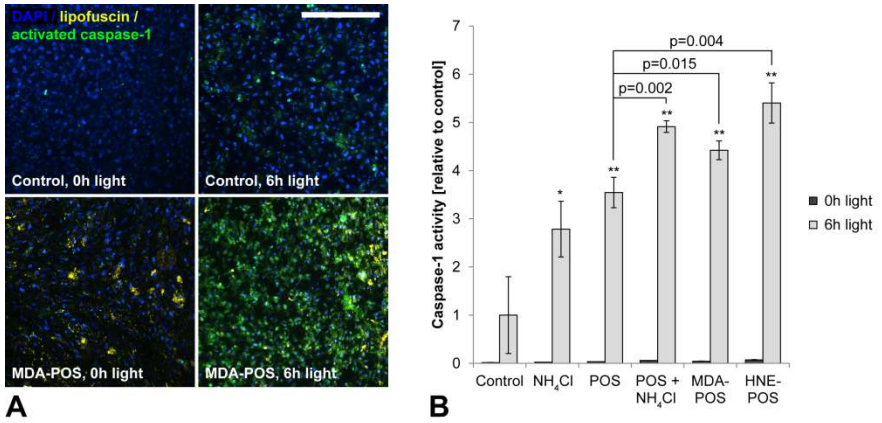

Figure IV.4: Lipofuscin-mediated photooxidative damage results in lysosomal membrane permeabilization with cytosolic leakage of lysosomal enzymes. (A) Intact lysosomes (red) and nuclei (green) were visualized in ARPE- 19 cells by means on acridine orange staining. (B) Lysosomal membrane permeabilization resulted in a loss of lysosomal staining that was quantified by flow cytometry. (C) Digitonin effect on ARPE-19 cells was titrated for maximum plasma membrane permeabilization (release of cytosolic LDH) and at the same time minimal lysosomal membrane permeabilization (release of lysosomal acid phosphatase, AP). (D) A digitonin concentration of 20 µg/ml was selected for separation of cytosolic and lysosomal cellular fractions, and cytosolic leakage of lysosomal enzymes was assessed by analyzing the activity of lysosomal marker enzyme acid phosphatase in the cytosolic fractions. Scale bar, 50 µm.

We detected significantly increased amounts of activated caspase-1 in lipofuscin-loaded, light-irradiated cells compared to controls. Moreover, secretion of inflammasome-regulated cytokines IL-1β and IL-18 was significantly increased in both pRPE and ARPE-19 cells as measured by ELISA (Fig. IV.5a,b). Similar to LMP, inflammasome activation increased with light dose and lipofuscin load asmeasured by autofluorescence. LMP by other means such as treatment with ciprofloxacin[123] or Leu-Leu-OMe[126] likewise induced inflammasome activation in RPE cells (Fig. IV.5c). Suppression of photooxidative damage by cell incubation with the singlet oxygen scavenger DABCO during irradiation resulted in a significant reduction of irradiation-induced IL-1β secretion (Fig. IV.5d), thus confirming that photooxidative damage is the critical mechanism underlying inflammasome activation in our experiments.

4.4.5 Inflammasome activation by lipofuscin phototoxicity is dependent on prior priming, activity of caspase-1, cathepsin B and cathepsin L, and expression of NLRP3

To further delineate the mechanism by which blue light irradiation induces inflammasome activation in RPE cells, we subjected cells to different inhibitor treatments before and during irradiation (Fig. IV.6). No inflammasome-mediated secretion of IL-1β and IL-18 was detectable when inflammasome priming by IL-1α prior to irradiation was omitted. Likewise, inhibition of caspase-1 activity by Z-YVAD-FMK suppressed inflammasome activation.

Finally, inhibition of the lysosomal proteases cathepsin B or cathepsin L resulted in a significant inhibition of IL-1β and IL-18 secretion, supporting a role of lysosomal enzyme leakage in inflammasome activation in our experiments. Next, we aimed to investigate whether inflammasome activation by lipofuscin phototoxicity is mediated by the NLRP3 inflammasome as opposed to other inflammasome subtypes. For this, we knocked down NLRP3 expression in lipofuscinloaded RPE cells by transfection with siRNA against NLRP3 prior to blue light irradiation (Fig. IV.7a). NLRP3 knockdown resulted in a significant reduction of irradiation-induced IL-1β secretion as compared to control cells transfected with nonspecific siRNA. These results demonstrate that inflammasome activation secondary to lipofuscin phototoxicity in RPE cells is mediated by NLRP3. To verify this result in a knockout model, we employed a NLRP3-deficient immortalized murine macrophage cell line[126] as NLRP3-deficient RPE cells were not available to us. Macrophages have been shown to generate lipofuscin secondary to POS phagocytosis in vitro similarly to RPE cells.[242] We confirmed lipofuscin accumulation in macrophages secondary to incubation with POS by fluorescence microscopy (Fig. IV.7b). Lipofuscin-loaded wild-type macrophages. responded to blue light irradiation similar to RPE cells, i.e., by inflammasome activation with increased IL-1βsecretion that was dependent on prior priming of the cells (Fig. IV.7c). In NLRP3 knockout cells, however, irradiation-induced IL-1β secretion was significantly suppressed compared to wildtype controls, consistent with the results of NLRP3 knockdown in human RPE cells.

4.5 Discussion

In the yet unresolved pathogenesis of AMD, oxidative damage and chronic immune response have been demonstrated to be centrally involved. However, the connection between these two mechanisms is unclear. Using an RPE cell culture model, we demonstrated for the first time that photooxidative stress by irradiation with blue light activates the NLRP3 inflammasome. This activation is mediated by permeabilization of lysosomal membranes with subsequent cytosolic leakage of lysosomal enzymes. It is amplified by the photosensitizer lipofuscin which accumulates in the RPE in vivo with age and has the highest concentration in the macula. Thus, the molecular mechanism of light-induced inflammasome activation in the RPE links key pathogenic factors of AMD and may provide new targets for therapeutic strategies. Multiple lines of evidence indicate that lipofuscin accumulation has adverse effects on RPE cell homeostasis and function.[243,244] Moreover, both in vitro and in vivo

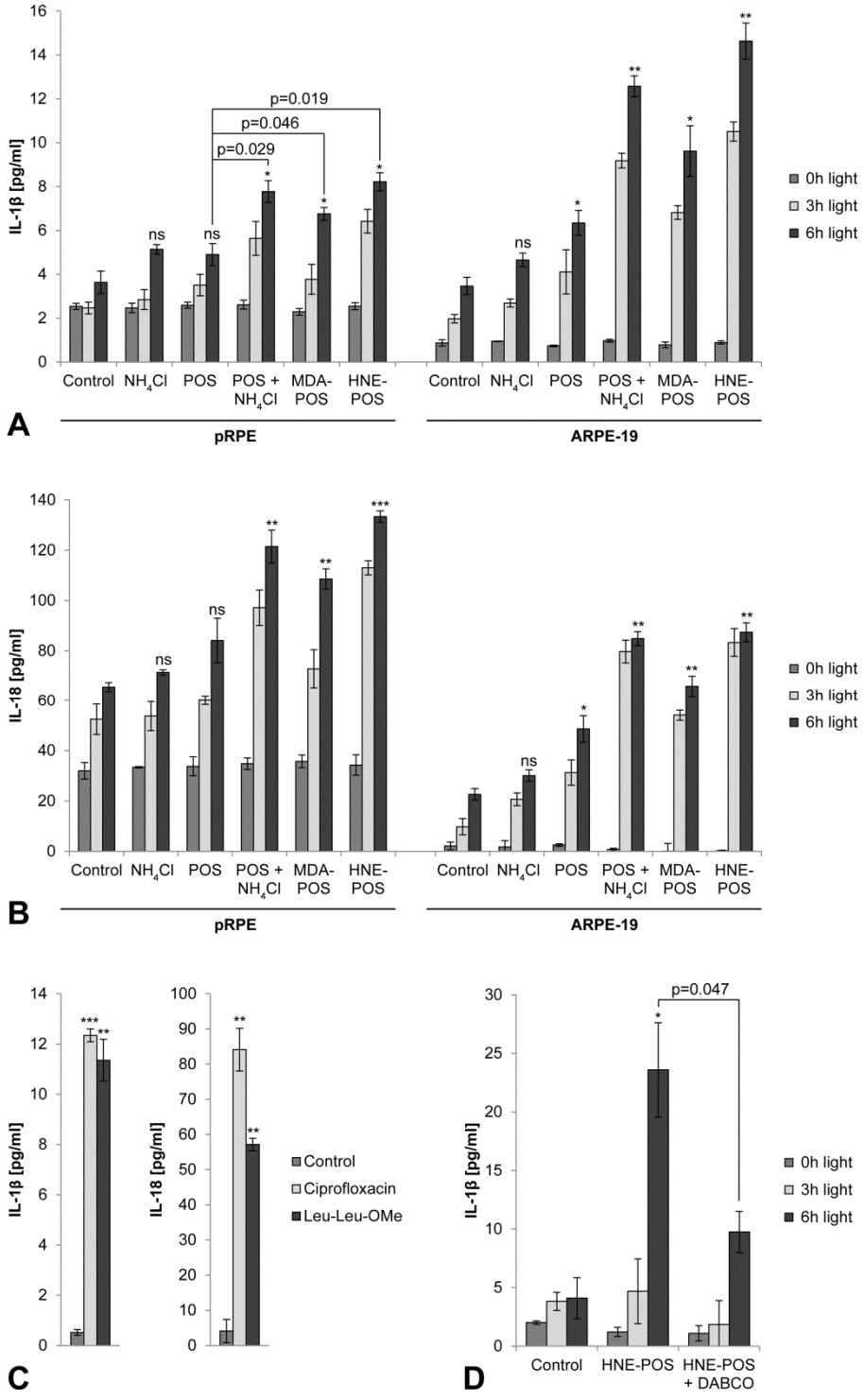

Figure IV.5: Lysosomal membrane permeabilization by lipofuscin phototoxicity results in inflammasome activation with secretion of IL-1β and IL-18. Inflammasome-mediated secretion of mature IL-1β (A) and IL-18 (B) was analyzed by ELISA in pRPE cells and ARPE-19 cells. (C) Lysosomal membrane permeabilization by ciprofloxacin and Leu-Leu-OMe in APRE-19 cells served as positive controls. (D) To assess the role of photooxidative damage in irradiation-induced inflammasome activation, ARPE- 19 cells were incubation with the singlet oxygen scavenger DABCO during blue light treatment.

studies demonstrated that lipofuscin accumulation increases the susceptibility of the RPE to light-induced cell damage and cell loss.[96–98] Based on this body of experimental data, a role of light-induced RPE cell damage in AMD pathogenesis has been postulated, and several clinical studies have investigated a possible association of visible light exposure and AMD development. However, results so far have been ambiguous with some studies demonstrating an association while others failed to do so. Among the positive results, the Chesapeake Bay Watermen Study reported a significant association between long-term visible or blue light exposure and geographic atrophy or disciform scarring.[100] In the Beaver Dam Study, sunlight exposure was significantly associated with early AMD.[99] Finally, the European Eye Study found a significant association of blue light exposure and neovascular AMD in patients with low antioxidant levels.[245] While the clinical data regarding the role of light damage in AMD pathogenesis remains controversial, the contribution of oxidative damage has been clearly established in interventional clinical studies such as the age-related eye disease study (AREDS).[3] In our experiments, we employed a model of blue light-induced photooxidative damage, enhanced by cellular loading with lipofuscin-like material, to study the effects of LMP in RPE cells. Different models for the in vitro study of lipofuscin effects in RPE cells have been described.[246] We and others have used A2E-loaded RPE cells as a model of lipofuscin phototoxicity in the past[97] as A2E is considered the major fluorophore of macular lipofuscin. However, recent data derived from human donor eyes has questioned this role.[247] In this study, we therefore employed a system of endogenous lipofuscin generation from phagocytosed POS in human RPE cells. Cellular lipofuscinogenesis was enhanced by POS modifications with products of lipid peroxidation such as HNE and MDA that result in lysosomal dysfunction by mechanisms described before in detail.[80,237,241] The levels of POS protein modifications used in our cell culture were quantified previously[80] and were chosen to correspond to the range of carbonyl modifications detected in human cells in vivo.[248] While this model was designed to closely resemble the in vivo situation, the composition of lipofuscin-like material generated in this model over a period of 7 days is likely to differ from the composition of lipofuscin generated in RPE cells in vivo over a human lifetime. However, the aim of this study was to investigate the consequences of light-induced LMP in RPE cells, and our model of lipofuscin generation was appropriate to achieve LMP by blue light irradiation, similar to what has been reported for RPE cells loaded with lipofuscin granules generated in human RPE in vivo.[96] Hence, potential differences in lipofuscin composition in our model compared to lipofuscin granules

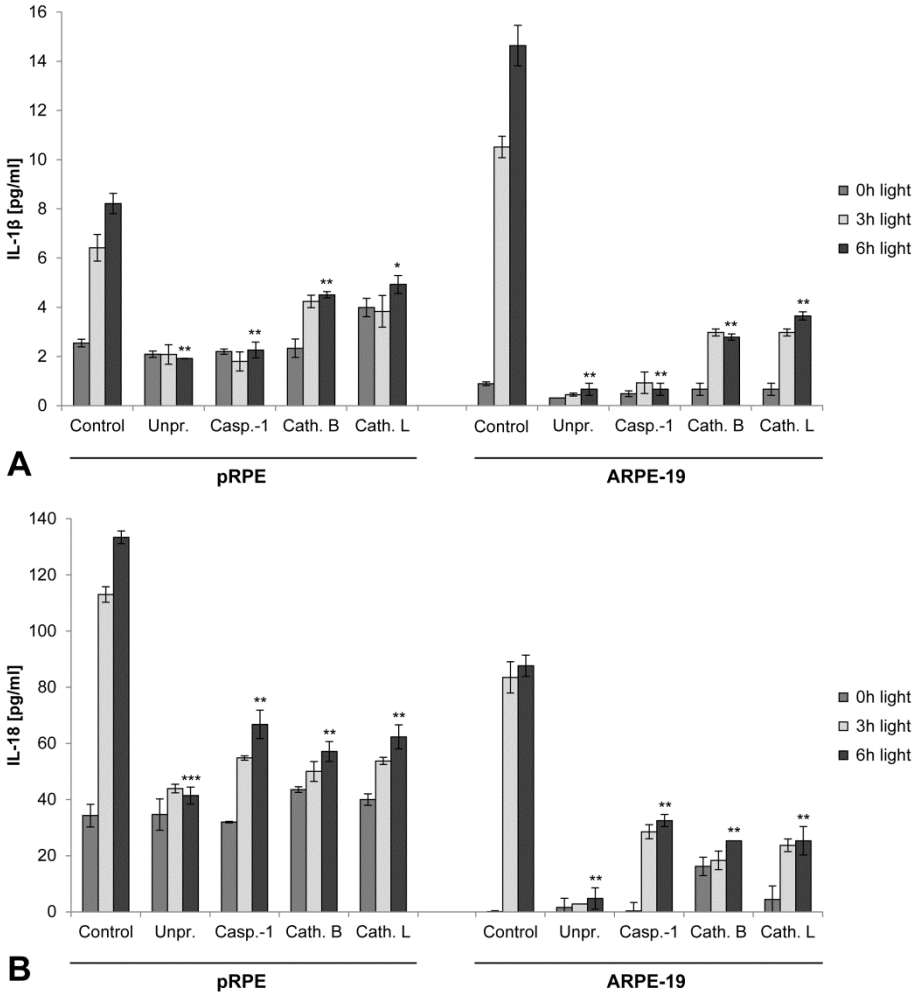

Figure IV.6: Inflammasome activation by lipofuscin phototoxicity is dependent on inflammasome priming and activity of caspase-1, cathepsin B, and cathepsin L. Secretion of IL-1β (A) and IL-18 (B) by pRPE cells and ARPE-19 cells was assessed by ELISA in primed cells incubated with HNEPOS (Control), unprimed cells incubated with HNE-POS (Unpr.), primed cells incubated with HNE-POS and caspase-1 inhibitor Z-YVAD-FMK (Casp.- 1), primed cells incubated with HNE-POS and cathepsin B inhibitor CA-074 (Cath. B), and primed cells incubated with HNE-POS and cathepsin L inhibitor ZFF-FMK (Cath. L).

from human RPE in vivo do not seem to affect light-induced LMP that was the focus of this study. In our experiments, the control group of RPE cells exhibited some degree of inflammasome activation following blue light irradiation even in the absence of POS, albeit considerable less compared to the treatment groups incubated with modified POS (Figs. IV.4 and IV.5). An explanation for this unexpected finding may be lipofuscin generation as a result of incomplete autophagy of endogenous cellular material in control cells [237] an effect that would be pronounced in cells cultured for several weeks in a confluent state as in our experiments. Indeed, fluorescence microscopy demonstrated low amounts of granules with

lipofuscin-like autofluorescence even in control cells (Fig. IV.1a). Consistent with this explanation, both lipofuscin accumulation and inflammasome activation increased further in cells treated with ammonium chloride in the absence of POS compared to control cells (Fig. IV.1b and IV.5), likely as a result of autophagy inhibition by ammonium chloride as previously described.[237] Secondary to light-induced LMP, we detected leakage of lysosomal enzymes into the cytosol of RPE cells. Inhibition of lysosomal proteases cathepsin B and L suppressed inflammasome activation associated with light-induced LMP. The finding of cathepsindependent NLRP3 inflammasome activation secondary to LMP is consistent with previous studies in silica crystalchallenged macrophages.[126] These results suggest a cytosolic substrate of cathepsin proteolytic activity as a critical component of NLRP3 inflammasome activation. However, the molecular mechanism by which cytosolic activity of cathepsins or other lysosomal enzymes induce inflammasome activation has not yet been resolved. Isolated HNE was reported to activate the NLRP3 inflammasome in RPE cells.[236] In our experiments, we used covalent modification of POS by HNE to induce lipofuscinogenesis. As POS were thoroughly washed following modification to remove unbound HNE, cells were not exposed to isolated HNE in our experiments. Furthermore, we did not observe inflammasome activation in cells incubated with HNE-modified POS without additional irradiation treatment (Fig. IV.5). This indicates that inflammasome activation by isolated HNE did not play a role in our study. Similarly, lipofuscin component A2E alone has been demonstrated to induce NLRP3 inflammasome activation in RPE cells.[235] The amount of A2E within the POS-derived lipofuscin in our experiments is unknown but likely to be small compared to RPE lipofuscin in vivo that accumulates over a lifetime. The low A2E content may explain why we did not see an effect of lipofuscin accumulation alone, without additional irradiation treatment, on inflammasome activation in our experiments (Fig. IV.5). These results suggest that direct, nonphototoxic effects of A2E did not contribute to inflammasome activation in our study. Proteins modified by lipid peroxidation products, such as carboxyethylpyrrole (CEP)-modified serum albumin, have been reported to be capable of providing the priming signal for subsequent NLRP3 inflammasome activation.[233] We did not investigate CEP-modified albumin in our study, but we did ot observe a priming effect of MDA- or HNE-modified POS proteins in our experiments. Indeed, we found that omission of IL-1α priming prevented inflammasome activation in RPE cells despite incubation of the cells with HNE-POS (Fig. IV.6). Similar results were obtained for MDA-POS (unpublished data). Thus, HNE- and MDA-modified proteins do not seem to be capable of inducing inflammasome priming in RPE cells.

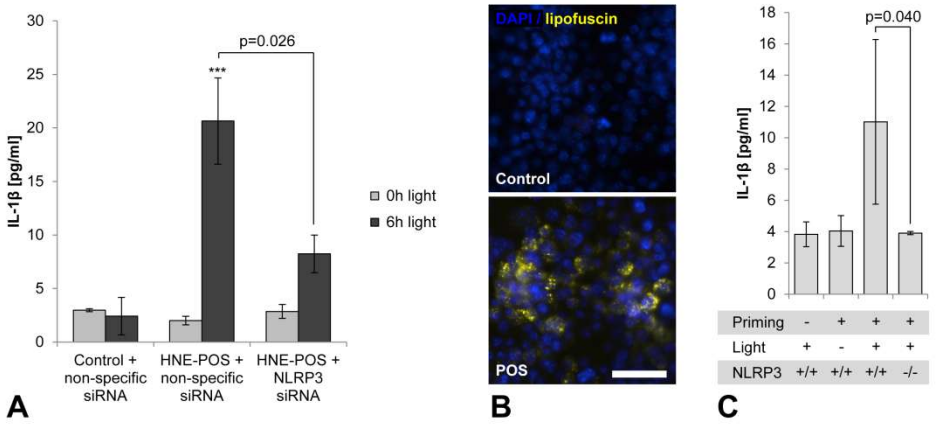

Figure IV.7: Inflammasome activity by lipofuscin phototoxicity is mediated by NLRP3. (A) In ARPE-19 cells incubated with HNE-POS prior to blue light irradiation, the effect of siRNA-mediated NLRP3 knockdown on IL-1β secretion was assessed as compared to control cells transfected with nonspecific siRNA. (B) Accumulation of lipofuscin-like material (yellow) following incubation with POS was documented by fluorescence microscopy in murine macrophages as a substitute model of RPE lipofuscinogenesis. (C) Following POS-induced lipofuscin accumulation, inflammasome priming with LPS, and subsequent blue light irradiation for 4 h, secretion of IL-1β by wild-type (NLRP3+/+) and NLRP3 knockout (NLRP3−/−) macrophages was analyzed by ELISA.Scale bar, 50µm.

In this study, we demonstrate that photooxidative damage to human RPE cells, intensified by accumulated lipofuscin, causes lysosomal membrane permeabilization and subsequent activation of the NLRP3 inflammasome by leaking lysosomal enzymes, resulting in secretion of inflammatory cytokines IL-1β and IL-18. These results identify blue light damage as a new mechanism of inflammasome activation and thus contribute to our understanding of light damage to the RPE. Moreover, this mechanism represents a novel molecular link between hallmark features of AMD pathogenesis such as photooxidative damage, innate immune response, and RPE cell dysfunction that may provide new therapeutic targets against this blinding disease.

V. Effects of Inflammasome Activation on Secretion of Inflammatory Cytokines and Vascular Endothelial Growth Factor by Retinal Pigment Epithelial Cells

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5.1 Abstract

PURPOSE: Activation of the NLRP3 inflammasome has been implicated in the pathogenesis of AMD. Lipofuscin phototoxicity activates the inflammasome in RPE cells by inducing lysosomal membrane permeabilization (LMP). We investigated the effects of LMP-induced inflammasome activation on the secretion of inflammation-related cytokines and **VEGF** by RPE cells.

METHODS: In primary human RPE cells and ARPE-19 cells, the inflammasome was activated by L-leucyl-L-leucine methyl ester (Leu-Leu-OMe)- or lipofuscin phototoxicity-induced LMP. Cytokine secretion was measured by protein dot blot and enzyme-linked immunosorbent assays. The polarization of cytokine secretion was assessed in RPE monolayers on permeable membranes. We analyzed the chemotactic and angiogenic effects of secreted cytokines on murine embryonic stem cell–derived microglia cells and human umbilical vascular endothelial cells, respectively.

RESULTS: Inflammasome activation in RPE cells was associated with caspase-1–dependent secretion of IL-1β, IL-6, IL-18, GM-CSF, and GRO (CXCL1/2/3), whereas constitutive secretion of VEGF was reduced. Secretion of IL-1β and IL-18 was highly polarized to the apical cell side. Incubation with conditioned media of inflammasome-activated RPE cells induced directed migration of microglia cells (11.0-fold increase) and diminished vascular endothelial cells proliferation (39.0% reduction) and migration (69.3% reduction) as compared with conditioned media of untreated control RPE cells.

CONCLUSIONS: Lysosomal membrane permeabilization–induced activation of the NLRP3 inflammasome in RPE cells results in apical secretion of inflammatory cytokines with chemotactic effects on microglia cells and reduced constitutive secretion of VEGF. Via these mechanisms, lipofuscin phototoxicity may contribute to local immune processes in the outer retina as observed in AMD.

5.2 Introduction

In the developed world, AMD is the most common cause for severe visual loss and legal blindness.[230] Age-related macular degeneration is a disease of the central retina that typically affects elderly people. Age-related macular degeneration is characterized by progressive degeneration of the RPE and secondary loss of photoreceptors in the macula leading to loss of central vision. Two late manifestations of AMD can be distinguished, atrophic AMD and neovascular AMD. Atrophic AMD causes slowly progressive central visual decline by RPE

cell degeneration known as geographic atrophy.[12]. In contrast, neovascular AMD is characterized by rapid visual loss secondary to VEGF-mediated ingrowth of choroidal neovascularizations (CNV).[249] Anti-VEGF treatment has proven highly effective in neovascular AMD and is now widely used clinically. In contrast, there is still no treatment available for atrophic AMD. Age-related macular degeneration is associated with oxidative and photooxidative damage of the RPE that is believed to be mediated at least in part by the phototoxic properties of lipofuscin that progressively accumulates in the RPE over a lifetime.[47,250] Further characteristics of the disease include the formation of extracellular deposits called drusen[5,251] and a chronic low-grade immune processes including complement activation in the sub-RPE space.[149,231] Thus, chronic innate immune activation plays a crucial role in AMD pathogenesis. Recent studies have shown that the NLRP3 inflammasome, a key mediator of the innate immune system, is activated in the RPE of patients with atrophic and neovascular AMD.[185,232] Clinical studies demonstrated increased intravitreal and systemic levels of the inflammasome-controlled cytokines IL-1β and IL-18 in AMD patients.[252,253] Based on these findings, a role of the NLRP3 inflammasome in AMD pathogenesis has been hypothesized. The NLRP3 inflammasome is a multiprotein complex, which induces caspase-1 activation resulting in secretion of inflammatory cytokines IL-1β and IL-18.[186,205] An initial priming signal in combination with a subsequent activation signal, such as reactive oxygen species or lysosomal membrane permeabilization (LMP), leads to assembly of NLRP3, ASC, and procaspase-1 to the active inflammasome. We previously have identified photooxidative damage, intensified by accumulated lipofuscin, with secondary LMP and enzyme leakage as a mechanism of NLRP3 inflammasome activation in human RPE cells.[254] Currently, there is only little data available regarding the profile of secreted cytokines in RPE cells following inflammasome activation as well on their paracrine effects. Doyle and coworkers[233] reported that incubation with inflammasomeregulated IL-18 reduces secretion of VEGF in RPE cells and suggested a protective effect against CNV formation. Consistently, they demonstrated increased laser-induced CNV formation in NLRP3 and IL-18 knockout mice.[233] In contrast, another inflammasome-regulated interleukin, IL-1β, has been shown to induce VEGF secretion in RPE cells18 and to promote laser-induced CNV formation in mice.[255] To elucidate the effects of inflammasome activation on pathologic processes in the outer retina, we induced inflammasome activation by LMP in human RPE cells and investigated the resulting secretion profile of inflammatory cytokines and VEGF as well as their secondary effects on microglial and vascular endothelial cells in vitro.

5.3 Material and Methods

5.3.1 Cell Culture

Human primary retinal pigment epithelial (pRPE) cells (H-RPE; Lonza, Cologne, Germany) were cultured as recommended by the manufacturer and used in experiments for a maximum of five cell culture passages. The human nontransformed RPE cell line ARPE-19 (ATCC CRL-2302; ATCC, Rockville, MD, USA) was cultured as previously reported.[237] Murine embryonic stem cell– derived microglia cells were a generous gift by Harald Neumann (Institute of Reconstructive Neurobiology, University of Bonn, Germany) and had been generated and cultured as described.[256] Human umbilical vein endothelial cells (HUVEC; Provitro, Berlin, Germany) were cultured as recommended by the manufacturer and used for experiments at culture passages 5 to 10.

5.3.2 Inflammasome Activation by L-leucyl-L-leucine methyl ester

For inflammasome activation, pRPE cells and ARPE-19 cells were primed with 4 ng/mL IL-1α (R&D Systems, Wiesbaden, Germany) for 48 hours as described by Tseng and coworkers.[185] Subsequently, cells were treated with 1 mM L-leucyl-L-leucine methyl ester (Leu-Leu-OMe; Bachem, Bubendorf, Switzerland) for 1.5 (pRPE) or 3 hours (ARPE-19) to induce LMP. Lysosomal membrane permeabilization was assessed by acridine orange staining as previously described.10,16 Briefly, cells were incubated with 5 IM acridine orange (AO; SigmaAldrich, Munich, Germany) for 30 minutes and washed with PBS immediately before beginning of the Leu-Leu-OMe treatment. For documentation of AO staining by fluorescence microscopy the rhodamine filter set (excitation 550 nm, emission 650 nm) and fluorescein filter set (excitation 502 nm; emission 526 nm) of an IX71 fluorescence microscope (Olympus, Hamburg, Germany) was used to detect intact lysosomes and nuclei, respectively. For inhibition experiments, RPE cells were treated with 20 IM of specific caspase-1 inhibitor Z-YVAD-FMK (BioVision, Munich, Germany) for 60 minutes or with 50 IM of specific cathepsin B inhibitor CA-074 (Calbiochem, Darmstadt, Germany) for 30 minutes prior to Leu-Leu-OMe treatment. Inflammasome Activation by Lipofuscin Phototoxicity The cell culture model used to induce LMP by lipofuscin phototoxicity has been described in detail.[254] Briefly, isolated porcine photoreceptor outer segments (POS) were incubated with 4-hydroxynonenal (HNE) to generate covalently modified POS (HNE-POS) that are stabilized against lysosomal degradation by RPE.[237,241] Cells were incubated with unmodified POS or HNE-POS (concentration equivalent to 4 lg total POS protein per cm2 cell growth area) daily for 7 days to induce lipofuscinogenesis. For inflammasome priming, 4 ng/mL IL-1α (R&D Systems,

Wiesbaden, Germany) was added to the mediafor the last 48 hours of POS incubation. Lipofuscin-loaded and primed cells were then irradiated with blue light-emitting diode (LED) light (wavelength, 455–460 nm; irradiance in our experimental setting, 0.8mW/cm2; XLamp XP-E royal blue; Cree, Durham, NC, USA) for 6 hours to induced LMP and inflammasome activation. Inhibition of caspase-1 or cathepsin B was performed immediately before blue light irradiation as described above.

5.3.3 Cytokine ELISA Analysis

For quantification of cytokine secretion, we employed specific ELISA assays against human IL-1β (BD OptEIA Human IL-1β ELISA Kit II; BD Biosciences, Heidelberg, Germany), human IL-18 (MBL Human IL-18 ELISA Kit; R&D Systems, Minneapolis, USA), and human VEGF (Human VEGF Quantikine ELISA Kit; R&D Systems) according to the manufacturers' instructions. The lower limit of detection of the IL-1β ELISA as determined by the manufacturer is 0.8 pg/mL, and thus well below the concentrations measured in treated cells in our experiments. For collection of media for cytokine analysis, cell culture media was replaced before the experimental treatment (LeuLeu-OMe or blue light irradiation) and collected thereafter. For VEGF measurements, the new media contained 1% fetal bovine serum as recommended by the ELISA manufacturer. In control groups, untreated cells were incubated for a time corresponding to the respective experimental treatment before media were collected.

5.3.4 Cytokine Dot Blot Analysis

Confluent ARPE-19 cells in 6-well plates were primed with IL- 1a and subsequently incubated with Leu-Leu-OMe in 1000 lL serum-free media. Conditioned media were collected immediately after treatment, and concentrations of 42 inflammationand angiogenesis-related cytokines were measured using a protein dot blot assay (RayBio Human Cytokine Antibody Array 3; RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. Appropriate films (Kodak BioMax Light Film; Sigma-Aldrich, Munich, Germany) were exposed to the membranes, and developed films were scanned (Perfection V700 Photo Scanner; Epson, Meerbusch, Germany) before image analysis by ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

5.3.5 Analysis of Polarization of Cytokine Secretion

Polarized RPE cell monolayers on permeable membranes were generated as described before.[257] Briefly, permeable membrane cell culture inserts (polyester membrane; diameter,

12 mm; pore size, 0.4 lm; Transwell-Clear; Corning, Kaiserslautern, Germany) were coated with 1.8 lg/cm2 laminin (SigmaAldrich) for 2 hours at 378C according to the manufacturer's recommendation. Postconfluent, stationary pRPE or ARPE-19 cells were seeded onto the membranes at a confluent density of 1.66 3 105 cells/cm2. Cells were cultured for another 4 weeks before use in experiments. Cell priming and Leu-LeuOMe treatment were performed as described above with addition of the Leu-Leu-OMe to both the apical and the basolateral media. Paracellular permeability of the RPE cell monolayer after Leu-Leu-OMe treatment was analyzed by means of marker dye leakage from the apical to the basolateral compartment using FITC-dextran (molecular weight, 20 kDa; Sigma-Aldrich) as described.[257]

5.3.6 Microglia Chemotaxis Assay

To obtain conditioned media, ARPE-19 cells were priming with IL-1α and then treated with Leu-Leu-OMe for 1 hour. Cells were washed to remove Leu-Leu-OMe and new media without Leu-Leu-OMe were added for another 2 hours. Control cells were primed with IL-1α but not treated with Leu-Leu-OMe and likewise incubated for 2 hours. Subsequently, conditioned media of treated and control cells were collected for use in the following experiments. For assessment of the chemotactic effects of secreted cytokines on microglia cells, 7 x 10^4 microglia cells in serumfree medium were added to the upper compartment of a permeable membrane cell culture insert (polycarbonate membrane; diameter, 24 mm; pore size, 8 lm; Transwell; Corning) while the RPE-conditioned media was applied to the lower compartment of the insert. In positive control experiments, unconditioned medium containing 1 lM chemotactic peptide N-formyl-methionine-leucine-phenylalanine (fMLP; Sigma-Aldrich) was applied to the lower compartment. For coculture experiments, ARPE-19 cells were cultured in the lower compartment, primed with IL-1α, and treated with LeuLeu-OMe for 1 hour, before medium was changed and the upper compartment of the cell culture insert containing the microglia cells was added. All experimental groups were incubated for 6 hours to allow for migration of the microglia cells. Subsequently, cells adherent to the upper side of the permeable membrane were mechanically removed using a cotton swab. Cells that had migrated through the membrane onto its lower surface were stained with crystal violet as described.[238] The dye was then eluted from the cells using 1% SDS. The number of transmigrated microglia cells was assessed by photometric quantification of the eluted dye.

5.3.7 Vascular Endothelial Cell Proliferation and Migration

Retinal pigment epithelium conditioned media were generated as described above for the microglia chemotaxis assay. The effect of conditioned media on vascular endothelial cell

proliferation was analyzed by BrdU cell proliferation assay (Merck, Darmstadt, Germany). Human umbilical vein endothelial cells were seeded onto 96-well plates at a subconfluent density of 1.6 x 10^4 cells/cm^2 and incubated for 6 hours to allow for cellular attachment. Subsequently, media was replaced by RPE cell conditioned media containing the BrdU label and incubated for another 24 hours. BrdU assay analysis was performed as recommended by the manufacturer. Vascular endothelial cell migration was assessed by scratch assay as described elsewhere.[258] In brief, confluent HUVEC monolayers were scraped with a 200-µL pipet tip to create a scratch of defined width. Cells were washed to remove detached cells before incubation with RPE conditioned media for 24 hours. Cell migration was documented by light microscopy (Olympus CKX41 microscope; Olympus) and the number of cells within the scratch area was counted. In each experiment, five wells were analyzed per treatment group.

5.3.8 Paracrine Cytokine Effects on VEGF Secretion

Conditioned media were collected from ARPE-19 cells after 3 hours of incubation with either Leu-Leu-OMe or medium alone. In addition, conditioned media were collected of lipofuscinloaded ARPE-19 cells following irradiation with blue light for 6 hours or following incubation without irradiation for 6 hours. Vascular endothelial growth factor content of conditioned media was measured by ELISA. Untreated ARPE-19 cells were incubated with conditioned media for 24 hours, and VEGF concentration was quantified again. The initial VEGF concentration was subtracted from the final VEGF concentration to allow for specific assessment of VEGF secretion during the 24- hours incubation time.

5.3.9 Statistical Analysis

Experiments were performed in duplicates (see Figs. V.1B, V.2, V.4F) as recommended by the assay manufacturers, triplicates (see Figs. V.3, V.4A–E), or quintuplicates (see Fig. V.4G), and results are presented as mean 6 SD. For statistical analysis, we employed 2-tailed unpaired Student’s t-test, and P less than 0.05 was considered statistically significant.

5.4 Results

5.4.1 Leu-Leu-OMe Induces LMP and Inflammasome

Activation in ARPE-19 and pRPE Cells We have demonstrated that blue light irradiation of lipofuscinloaded RPE cells activates the NLRP3 inflammasome secondary to induction of LMP and cytosolic leakage of lysosomal enzymes.16 In the current study, we employed a

model of chemically induced LMP by Leu-Leu-OMe that has been demonstrated to likewise result in inflammasome activation in ARPE-19 cells.10 To assess lysosomal membrane integrity, cells were labeled with acridine orange, a fluorescent dye that both intercalates into DNA (green fluorescence) and stains intact lysosomes (red fluorescence). In both ARPE-19 cells and pRPE cells, we observed a marked loss of lysosomal staining after incubation with Leu-Leu-OMe compared with untreated controls, indicating effective induction of LMP by Leu-Leu-OMe in both cell types (Fig. V.1A). When RPE cells were primed with IL-1α as described by Tseng and coworkers,[185] LMP induction by Leu-Leu-OMe resulted in inflammasome activation with significantly increased secretion of inflammatory cytokines IL-1β and IL-18 in both ARPE-19 and pRPE cells (Fig. V.1B). Inhibition of the inflammasome component caspase-1 by Z-YVAD-FMK suppressed the release of IL-1β and IL-18. Leakage of lysosomal enzymes, particularly cathepsin B, has been described to be involved in NRLP3 inflammasome activation secondary to LMP.[126,185] Indeed, inhibition of cathepsin B by CA-074 also suppressed IL-1β and IL-18 release. These findings demonstrate that Leu-Leu-OMe-induced LMP induces caspase-1- and cathepsin B–dependent inflammasome activation in both ARPE-19 and pRPE cells.

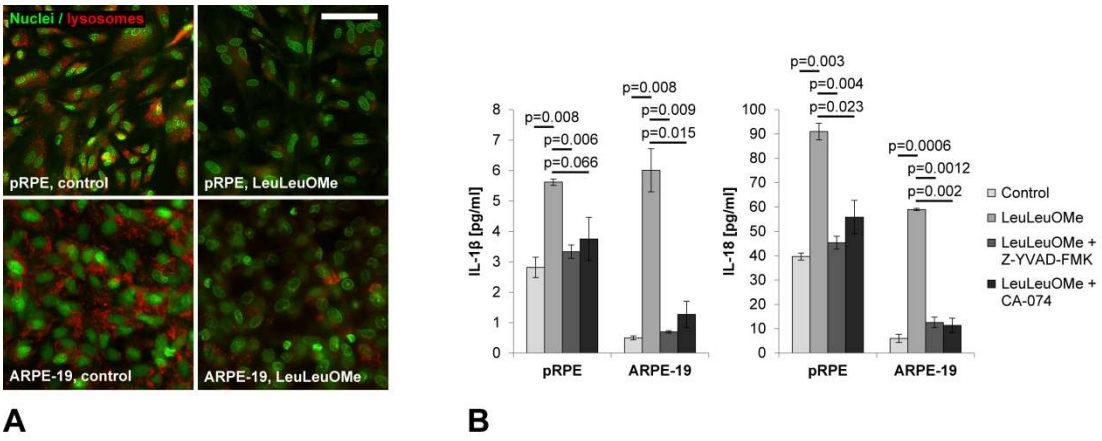

Figure V.1: Leu-Leu-OMe induces LMP and inflammasome activation in RPE cells. (A) Acridine orange staining visualizes LMP in pRPE cells and ARPE-19 cells by loss of red lysosomal staining after incubation with Leu-Leu-OMe. Nuclei are labeled green by acridine orange. Scale bar: 200 µm. (B) Interleukin-1b and IL-18 secretion was analyzed by ELISA in IL-1α–primed RPE cells following Leu-Leu-OMe treatment. Caspase-1 and cathepsin B were inhibited in Leu-Leu-OMe–treated cells by Z-YVAD-FMK and CA-074, respectively.

5.4.2 Profile of Secreted Cytokines

Secondary to Inflammasome Activation in RPE Cells After detecting an increased secretion of the inflammasomeregulated cytokines IL-1β and IL-18, we performed a screening analysis

to identify potential additional cytokines induced by inflammasome activation in RPE cells. For this, protein levels of 42 inflammation- and angiogenesis-related cytokines were analyzed in conditioned media of IL-1α–primed and Leu-Leu OMe–treated ARPE-19 cells by dot blot analysis. Leu-Leu-OMe treatment resulted in significantly increased secretion of GMCSF (2.8-fold increase, P = 0.027), GRO (CXCL1/2/3; 5.2-fold, P = 0.020), and IL-6 (13.6-fold, P = 0.031) compared with an IL- 1a–primed but not Leu-Leu-OMe–treated control group (Fig. V.2). These results were found consistently in two independent experiments. Inflammasome inhibition by incubation with the caspase-1–specific inhibitor Z-YVAD-FMK partially suppressed the Leu-Leu-OMe–induced increase in secretion of GM-CSF, GRO, and IL-6. In summary, Leu-Leu-OMe–induced LMP in RPE cells resulted in a caspase-1–dependent release of IL-1β, IL-18,GM-CSF, GRO, and IL-6.

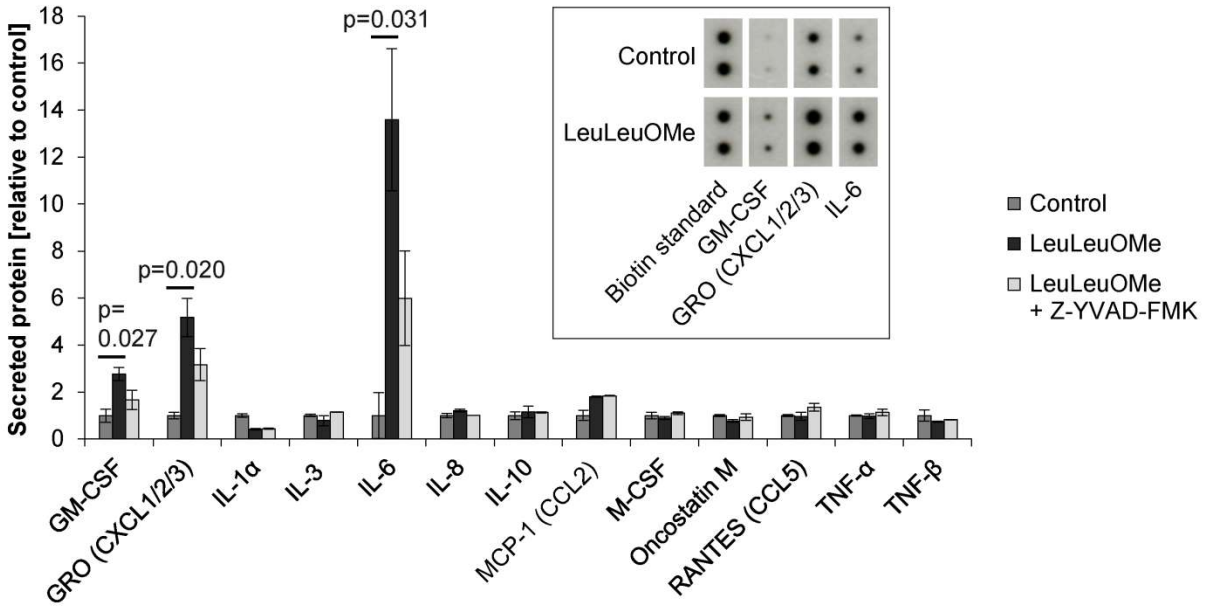

Figure V.2: Profile of secreted cytokines following LMP-induced inflammasome activation in RPE cells. Conditioned media of Leu-Leu-OMe–treated ARPE-19 cells was screened for 42 inflammation- and angiogenesis-related cytokines by dot blot analysis. Results for detected cytokines are shown. Significantly increased secretion was observed for GM-CSF, GRO (CXCL1/2/3), and IL-6. Caspase-1 inhibition by Z-YVAD-FMK partially suppressed this effect. The inset shows consistent results from a second, independent experiment.

5.4.3 Polarization of Cytokine Secretion by RPE Cells and Chemotactic Effects on Microglia Cells

The effects of secreted inflammatory cytokines on adjacent cells and tissues may differ considerably depending on whether the secretion is directed to the apical (neuroretinal) or

basolateral (choroidal) side of the RPE monolayer. To identify the predominant direction of cytokine secretion after inflammasome activation we cultured ARPE-19 and pRPE cells on permeable membranes. We demonstrated previously that the culture conditions employed result in the formation of a polarized cell monolayer with apical microvilli and intercellular tight junctions.[257] To verify that Leu-Leu-OMe treatment does not compromise RPE barrier function, we incubated RPE cells with the marker dye FITC-dextran of a molecular weight of 20 kDa, similar to that of IL-1β (17 kDa). FITC-dextran was added in the apical cell culture compartment, and dye leakage into the basolateral compartment was monitored during treatment with Leu-Leu-OMe. The assay demonstrated that the ARPE-19 cell monolayers almost completely prevented leakage between the two compartments and that effects of Leu-Leu-OMe treatment on this barrier function were minimal (Fig. V.3A). We then treated polarized monolayers of pRPE cells and ARPE-19 cells on permeable membranes with Leu-Leu-OMe added to both the apical and basolateral media to induce cytokine secretion. Unlike in Figure 1, secretion levels in Figure 3 are displayed as total cytokine mass instead of cytokine concentration to account for different volumes of the apical and basolateral compartments of the cell culture insert. The cells exhibited a significant increase in IL-1β secretion that was predominantly directed to the apical side (Fig. V.3B). Apical secretion of IL-1β in pRPE cells and ARPE-19 cells accounted for at least 69% and 75% of total IL-1β secretion, respectively. The lower IL-1β levels in pRPE cells compared with ARPE-19 cells may correspond to the different durations of Leu-Leu-OMe treatment of 1.5 and 3 hours, respectively. Similarly to IL-1β, IL- 18 in ARPE-19 cells was predominantly secreted to the apical side, with at least 92% of total secreted IL-18 detectable in the apical medium (Fig. V.3C). Of note, IL-1β and IL-18 in the basolateral medium were almost exclusively detectable in the group treated with 1.0 mM Leu-Leu-OMe that was also the only group that demonstrated some compromise of RPE barrier function in FITC-dextran leakage assay. In contrast, the 0.5 and 0.75 mM Leu-Leu-OMe– treatment groups exhibited both intact barrier function and near-complete lack of cytokines in the basolateral medium. It thus appears possible that cytokines in the 1.0 mM Leu-LeuOMe–treatment group may have reached the basolateral medium by transcellular leakage rather than basolateral secretion and that IL-1β and IL-18 release after Leu-Leu-OMe treatment occurs in fact almost entirely to the apical side. As apical secretion of inflammatory cytokines in vivo may affect retinal microglia activation and recruitment, we assessed the effects of secreted cytokines on microglia migration in vitro. For this assay, we placed murine embryonic stem cell–derived microglia cells in the upper compartment of a permeable membrane cell culture insert. The number of

microglia cells that transmigrated through the membrane was significantly increased (11.0-fold, P = 0.0003) when conditioned media of Leu-Leu-OMe–treated ARPE-19 cells was applied to the lower compartment as compared with conditioned media of untreated control cells (Fig. V.3D). Cocultures of Leu-Leu-OMe–treated ARPE-19 cells in the lower compartment likewise resulted in a chemotactic effect on microglia cells in the upper compartment. The observed effects were comparable to that of the chemotactic peptide fMLP that was used as a positive control. Together, these results suggest that secretion of inflammatory cytokines by RPE cells secondary to inflammasome activation is directed predominantly to the apical side, corresponding to the neuroretinal side in vivo, and that the secreted cytokines exert a chemotactic effect on microglial cells.

5.4.4 Effects of Inflammasome Activation on VEGF Secretion by RPE Cells and Secondary Effects on Vascular Endothelial Cells

Vascular endothelial growth factor plays a central role in CNV formation in neovascular AMD. Inflammasome activation has been suggested to be involved in this process, although the reported effects of different inflammasome-controlled cytokines on VEGF secretion and angiogenesis are divergent.[233,255,259] Therefore, we investigated the effects of LMP-induced inflammasome activation on VEGF secretion in RPE cells. Interestingly, the constitutive secretion of VEGF was significantly reduced in both pRPE and ARPE-19 cells following inflammasome activation by Leu-Leu-OMe (Figs. V.4A, V.4B). Inhibition of caspase-1 by Z-YVAD-FMK reversed this effect. When LMP was induced in ARPE-19 cells by lipofuscin-mediated photooxidative damage as described,[254] we likewise detected significantly reduced VEGF secretion (Fig. V.4C). These findings demonstrate that inflammasome activation in RPE cells secondary to LMP, both by Leu-Leu-OMe and by lipofuscin phototoxicity, results in a reduction of constitutive VEGF secretion. Inflammasome-regulated cytokine IL-18 has been shown to reduce VEGF secretion in RPE cells.17 We therefore sought to investigate whether the reduction of VEGF secretion observed in our experiments was a direct effect of inflammasome activation or rather a secondary effects mediated by secreted IL-18. To test for this, we incubated untreated RPE cells with conditioned media of Leu-Leu-OMe–treated RPE cells or conditioned media of untreated control cells (Fig. V.4D). Vascular endothelial growth factor content of conditioned media before incubation was subtracted from VEGF content after incubation to allow for a selective quantification of VEGF secretion by the incubated RPE cells. With this experimental design, we did not detect a significant effect of conditioned media on VEGF secretion. When LMP was induced by lipofuscin phototoxicity

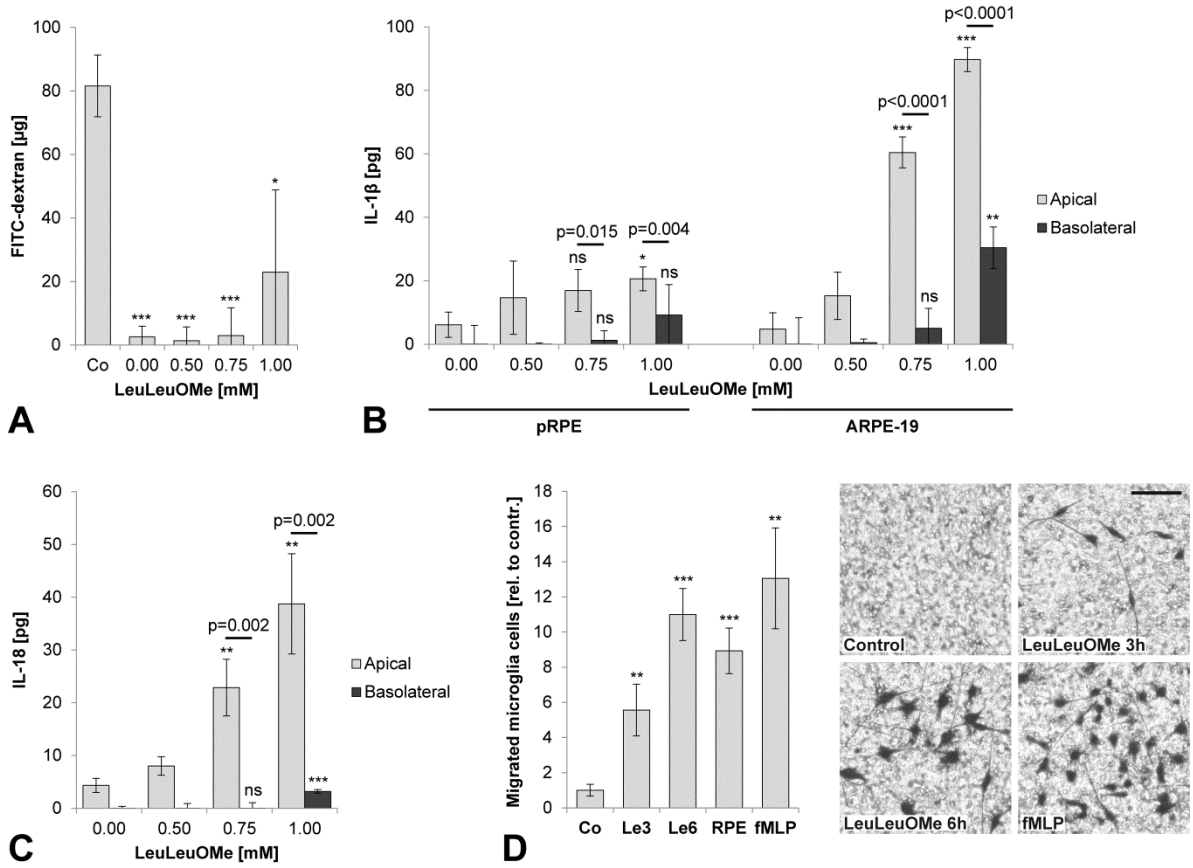

Figure V.3: Polarization of inflammasome-related cytokine secretion in RPE cells and chemotactic effects on microglia cells. (A) The effect of LeuLeu-OMe on barrier function of ARPE-19 monolayers was assessed by permeability assay. For this, a marker dye (FITC-dextran, 20 kDa) with a molecular weight corresponding to that of IL-1β (17 kDa) was added to the apical compartment only, and dye leakage into the basolateral compartment was measured following Leu-Leu-OMe treatment. (B) Polarized monolayers of pRPE cells and ARPE-19 cells on permeable membranes were treated with Leu-Leu-OMe for 1.5 and 3 hours, respectively. Subsequently, separate analysis of IL-1β in the apical and basolateral media revealed that the cytokine was predominantly secreted to the apical cell side. Induction of cytokine secretion by Leu-Leu-OMe was dose-dependent, and IL-1β secretion to the basolateral side was detectable only at higher concentrations of Leu-Leu-OMe. (C) Similarly, secretion of IL-18 in ARPE-19 cells occurred predominantly toward the apical side. (D) To assess chemotactic effects of the released cytokines, murine embryonic stem cell–derived microglia cells were placed in the upper compartment of a permeable membrane cell culture insert. Migration of microglia cells across the membrane into the lower compartment was assessed following application of the following substances or cells to the lower compartment for 6 hours: conditioned media of untreated control ARPE-19 cells (Co), conditioned media of untreated ARPE-19 cells for the first 3 hours and conditioned media of LeuLeuOMe-treated ARPE-19 cells for the last 3 hours (Le3), conditioned media of LeuLeuOMe-treated ARPE-19 cells for the entire 6 hours (Le6), Leu-Leu-OMe–treated ARPE-19 cells (RPE), and unconditioned media containing the chemotactic peptide fMLP as positive control (fMLP). Migration of microglia cells onto the lower side of the permeable membrane was quantified by crystal violet assay and documented by light microscopy. Scale bar: 100 µm. Significance levels as compared with the respective controls are indicated *P < 0.05, **P < 0.01, and ***P < 0.001.

instead of Leu-Leu-OMe, we likewise did not find VEGF secretion to be significantly affected by conditioned media (Fig. V.4E). These results suggest the reduced VEGF secretion observed in our experiments to be a direct effect of inflammasome activation, rather than a secondary effect mediated by released cytokines such as IL-18. Inflammasome-regulated IL-1β has been reported to exert angiogenic effects.[255,260] Although our experiments demonstrated that inflammasome-induced release of IL-1β by RPE cells occurs predominantly toward the apical side, we also detected a significant increase in basolateral secretion (Fig. V.3B) that could contribute to choroidal angiogenesis in AMD. To determine the prevailing effect on angiogenesis of combined increased IL-1β release and reduced VEGF secretion as observed after inflammasome activation in RPE cells, we incubated HUVEC with conditioned media of Leu-Leu-OMe– treated RPE cells. Measurements by BrdU assay revealed that incubation with conditioned media of Leu-Leu-OMe–treated cells reduced vascular endothelial cell proliferation by 61% compared with conditioned media of untreated control cells (P ¼ 0.0013; Fig. V.4F). Likewise, analysis by scratch assay demonstrated a reduction of vascular endothelial cell migration by 31% (P ¼ 0.0009; Fig. V.4G). Thus, conditioned media of RPE cells after inflammasome activation reduced vascular endothelial cell proliferation and migration compared with conditioned media of untreated control RPE cells, consistent with the observed reduction in constitutive VEGF secretion.

5.5 Discussion

Various lines of evidence indicate that the chronic innate immune response in the sub-RPE space that is detectable as both local deposition and increased systemic levels of activated complement components represents a key pathogenetic factor in AMD.[149,231] In a study examining AMD patients with the AMD risk polymorphism of the complement factor H (CFH) gene, increased systemic concentrations have also been reported for the cytokine IL-18, which represents a product of inflammasome activation.[261] Inflammasome activation in the RPE has been detected in patients with both atrophic and neovascular AMD,[185,232] and increased intravitreal and systemic levels of the inflammasome-controlled cytokines IL-1β and IL-18 in AMD patients have been reported.[252,253] However, clinical data regarding inflammasome activation in AMD is still sparse, and more research is needed on the potential contribution of the inflammasome to AMD pathogenesis. Also, the signal for inflammasome activation in AMD is yet unknown, and substances that have been suggested

to provide this signal include drusen components such as C1q[233] and amyloid-β,[234] Alu RNA accumulating secondary to DICER1 deficiency,[232] the lipofuscin component N-retinylidene-N-retinyl-ethanolamine (A2E)[235] and the lipid peroxidation product 4-hydroxynonenal (HNE).[236] A well-established mechanism of inflammasome activation in various cell types is induction of LMP, for example, by viruses, bacteriotoxins, and phagocytosed crystalline substances.[186] Inflammasome activation by LMP has also been reported to occur in RPE cells.[185] In the aging RPE, lipofuscin accumulates within the lysosomal compartment, and we and others have demonstrated that photoreactive properties of lipofuscin induce LMP in RPE cells.[96,97] We also demonstrated that lipofuscin phototoxicity, via induction of LMP and cytosolic leakage of lysosomal enzymes, results in inflammasome activation in RPE cells.[254] These results make it conceivable that with age, progressive accumulation of lipofuscin together with a declining defensive capacity against photooxidative stress may trigger inflammasome activation in the RPE that contributes to the development of AMD. In contrast to the mechanisms of inflammasome activation in the RPE, the consequences of this mechanism have been investigated less intensively so far, in particular with regard to AMD pathogenesis. In this study, we addressed this question by analyzing the profile of cytokines that are secreted by RPE cells following inflammasome activation. For this, we used an established model for LMP-induced inflammasome activation that employs Leu-Leu-OMe for chemical destabilization of lysosomes.[126,185] Tseng and coworkers[185] demonstrated that incubation of ARPE-19 cells with Leu-Leu-OMe as also employed in our study induces characteristic features of inflammasome activation such as caspase-1, maturation and release of IL-1β, and cell death by pyroptosis.[185] Using the same model in pRPE and ARPE-19 cells, we observed a significant and caspase-1–dependent increase in secretion of the inflammation-related cytokines IL-1β, IL-6, IL- 18, GM-CSF, and GRO family cytokines (CXCL1, CXCL2, CXCL3) secondary to LMP-induced inflammasome activation in RPE cells. The cytokines IL-1β and IL-18 are directly regulated by the inflammasome, and thus their increased secretion is most likely a direct result of inflammasome activation. In contrast, IL-6, GM-CSF, and GRO are not considered to be controlled by the inflammasome but are rather upregulated secondary to IL-1β or IL-18 activity. In particular, IL-1β has been demonstrated to induce secretion of IL-6, GM-CSF, and GRO in RPE cells.[262–265] The IL-1β concentrations measured in inflammasomeactivated RPE cells in our experiments are in the range of those determined by previous studies.[233,235,254] Likewise, the concentrations of IL-18 measured in our study correspond to the range of previously reported levels.[232,236,254] Secretion of IL- 1β was

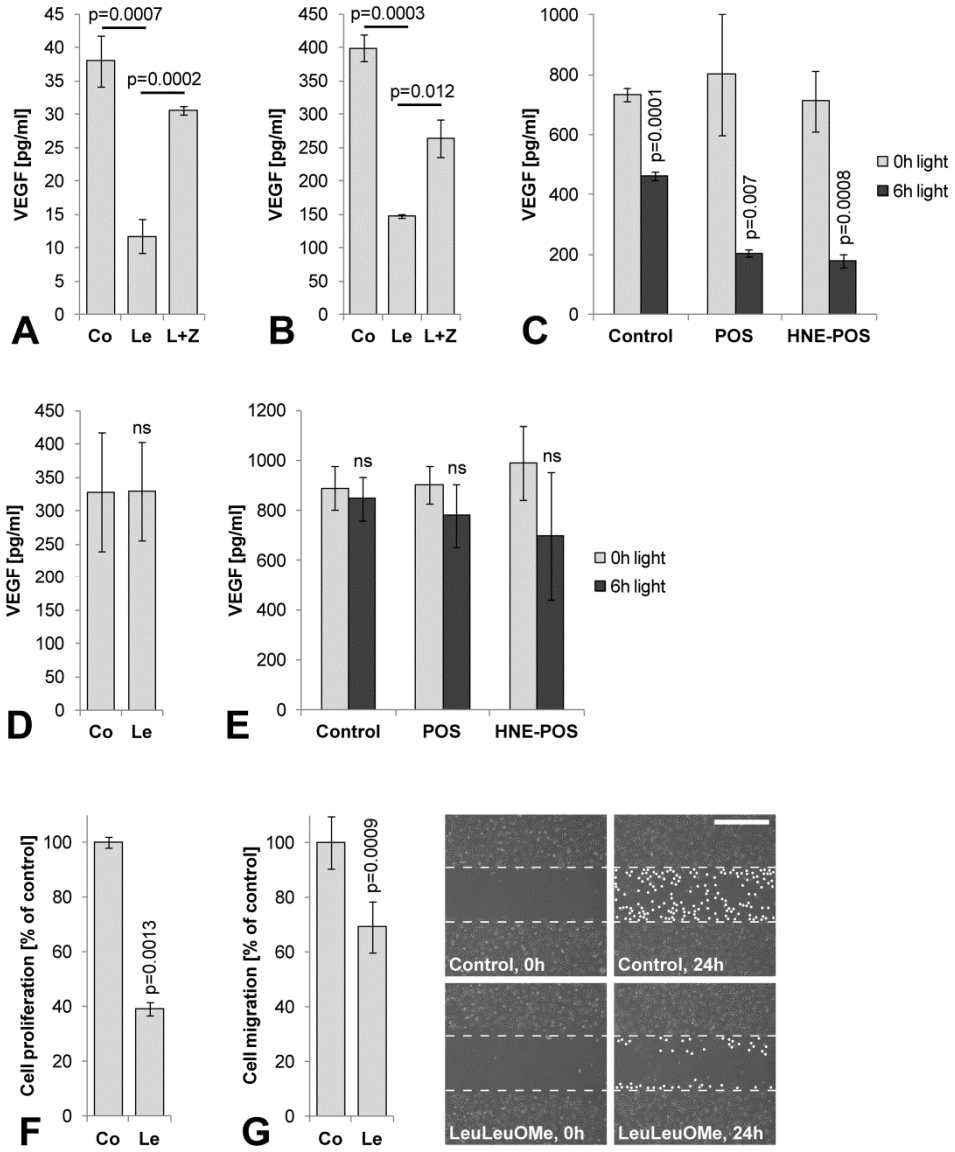

Figure V.4: Effect of inflammasome activation on RPE cell VEGF secretion and vascular endothelial cell proliferation and migration. For induction of inflammasome activation by LMP, (A) pRPE cells and (B) ARPE-19 cells were treated with Leu-Leu-OMe alone (Le) or Leu-Leu-OMe and the caspase-1 inhibitor Z-YVAD-FMK (LþZ). Control cells (Co) were left untreated. In both cell types, inflammasome activation resulted in a significant reduction of constitutive VEGF secretion that was dependent on caspase-1 activity. (C) Lysosomal membrane permeabilization induced by lipofuscin-mediated photooxidative damage similarly reduced VEGF secretion in ARPE-19 cells. For this, low or high lipofuscin accumulation was induced by incubation with native photoreceptor outer segments (POS) and HNE-modified POS (HNE-POS), respectively, and lipofuscin-loaded cells were irradiated with blue light for 6 hours. Incubation of untreated ARPE-19 cells with conditioned media of ARPE-19 cells subjected to either (D) Leu-Leu-OMe treatment or (E) lipofuscin phototoxicity did not result in a significant reduction of VEGF secretion, suggesting a direct effect of inflammasome activation on VEGF secretion rather than an indirect effect mediated by the secreted interleukins. The effect of inflammasome-induced cytokines on human vascular endothelial cell proliferation and migration was assessed by (F) BrdU and (G) scratch assays, respectively. Consistent with the observed reduction in VEGF secretion, incubation with conditioned media of Leu-Leu-OMe–treated ARPE-19 cells resulted in a significant reduction of vascular endothelial cell proliferation and migration as compared with conditioned media of untreated control cells. Scale bar: 250 µm.

significantly higher in polarized RPE cells (Fig. V.3B) compared with unpolarized cells (Fig. V.1B). The observation that polarization increases cytokine expression in RPE cells has been described before. For example, secretion of pigment epithelium-derived factor (PEDF) and VEGF has been reported to be increased 34-fold and 6-fold, respectively, in polarized compared with unpolarized RPE cells.[266] Polarization may thus likewise be the cause for the increased secretion of IL-1β observed in our polarized RPE cell experiments. The identified cytokines may contribute to RPE cell pathology in AMD in various ways. IL-18 has been reported to exert a direct cytotoxic effect on RPE cells in vivo,[232] although this observation has been questioned by others.[267] In vitro, no cytotoxic effects on RPE cells have been observed for IL-18 concentrations of up to 10 lg/mL.39 This concentration is five orders of magnitude higher than the IL-18 concentrations measured in conditioned media in our experiments (Fig. V.1B). Therefore, cytotoxic effects of IL-18 in our experiments are notto be expected. Similar to IL-18, IL-6 was described as capable of inducing degeneration of RPE cells.[268] In addition to their direct effects on RPE cells, the identified cytokines could induce activation and recruitment of microglia cells, monocytes, and macrophages, which may exert secondary effects on the RPE. We found that secretion of IL-1β and IL-18 was predominantly directed toward the apical side of the RPE monolayer, corresponding to the neuroretinal side in vivo. This is in accordance with the results of previous studies that likewise reported a predominantly apical secretion of inflammatory cytokines by RPE cells following induction by various stimuli.[269–271] Retinal cell populations that may be affected by the observed apical cytokine secretion include resident microglia cells and infiltrating macrophages. Indeed, activation of these cell types and their migration into the subretinal space has been observed in AMD patients and animal models and have been discussed as a factor contributing to AMD pathogenesis.[35,272,273] Our experiments demonstrated a significant chemotactic effect of conditioned media from Leu-Leu-OMe– treated RPE cells on microglia cells, supporting a role of inflammasome-related cytokines in microglia recruitment. Via the apically polarized secretion of inflammatory cytokines and the chemotactic effects on microglia cells, LMP-mediated inflammasome activation in the RPE as observed secondary to lipofuscin phototoxicity may contribute to microglia/macrophages activation and recruitment in vivo in retinal pathologies such as AMD. For the evaluation of the inflammasome as a potential new therapeutic target in neovascular AMD, the knowledge of downstream effects of inflammasome activation is crucial. However, previous reports on the effects of inflammasome activation on CNV formation are ambiguous. The two classical inflammasome-induced cytokines, IL-1β and IL-18, have been reported to induce and inhibit

VEGF secretion by RPE cells, respectively.[233,259] Similarly, IL-1β and IL-18 have been demonstrated to promote and suppress CNV formation in mice, respectively,[233,255] whereas others did not find CNV formation to be affected by IL-18.[274] We sought to elucidate the angiogenesisrelated effects of inflammasome activation in the RPE. Interestingly, we found that LMP-induced inflammasome activation in RPE cells does not result in an increase but rather in a significant reduction of the cells' constitutive secretion of VEGF. Consistently, conditioned media of RPE cells following inflammasome activation reduced proliferation and migration of vascular endothelial cells compared with conditioned media of untreated control cells. In addition to the reduction in VEGF secretion, the released IL-18 may add to this effect due to its suggested antiangiogenic properties.[233] While our experiments demonstrate reduced VEGF secretion by RPE cells following inflammasome activation, inflammatory cytokines released by RPE cells after inflammasome activation in vivo may exert indirect angiogenic effects via other cell types such as recruited microglia cells.[35] Both a priming signal and an activation signal are required for inflammasome activation. In IL-1α–primed RPE cells, we found that LMP induced by lipofuscin phototoxicity results in activation of the NLRP3 inflammasome[254] and in the current study this mechanism was associated with a significant reduction in constitutive VEGF secretion. In contrast, the isolated lipofuscin component A2E has been shown to induce VEGF secretion in unprimed ARPE-19 cells.[275] After priming of ARPE-19 cells with IL-1α, A2E treatment resulted in both inflammasome activation and induction of VEGF secretion.[235] Similar to lipofuscin, phototoxicity of A2E has been shown to induce LMP[97] and blue light irradiation of unprimed A2E-loaded ARPE-19 cells induced an increase in VEGF secretion.[276] Whether A2E phototoxicity in primed cells results in a reduction of VEGF secretion similar to our results for lipofuscin phototoxicity still needs to be investigated. Following up on our previous investigation describing the mechanism of LMP-induced inflammasome activation induced by lipofuscin-mediated photooxidative damage in RPE cells, our current study identifies the cytokine profile secreted by RPE cells following LMP-induced inflammasome activation. While secretion of VEGF is suppressed after inflammasome activation, several inflammatory cytokines are significantly induced and secreted predominantly to the apical RPE side. Via this mechanism, photooxidative damage to the RPE may trigger local immune processes such as activation and recruitment of retinal microglia/macrophages, and thus contribute to the chronic innate immune response in AMD.

VI. Complement component C5a primes retinal pigment epithelial cells for inflammasome activation by lipofuscin-mediated photooxidative damage

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6.1 Abstract

Complement activation, oxidative damage, and activation of the NLRP3 inflammasome have been implicated in retinal pigment epithelium (RPE) pathology in age-related macular degeneration (AMD). Following priming of RPE cells, the NLRP3 inflammasome can be activated by various stimuli such as lipofuscin-mediated photooxidative damage to lysosomal membranes. We investigated whether products of complement activation are capable of providing the priming signal for the inflammasome in RPE cells. Incubation of primary human RPE cells and ARPE-19 cells with complement-competent human serum resulted in upregulation of C5a receptor, but not C3a receptor. Furthermore, it induced expression of pro-IL-1β and enabled IL-1β secretion in response to lipofuscin phototoxicity, thus indicating inflammasome priming by human serum. Complement heat-inactivation, C5 depletion, and C5a receptor inhibition suppressed the priming effect of human serum whereas recombinant C5a likewise induced priming. Conditioned media of inflammasomeactivated RPE cells provided an additional priming effect that was mediated by the IL-1 receptor. These results indicate that complement activation product C5a represents a priming signal for RPE cells that allows for subsequent inflammasome activation by stimuli such as lipofuscin-mediated photooxidative damage. This molecular pathway provides a functional link between key factors of AMD pathogenesis including lipofuscin accumulation, photooxidative damage, complement activation, and RPE degeneration and may provide novel therapeutic targets in this disease.

6.2 Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in all industrialized countries.[230] For the majority of patients, in particular those affected by the intermediate stage and the atrophic late stage of the disease, there is currently no effective treatment available. Elucidating the still unresolved pathogenesis of this multifactorial, complex disease will help to identify potential targets for therapeutic intervention. The retinal pigment epithelium (RPE), a monolayer of post-mitotic support cells essential for photoreceptor function, is primarily affected by AMD. Oxidative/photooxidative damage to the RPE contributes to AMD, and antioxidative treatment has been demonstrated to slow disease progression in clinical trials.[3] This damage is believed to be mediated at least in part by the photoreactive properties of lipofuscin and lipofuscin component A2E that accumulate in the macular RPE in large amounts over a lifetime.[277,278] In addition, several lines of

evidence indicate that processes of the innate immune system play a critical role in the pathogenesis of AMD. Activated components of the complement system such as C3a and C5a are detectable both locally in the sub-RPE space and systemically in plasma of AMD patients. [149,231] Genetic polymorphisms in several complement components and regulators such as CFH, C2, C3, and CFB are strongly associated with AMD.[279] Another part of the innate immune system, the NLRP3 inflammasome, has recently been proposed to also contribute to AMD pathogenesis. Activation of the NLRP3 inflammasome in RPE cells was demonstrated in both atrophic and neovascular AMD,[185,232] and increased intravitreal and systemic levels of the inflammasome activation products IL-1β and IL-18 have been reported in AMD patients.[252,253] The inflammasome protein complex serves as an intracellular sensor for various signals of cell damage. [186] Its activation results in the secretion of highly proinflammatory cytokines such as IL-1β and IL-18 and eventually in cell death by pyroptosis. Activation of the NLRP3 inflammasome is a two-step process that requires an initial priming signal and a subsequent activation signal. [186] The priming signal results in NF-κB–dependent transcriptional induction of NLRP3 and pro-IL- 1β. The activation signal subsequently triggers assembly of NLRP3 and other protein components into the active inflammasome protein complex that results in caspase-1- mediated cleavage of pro-IL-1β and pro-IL-18 into mature IL-1β and IL-18. Several substances have been suggested to provide the inflammasome activation signal in AMD including drusen components such as C1q[233] and amyloid-beta,[234] Alu RNA accumulation secondary to DICER1 deficiency,[232] the lipofuscin component N-retinylidene-N-retinylethanolamine (A2E),[235] and the lipid peroxidation product 4-hydroxynonenal (HNE).[236] We have recently suggested an additional mechanism by demonstrating that photooxidative damage to the RPE, enhanced by accumulated lipofuscin, can activate the NLRP3 inflammasome by inducing lysosomal membrane permeabilisation and cytosolic leakage of lysosomal enzymes.[254,280] In contrast to inflammasome activation, the mechanism of inflammasome priming in AMD has been little investigated so far. Interestingly, a recent study in patients with early or intermediate AMD demonstrated the CFH risk genotype to be associated with significantly increased plasma levels of the inflammasomeregulated cytokine IL-18, suggesting a role for activated complement components like C3a and C5a in inflammasome activation in AMD.[261] Inflammasome priming by complement activation products has also been proposed in the context of other diseases such as atherosclerosis and gout.[281,282] In this study, we investigated the capacity of activated complement components to prime human RPE cells for inflammasome activation by lipofuscin-mediated photooxidative damage.

6.3 Experimental Procedures

6.3.1 Cell culture and treatments.

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 (FBS) and were used in experiments for a maximum of 6 cell culture passages. The human non-transformed RPE cell line ARPE-19 (CRL-2302; ATCC, Rockville, MD, USA) was cultured as previously reported using medium containing 10% heat-inactivated FBS.[237] For inflammasome priming, culture medium was exchanged by FBS-free medium supplemented with the indicated priming agents as described below. For analysis of C5a receptor (C5aR) expression, cells were treated with 50 ng/ml C5a (R&D Systems, Wiesbaden, Germany).[283,284] Cathepsin B inhibitor CA-074 (Merck/Calbiochem, Darmstadt, Germany) and cathepsin L inhibitor Z-FF-FMK (Merck/Calbiochem) were used at a concentration of 10 µM each for 1 h prior to and during irradiation treatment. For inhibition of caspase-1, we applied 10 µM of Z-YVAD-FMK (BioVision, Munich, Germany) 30 min prior to and during irradiation. Binding to C5aR was blocked using 0.5 µM of an inhibitory mouse monoclonal IgG antibody directed against human C5aR (clone S5/1; Biolegend, Fell, Germany). The drug anakinra (Kineret; Swedish Orphan Biovitrum, Langen, Germany) was used at a concentration of 100 ng/ml to inhibit the IL- 1 receptor (IL1R).

6.3.2 Immunocytochemistry and western blot analysis

For immunocytochemical detection of ZO-1 and C5aR, cells were stained with a rabbit polyclonal anti human ZO-1 antibody (Life Technologies, Darmstadt, Germany) and a mouse monoclonal anti human C5aR antibody (clone S5/1; Biolegend, Fell, Germany), respectively. Cells were fixed with 4% paraformaldehyde for immunocytochemistry. No cell permeabilisation agent was applied prior to immunodetection of C5aR to limit this staining to cell membrane proteins. For western blot analysis of C5aR and pro-IL- 1β, we employed a mouse monoclonal anti human C5aR antibody (clone S5/1; Biolegend, Fell, Germany) and a goat polyclonal anti human IL-1β antibody (R&D Systems, Wiesbaden, Germany), respectively. Cells were lysed using RIPA buffer, and total protein content of cell lysates was quantified by Bradford assay (Sigma-Aldrich, Munich, Germany). Equal amounts of 50 µg total protein per sample were separated by electrophoresis in 4-12% SDS–polyacrylamide gels (Lonza, Cologne, Germany) prior to transfer onto nitrocellulose membranes (Thermo Scientific) and subsequent immunodetection.

6.3.3 RT-PCR and quantitative real-time PCR

For conventional RT-PCR, isolation of total RNA from RPE cells and reverse transcription into cDNA was carried out with the Power SYBR Green Cells-to-Ct Kit (Life Technologies, Darmstadt, Germany) as recommended by the manufacturer. PCR was performed with 40 cycles using the KAPA2G Fast PCR Kit (PEQLAB Biotechnologie, Erlangen, Germany). The primers used for detection of C5aR, C3a receptor (C3aR), and C5a-like receptor 2 (C5L2) have been described.[285] For human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we employed the sense primer 5´-CTCTGCTCCTCCTGTTCGAC-3´ and the antisense primer 5´-GCGCCCAATACGACCAAATC-3´. PCR products were run on 2% agarose gel with a 100 bp DNA ladder marker (Sigma-Aldrich, Munich, Germany). The negative control contained all PCR components but no cDNA template. Quantitative real-time PCR (qPCR) was performed using again the Power SYBR Green Cells-to-Ct Kit (Life Technologies, Darmstadt, Germany) according to the manufacturer's protocol on a real-time PCR system (LightCycler 480 II; Roche, Basel, Switzerland) with the primers described above. The amount of target mRNA in test samples was normalized to GAPDH, and the comparative Ct method was used to evaluate gene expression.

6.3.4 Inflammasome priming

For inflammasome priming, cells were treated with the indicated priming agents during the last 48 h of incubation with photoreceptor outer segments (POS). For priming with interleukins, cells were treated with 4 ng/ml recombinant human IL-1α (R&D Systems, Wiesbaden, Germany)[185] or 50 pg/ml recombinant human IL-1β (R&D Systems). For priming with normal human serum (NHS), full blood was drawn from a healthy donor into anticoagulant-free tubes. Blood samples were sedimented at room temperature for 30 minutes. Serum was separated by centrifugation (2000g, 5 minutes) and immediately stored at -80°C. Heat inactivation of complement components was performed by incubating NHS in a water bath of 56°C for 30 min. For inflammasome priming, NHS, heat-inactivated NHS, or C5-depleted human serum (Sigma-Aldrich, Munich, Germany) was added to FBS-free cell culture media at a concentration of 25% each. In an additional treatment group, cell culture media containing C5-deficient serum were resupplemented with 50 ng/ml C5a (R&D Systems, Wiesbaden, Germany).

6.3.5 Inflammasome activation

Following inflammasome priming, inflammasome activation by lipofuscin-mediated photooxidative damage was induced in RPE cells as previously described.[254] Briefly, isolated

porcine POS were covalently modified with the lipid peroxidation product HNE (5 mM) to stabilize them against lysosomal degradation.[241] 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.[237] During the last 48 h of POS treatment, cells were co-incubated with the respective priming agent as indicated. Subsequently, medium was changed, and cells were irradiated with blue light (peak wave length, 448 nm; irradiance, 0.8 mW/cm²) for the indicated times of up to 6 h to induce photooxidative lysosomal membrane permeabilisation and subsequent NLRP3 inflammasome activation.[254] Irrespective of the duration of irradiation, media were collected 6 h after the medium change in all treatment and control groups. Secretion of IL-1β secondary to inflammasome activation was measured by specific ELISA (BD OptEIA Human IL-1β ELISA Kit II; BD Biosciences, Heidelberg, Germany). To analyze loss of plasma membrane integrity secondary to inflammasome-mediated cell death, we assessed lactate dehydrogenase (LDH) release (Cytotoxicity Detection Kit; Roche, Mannheim, Germany).

6.3.6 Priming by conditioned media.

Conditioned media were obtained from ARPE-19 cells following inflammasome activation by lipofuscin phototoxicity. This was induced by HNE-POS incubation, IL-1α priming, and blue ight irradiation as described above. Before the start of irradiation, cells were washed and medium was changed. After 6 h of irradiation, conditioned media were collected for use in priming experiments. In control experiments, inflammasome activation was achieved by treatment with L-leucyl-L-leucine methyl ester (Leu-Leu-OMe).[185] For this, ARPE-19 cells were primed by IL-1α, incubated with 1mM Leu-Leu-OMe (Bachem, Bubendorf, Switzerland) for 1 h, washed, and incubated with new medium without Leu-Leu-OMe for another 2 h before conditioned media were collected. For inflammasome priming by conditioned media, treatment-naive cells were incubated with conditioned media of HNE-POS/blue lighttreated or Leu-Leu-OMe-treated cells for 48 h.

6.3.7 Statistical Analysis

Experiments were performed in duplicates (Fig. VI.3A, VI.3C, VI.4A, VI.4C, VI.6A, VI.6B) following the assay manufacturer's recommendation or in triplicates (Fig. VI.2, VI.3B, VI.3D, VI.4B, VI.4D). Results are presented as mean ± standard deviation. Statistical analysis was performed using paired (Fig. VI.2) or unpaired (Fig. VI.3, VI.4, VI.6) two-tailed Student's t tests (Microsoft Excel 2013; Microsoft, Redmond, WA, USA). Differences were considered statistically significant at $p<0.05$. In experiments with multiple group comparisons (Fig. VI.3,

VI.4), significant differences were confirmed by additional analysis using one-way ANOVA with post-hoc analysis by Tukey's range test (GraphPad InStat 3.06, GraphPad Software, La Jolla, CA, USA).

6.4 Results

6.4.1 Anaphylatoxin receptors C3aR, C5aR, and C5L2 are constitutively expressed by human RPE cells.

C3a and C5a represent the two dominant anaphylatoxins during complement activation. C3a binds to C3aR. C5a is a ligand for both C5aR and C5L2 with most biological effects being mediated by C5aR. Expression of C5aR and C3aR has been demonstrated in ARPE-19 cells.[241,283,285] Expression of C5aR in RPE cells has also been detected by immunohistochemistry of human donor eyes where it was found to be localized predominantly on the basolateral cell side.[286] We extended these previous studies by investigating the expression of C3aR, C5aR, and C5L2 on ARPE-19 and pRPE cells. Under the culture conditioned employed in our experiments, both cell types exhibited characteristics of differentiated RPE cells including epithelial monolayer formation, hexagonal cell morphology, and intracellular ZO-1-positive tight junctions (Fig. VI.1A). In both RPE cell types, we detected constitutive expression of all three anaphylatoxin receptors (Fig. VI.1B).

6.4.2 C5aR, but not C3aR, is upregulated following incubation with activated complement.

In normal human serum (NHS) in vitro, complement activation occurs rapidly when incubated at 37°C, and thus complementcompetent NHS in vitro is a rich source for complement activation products even without addition of complement activators such as zymosan.[287] In contrast, heating of NHS to 56°C for 30 min inactivates complement components and prevents complement activation but preserves the activity of other less heat-labile serum proteins. To assess the effects of activated complement components on anaphylatoxin receptors in human RPE cells, we measured expression of C5aR and C3aR in ARPE-19 cells and pRPE cells after incubation with complement-competent NHS and heatinactivated NHS (HI-NHS) by qPCR analysis. Studies investigating the time course of C5aR expression in ARPE-19 cells following stimulation with inflammatory cytokines reported a upregulation with a maximum after 6 hours for mRNA expression and after 24 hours for cell surface protein expression.[283] We likewise found that incubation of ARPE-19 cells with complement-

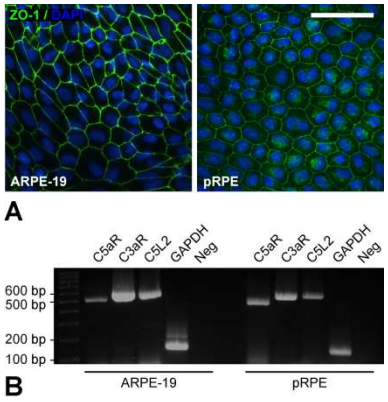

Figure VI.1: Anaphylatoxin receptors C5aR, C3aR, and C5L2 are constitutively expressed by human RPE cells. (A) human RPE cell line ARPE-19 cells and primary human RPE cells (pRPE) were used for expression analysis. Both RPE cell types exhibited RPE-characteristic morphology in cell culture, including epithelial cell monolayer formation with polygonal cell shape and intercellular tight junction formation as evident from immunostaining of ZO-1 (green). Nuclei were visualized by DAPI staining (blue). Scale bar, 50 µm. (B), expression of C5aR (PCR product, 500 bp), C3aR (585 bp), and C5L2 (585 bp) in RPE cells was analyzed by RT-PCR. Expression of GAPDH (121 bp) was used as a positive control. PCR reaction mix without cDNA template served as negative control (Neg).

competent NHS induced a significant upregulation of C5aR expression (p=0.007) with a peak 6-fold induction after 6 hours (Fig. VI.2A). Heat inactivation of complement components completely prevented the effect of NHS on C5aR expression (Fig. VI.2B). In contrast to C5aR, expression of C3aR was not significantly affected by incubation with complement-competent NHS (Fig. VI.2C). Similar to NHS, recombinant C5a induced a maximum 6-fold upregulation of C5aR expression in ARPE-19 cells (p=0.011) after 6 hours (Fig. VI.2D). This result was confirmed in pRPE cells which likewise exhibited a significant upregulation of C5aR epression (p=0.0097) following incubation with C5a (Fig. VI.2E). Western blot analysis performed in ARPE-19 cells at different time points up to 24 hours after beginning of an incubation with C5a for 6 hours confirmed that the observed mRNA induction resulted in increased C5aR protein expression (Fig. VI.2F). Similarly, immunocytochemistry performed without cell permeabilisation demonstrated increased C5aR cell surface staining in ARPE-19 cells 24 hours following start of a C5a incubation for 6 hours (Fig. VI.2G). Our findings indicate that human RPE cells respond to incubation with activated complement components by upregulation of C5aR mRNA expression whereas expression of C3aR is not affected. These results suggest C5aR as a mediator of complement effects on RPE cells and triggered us to further investigate C5a as a potential priming signal for the NLRP3 inflammasome in RPE cells.

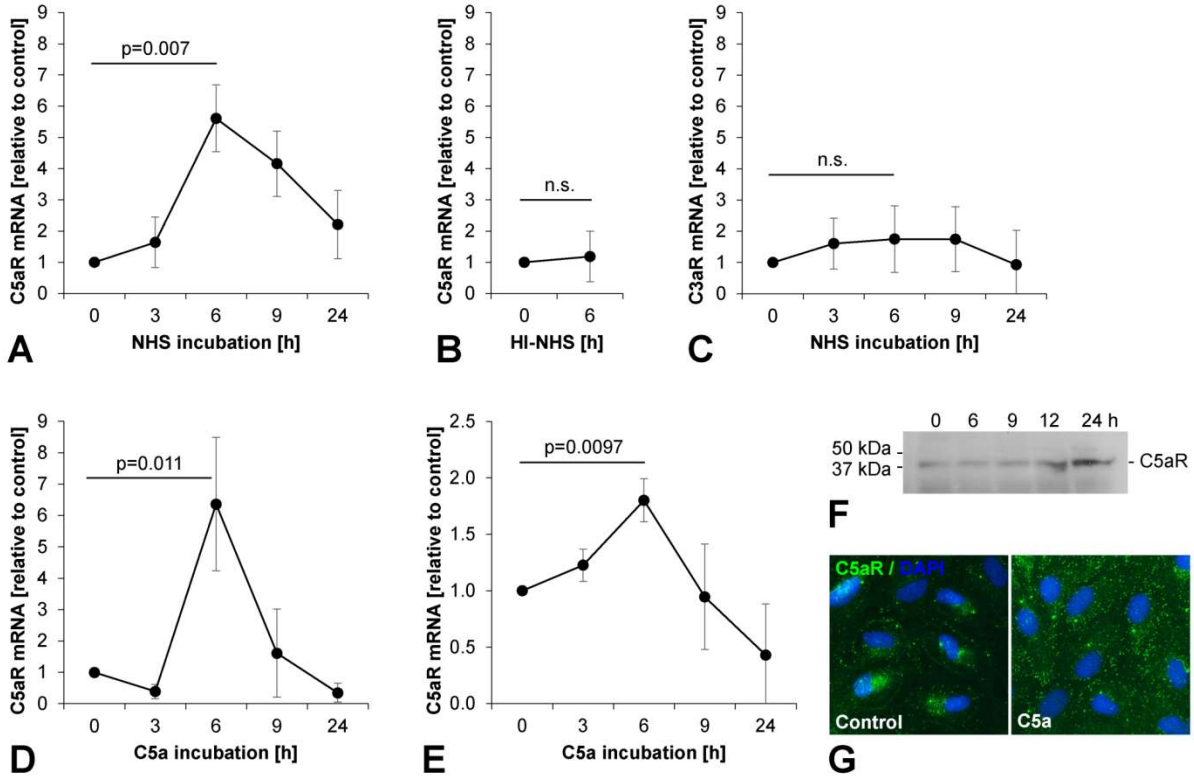

Figure VI.2: C5aR, but not C3aR, is up-regulated following incubation with activated complement. Using qPCR, we analyzed expression kinetics of (A) C5aR in ARPE-19 cells incubated with complement-competent NHS, (B) C5aR in ARPE-19 cells incubated with heat-inactivated NHS, (C) C3aR in ARPE-19 cells incubated with complement-competent NHS, (D) C5aR in ARPE-19 cells incubated with recombinant human C5a, and (E) C5aR in pRPE cells incubated with recombinant human C5a. Target mRNAs were normalized to GAPDH. Experiments were performed in triplicates, and results are presented as mean standard deviation. F, increased expression of C5aR protein (37 kDa) following priming with C5a was detected by (F) Western blot analysis and (G) immunocytochemistry in ARPE-19 cells.

6.4.3 NHS primes RPE cells for inflammasome activation by lipofuscin-mediated photooxidative damage.

We previously demonstrated that the in vitro model of lipofuscin-mediated photooxidative damage in RPE cells employed in this study results in activation of the NLRP3 inflammasome with activation of caspase-1 and subsequent release of IL-1β and IL-18.[254,280] For our experiments, ARPE-19 cells and pRPE cells were incubated with unmodified POS or POS modified with the lipid peroxidation product HNE (HNE-POS) to induce intracellular accumulation of low and high levels of lipofuscin-like material, respectively. Subsequently, lipofuscin-loaded RPE cells were irradiated with blue light for up to 6 h. Inflammasome activation was assessed by means of inflammasome-regulated IL-1β secretion and inflammasome-induced pyroptotic cell death in both ARPE-19 cells (Fig. VI.3A, B) and pRPE

cells (Fig. VI.3C, D). Without prior inflammasome priming, no inflammasome activation was detectable in RPE cells following blue light irradiation. In contrast, in positive control cells treated with the priming agent IL-1α,[185] blue light irradiation resulted in significant inflammasome activation with IL-1β secretion and pyroptosis. Inflammasome activation increased with light dose. Incubation with NHS exerted a strong priming effect similar to IL-1α. Inflammasome activation of NHSprimed cells was dependent on activity of the inflammasome component caspase-1 and the lysosomal enzyme cathepsin B, consistent with the previously reported mechanism of inflammasome activation by lysosomal enzyme leakage.[254] There was good agreement between results in ARPE-19 cells and pRPE cells. The data demonstrate that complementcompetent NHS contains a factor capable of providing the priming signal for subsequent inflammasome activation in RPE cells.

6.4.4 Complement component C5a is the active priming agent in NHS.

To identify the active priming agent in NHS, different complement components were inhibited during priming, and subsequent inflammasome activation by lipofuscin/blue light treatment was assessed again by means of IL-1β secretion and cell death in ARPE-19 cells (Fig. VI.4A, B) and pRPE cells (Fig. VI.4C, D). First, we inactivated all complement components in NHS by heating. We found that the priming effect of NHS was completely suppressed after heat inactivation, suggesting that heat-labile serum components such as complement components mediate inflammasome priming by NHS. To further delineate the responsible complement component in NHS, cells were treated with a C5aR inhibitor during priming with NHS with resulted in a significant reduction of the priming effect. Likewise, depletion of C5 prevented the priming effect of NHS. Supplementation with recombinant C5a resulted in complete restoration of the priming capacity of C5-depleted NHS. To confirm that the observed IL-1β secretion is indeed a result of inflammasome priming we analyzed the induction of pro-IL-1β protein expression that represents a key element of inflammasome priming. In agreement with our results regarding IL-1β secretion, pro-IL-1β protein expression in ARPE-19 cells was strongly induced by incubation with IL-1α, NHS, or C5a as compared with control cells incubated with heat-inactivated NHS or C5-depleted NHS (Fig. VI.5). As previous studies demonstrated that ARPE-19 cells, unlike other cell types, constitutively express NLRP3 even in unprimed conditions and do not induce NLRP3 expression after priming[185] we did not include NLRP3 in this analysis. In summary, our results identify the complement component C5a as the active priming agent in NHS and demonstrate that C5a is capable of priming human RPE cells for inflammasome activation by lipofuscin-mediated photooxidative damage.

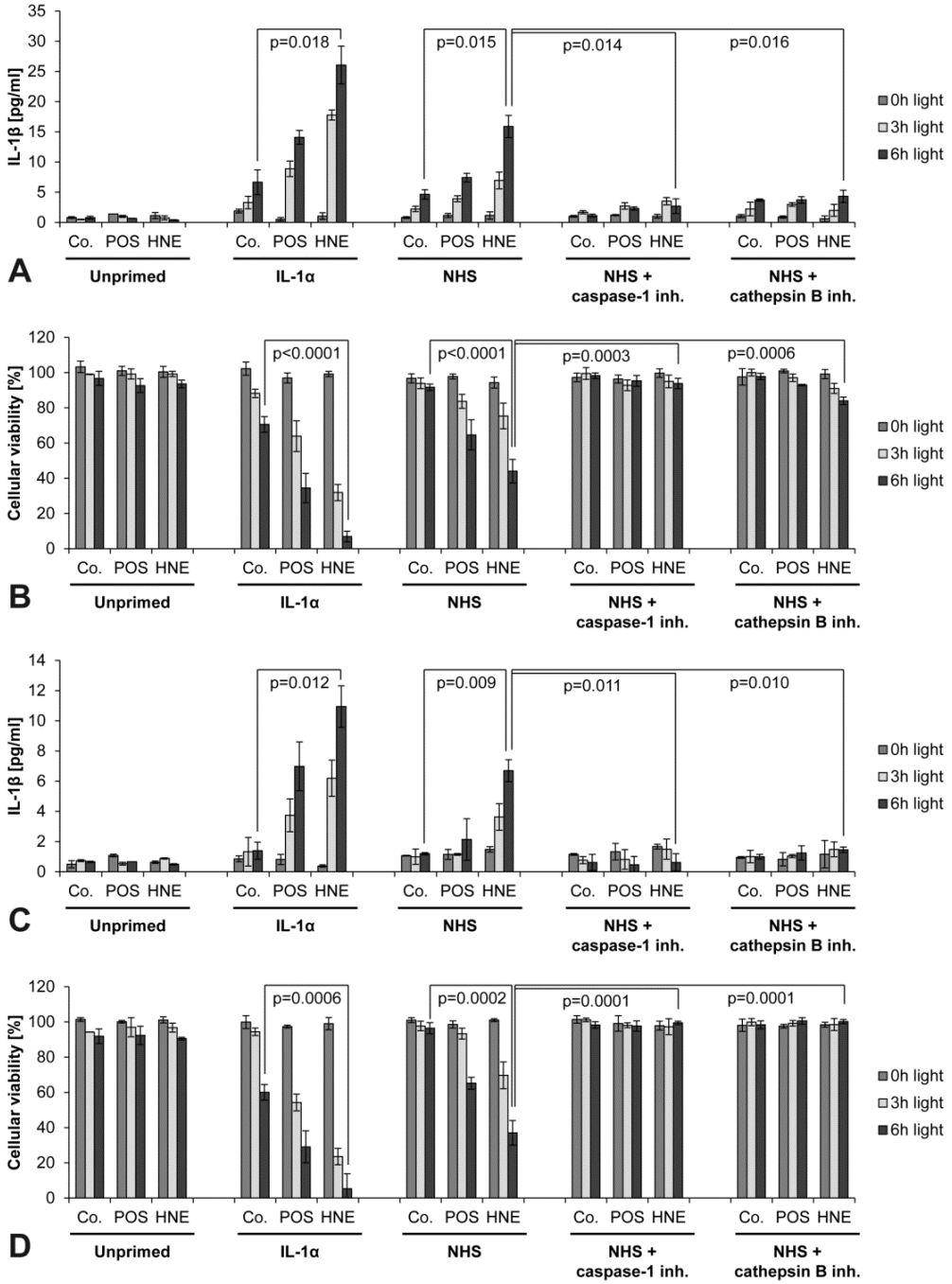

Figure VI.3: NHS primes RPE cells for inflammasome activation by lipofuscin-mediated photooxidative damage. ARPE-19 cells (A, B) and pRPE cells (C, D) were incubated with unmodified POS (POS) or HNE-modified POS (HNE) to induce lipofuscinogenesis whereas control cells were incubated without POS (Co.). Lipofuscin-loaded cells were primed for inflammasome activation by treatment with IL-1, complement-competent normal human serum (NHS), NHS, and caspase-1 inhibitor Z-YVAD-FMK, or NHS and cathepsin B inhibitor CA-074. Subsequently, inflammasome activation was induced by cell irradiation with blue light for 3 or 6 h. Inflammasome activation under these experimental conditions was measured by means of inflammasome-regulated secretion of IL-1 (A, C) and inflammasome-induced cytotoxicity (LDH release) (B, D). Experiments were performed in duplicates (A, C) following the assay manufacturer's recommendation or in triplicates (B, D), and results are presented as mean S.D.

6.4.5 Inflammasome priming in RPE cells is enhanced by a paracrine amplification loop.

Inflammasome priming involves induction of IL-1β expression, and IL-1β is known to induce ist own expression by an auto-/paracrine amplification loop.[288,289] Therefore, we investigated whether inflammasome activation in RPE cells results in paracrine priming of the inflammasome in neighboring RPE cells. To test for this, we performed priming experiments with conditioned media of cells following inflammasome activation. First, ARPE-19 cells were incubated with HNE-POS to induce lipofuscin accumulation, primed with IL-1α, and subsequently irradiated with blue light to trigger inflammasome activation as described above. As a positive control, inflammasome activation was induced in ARPE-19 cells by IL-1α priming and subsequent treatment with Leu-Leu-OMe[185] We confirmed that inflammasome activation resulted in a significant induction of IL-1β secretion in both treatment groups (Fig. VI.6A). Conditioned media of lipofuscin/blue lighttreated and Leu-Leu-OMe-treated RPE cells were collected for further priming experiments. In a second step, new, untreated ARPE-19 cells were loaded with lipofuscin by HNE-POS treatment, incubated with the collected conditioned media for 48 h, thoroughly washed to remove any IL-1β contained in the conditioned media, and irradiated with blue light. Subsequently, inflammasome activation was assessed by measuring IL-1β secretion. In these experiments, conditioned media derived from both lipofuscin/blue light-treated and LeuLeu-OMe-treated cells exerted a strong priming effect that enabled inflammasome activation (Fig. VI.6B). However, when cells were coincubated with the IL1R-inhibitory drug anakinra during priming with conditioned media from lipofuscin/blue light-treated cells, the priming effect was significantly reduced ($p=0.020$). This indicates that the priming effect of conditioned media is mediated by an IL1R ligand such as IL-1β. Indeed, incubation of RPE cells with recombinant IL-1β alone instead of conditioned media likewise resulted in a strong priming effect. Additional analysis of inflammasome priming by means of pro-IL-1β protein expression produces results consistent with IL-1β secretion measurements (Fig. VI.6C). As described above, conditioned media were collected from ARPE- 19 cells following priming with IL-1α, treatment with HNE-POS, and irradiation with blue light. Incubation of new, untreated ARPE-19 cells with the conditioned media resulted in marked induction of pro-IL-1β protein expression as compared with unprimed control cells incubated with unconditioned media. The induction of pro-IL-1β was partially suppressed when cells were co-incubate with the IL1R inhibitor anakinra during priming.

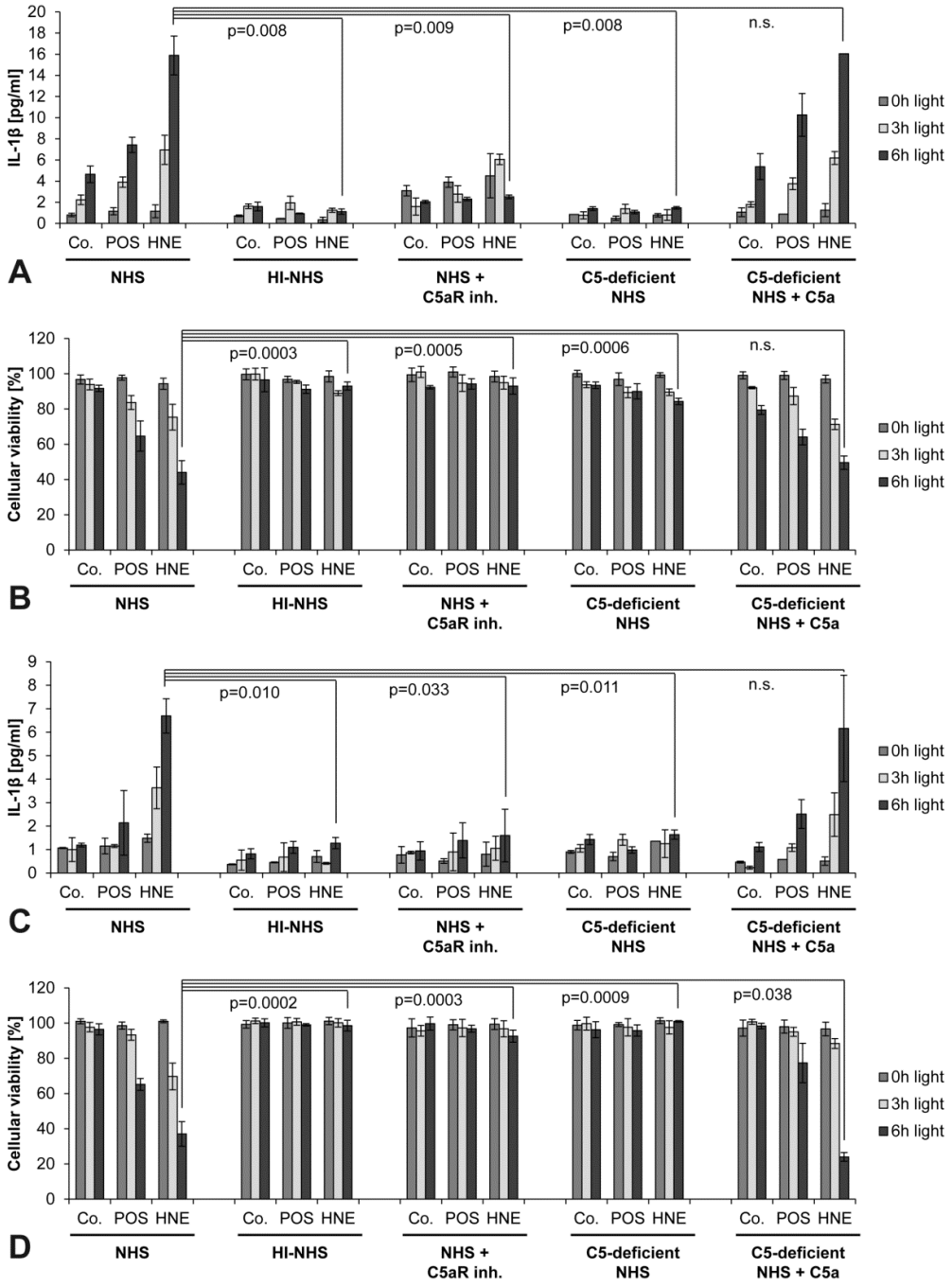

Figure VI.4: Complement component C5a is the active priming agent in NHS. ARPE-19 cells (A,B) and pRPE cells (C,D) were examined. Treatment groups and labels are identical to Fig. VI.3. However, inflammasome priming in this experiment was performed by incubation with complement-competent normal human serum (NHS), heat-inactivated human serum (HI-NHS), NHS and a C5aR inhibitory antibody, C5-deficient NHS, or C5-deficient NHS and recombinant human C5a. Inflammasome activation was assessed be means of secretion of IL-1 (A,C) and cytotoxicity (B,D). Experiments were performed in duplicates (A,C) following the assay manufacturer's recommendation or in triplicates (B,D), and results are presented as mean S.D.

Together, these results indicate that during inflammasome activation in RPE cells in vitro, inflammasome-regulated cytokines such as IL-1β initiate a paracrine amplification loop of inflammasome priming that is amendable to intervention by IL1R-inhibitory drugs.

6.5 Discussion

Blue light irradiation of RPE cells in the presence of oxygen results in generation of reactive oxygen species in a lipofuscindependent manner.[277] and subsequent permeabilisation of lysosomal membranes by oxidative damage.[96,97] We have previously shown that lysosomal membrane permeabilisation by lipofuscin-mediated photooxidative damage activates the NLRP3 inflammasome in primed RPE cells.[254,280] This mechanism may underlie the inflammasome activation observed in the RPE of AMD patients.[185,232] and may contribute to RPE pathology in this disease. Activation of the NLRP3 inflammasome is a posttranscriptionally regulated event mediated by assembly of inflammasome components and subsequent proteolytic maturation of interleukin precursors. In most cells, however, inflammasome component NLRP3 and interleukin precursor proIL-1 are not expressed constitutively or only to low amounts. Therefore, inflammasome activation requires a prior priming signal to induce expression of these proteins. Most previous studies including our own investigated the mechanisms of inflammasome activation in RPE cells by utilizing well-established priming agents such as LPS and IL-1α.[185,235,236,254,280] However, the relevance of these substances as priming agents of the RPE in vivo in the context of AMD is unclear. We therefore investigate activated complement components as potential priming agents in RPE cells. Chronic complement activation is associated with AMD, and activated complement components like C3a and C5a are deposited in the sub-RPE space in AMD.[149] Thus, RPE cells are in constant, direct contact with these bioactive substances that, therefore, represent candidates for the inflammasome priming signal in AMD via anaphylatoxin receptors such as C5aR that is expressed on the basolateral side of the RPE.[286] Indeed, AMD patients with the CFH risk genotype exhibit significantly increased systemic levels of the inflammasomeregulated cytokine IL-18 as compared with AMD patients without the CFH risk genotype, supporting a role for activated complement components in inflammasome activation in AMD.[261] In other autoinflammatory diseases such as atherosclerosis and gout, inflammasome priming by complement activation products has likewise been proposed.[281,282] To elucidate the role of complement activation products in inflammasome activation in AMD, we studied the capacity of activated complement components to provide the priming signal in

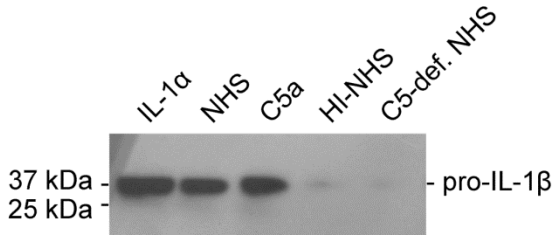

Figure VI.5: Priming by C5a induces expression of pro-IL-1β protein. Expression of pro-IL-1β protein (36 kDa) was assessed in ARPE-19 cells following priming with IL-1α, complement-competent normal human serum (NHS), recombinant human C5a, heat inactivated human serum (HI-NHS), or C5-deficient NHS.

human RPE cells for subsequent NLRP3 inflammasome activation by lipofuscin-mediated photooxidative damage. Our experiments were performed in the human RPE cell line ARPE-19 and primary fetal human RPE cells. In both cell types be detected constitutive expression of the anaphylatoxin receptors C5aR, C3aR, and C5L2. Employing inflammasome-regulated IL-1β secretion and inflammasome-mediated pyroptotic cell death as measures for inflammasome activation, we demonstrated distinct priming effects for activated complement in human serum as well as for recombinant C5a. Complement heatinactivation, C5 depletion, and C5a receptor inhibition suppressed the priming effect of human serum, indicating that C5a represents the active priming agent in complement-activated human serum. Priming by C5a enabled subsequent inflammasome activation by lipofuscin-mediated photooxidative damage. Inflammasome activation was dependent on activity of caspase-1 and cathepsin B. Unlike the priming signal, complement-activated serum and C5a were found to be unable to provide the activation signal and, thus, to directly induce inflammasome activation in RPE cells which is consistent with previous reports.[290] Proteins covalently modified with the lipid peroxidation product carboxy-ethylpyrrole (CEP) have been reported to prime the NLRP3 inflammasome via TLR2.[233] However, subsequent reports have questioned the priming ability of CEP-adducted proteins and rather found it to potentiate inflammasome priming by other signals.[291] In our experiments, we did not observe a priming effect of HNE-adducted POS in RPE cells. For example, inflammasome activation was not inducible in cells incubated with HNE-POS alone (Fig. VI.3, unprimed group) but only in cells co-incubated with the priming agent IL-1α (Fig. VI.3, IL-1α group). In similar experiments, we determined that POS modified by malondialdehyde (MDA) likewise did not induce inflammasome priming in RPE cells (data not shown). Thus, proteins modified by the lipid peroxidation products HNE and MDA do not seem to represent inflammasome priming signals for the RPE. While immunological and inflammatory processes are usually believed to contribute to AMD pathogenesis in a detrimental way, the role of inflammasome activation in AMD is still

controversial and may vary depending on disease stage and subtype. Inflammasome activation in retinal microglial cells and macrophages has been suggested to reduce choroidal neovascularization via IL-18 and thus to be protective in neovascular AMD.[233,292] In contrast, inflammasome activation in RPE cells has been reported to result in RPE degeneration which may contribute to the development of atrophic AMD.[232,293] While patients with neovascular AMD can be effectively treated with VEGF blocking drugs, no effective therapeutic options are currently available for atrophic AMD. Therefore, the unmed need for identification of potential pharmaceutical targets in atrophic AMD is of crucial importance for clinical ophthalmology. Inhibitors of C5 and C5a as well as of complement components upstream of C5a generation such as CFD and C3 are currently evaluated in clinical studies in patients with AMD. In our study, inhibition of the C5a/C5aR axis reduced inflammasome activation by lipofuscin phototoxicity in RPE cells.

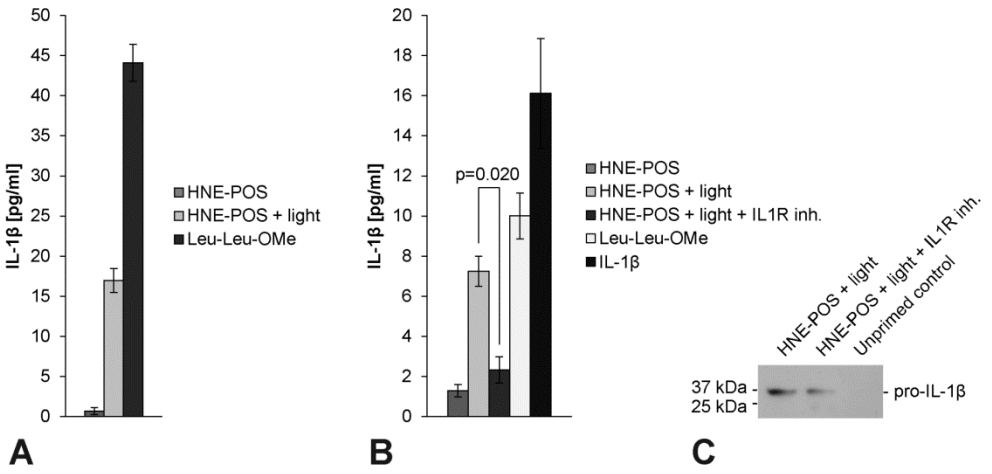

Figure VI.6: Inflammasome priming in RPE cells is enhanced by a paracrine amplification loop. A, inflammasome activation was induced in IL-1-α primed ARPE-19 cells by HNE-POS/blue light treatment or incubation with Leu-Leu-OMe. Cells treated with HNE-POS but without blue light irradiation served as controls. Conditioned medium of these cells was collected. B, subsequently, new ARPE-19 cells were primed with conditioned medium of HNE-POS-treated control cells, conditioned medium of HNE-POS/blue light-treated cells, conditioned medium of HNE-POS/blue light-treated cells, and the IL1R inhibitory drug anakinra, conditioned medium of Leu-Leu-OMe-treated cells or recombinant human IL-1β. Inflammasome activation in all treatment groups was induced by HNE-POS/blue light treatment and analyzed by means of IL-1 secretion. C, expression of pro-IL-1β protein (36 kDa) was assessed in ARPE-19 cells following priming with either conditioned medium of HNE-POS/blue light-treated cells or conditioned medium of HNE-POS/blue light-treated cells and co-incubation with the IL1R inhibitor anakinra, as well as in unprimed control cells. Experiments (A, B) were performed in duplicates following the assay manufacturer's recommendation, and results are presented as mean S.D.

This result supports the rational for therapeutic complement inhibition in atrophic AMD. Moreover, our results suggest the presence of a paracrine amplification loop of inflammasome priming in RPE cells via IL1R. Treatment by the IL1Rinhibitory drug anakinra significantly reduced inflammasome activation in our in vitro experiments, thus providing another potential treatment strategy. Finally, direct therapeutic interference with inflammasome activation has been demonstrated to be effective in vivo, for example using small molecules that provide specific inhibition of NLRP3,[294] and could be tested in future clinical trials for AMD. In summary, our study identifies complement component C5a as a priming agent for the inflammasome in RPE cells that enables subsequent NLRP3 inflammasome activation by stimuli such as lipofuscin-mediated photooxidative damage. This molecular pathway links hallmark events of AMD pathogenesis including complement activation, lipofuscin accumulation, oxidative damage, and RPE degeneration and may provide novel treatment targets. Inflammasome-inhibiting therapeutics may serve as a potential future means of prevention and treatment of atrophic AMD.

VII. 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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7.1 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 h) 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 h 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.

7.2 Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in all industrialized countries.[230] 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 plays an important pathophysiological role.[295] Recent studies suggest that the NLRP3 inflammasome also contributes to RPE cell death secondary to AMD.[185,296] Indeed, NLRP3 inflammasome activation has been demonstrated in RPE cells

affected by AMD,[185,232] and increased intravitreal and systemic levels of the inflammasome activation products IL-1β and IL-18 have been reported in AMD patients.[252,253] 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.[254] Inflammasome activation in RPE cells requires a prior priming signal that can be provided by complement activation product C5a.[297] 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.[298] 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.

7.3 Methods

7.3.1 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% CO_2 in air.

7.3.2 Induction of photooxidative damage

Lipofuscin-mediated photooxidative damage was induced in RPE cells using experimental conditions described previously in detail.[254] 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.[241] These treatment parameters result in a modification of 48 nmol/mg protein as previously determined by HNE-specific ELISA[299] and were chosen to correspond to the range of carbonyl modifications detected in aged human cells in vivo[300] 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.[237] Subsequently, cells were irradiated with blue light (dominant wave length, 448 nm; irradiance, 0.8 mW/cm2) as described previously.[254] In all experiments, duration of irradiation was 3 h for pRPE cells and 6 h for ARPE-19 cells.

7.3.3 L-Leucyl-leucine-methyl Ester (Leu-Leu-OMe) Treatment

L-Leucyl-leucine-methyl ester (Leu-Leu-OMe) induces lysosomal membrane permeabilization (LMP) and inflammasome activation in RPE cells similar to lipofuscin phototoxicity.[185,280] We treated cells with 1 mM Leu-Leu-OMe (Bachem, Bubendorf, Switzerland) for 3 h.

7.3.4 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 h prior to irradiation or Leu-Leu-OMe treatment.

7.3.5 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.

7.3.6 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.

7.3.7 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).

7.3.8 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.

7.3.9 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 h of irradiation, conditioned media were collected. New, treatment-naive cells were primed with the conditioned media for 48 h. 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.

7.3.10 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 ZFF-FMK (Merck/Calbiochem) were used at a concentration of 10 µM each for 1 h prior to and during irradiation treatment.

7.3.11 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 $p < 0.001$.

7.4 Results

7.4.1 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[80,237] and that subsequent blue light irradiation results in photooxidative damage to lysosomal membranes and cell death.[254] Substances such as IL-1α or C5a represent priming signals for the inflammasome in RPE cells that induce expression of pro-IL-1β.[185,297] In primed RPE cells, LMP by photooxidative damage results in activation of the NLRP3 inflammasome with release of IL-1β and IL-18.[254,301] To investigate the effect of inflammasome priming on the extent of photooxidative damage-induced RPE cell death, we analyzed the timecourse of cell death-associated loss of plasma membrane integrity (LDH release) over 48 h in ARPE-19 cells treated by 6 h of irradiation (Fig. VII.1A) and pRPE cells irradiated for 3 h (Fig. VII.1B). At the beginning of the timecourse, all treatment groups exhibited full viability, including the HNEPOS 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 h preceding the experiment. This indicates that none of these treatments alone had an effect on cell viability without irradiation. Likewise, irradiation alone did not significantly affect cell viability in lipofuscin-free unprimed control cells. 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 h 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 h 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 h after the start of the 6 h 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 ($p = 0.0002$; Fig. VII.1A). Similarly, priming by C5a and IL-1α reduced viability in lipofuscin-loaded pRPE cells 24 h after the start of the 3 h irradiation from 65.3% to 33.1% ($p = 0.004$) and 22.6% ($p = 0.003$), respectively (Fig. VII.1B). We have previously demonstrated that inflammasome-induced IL-1β release is stronger following IL-1α priming compared to C5a priming,[254] 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-

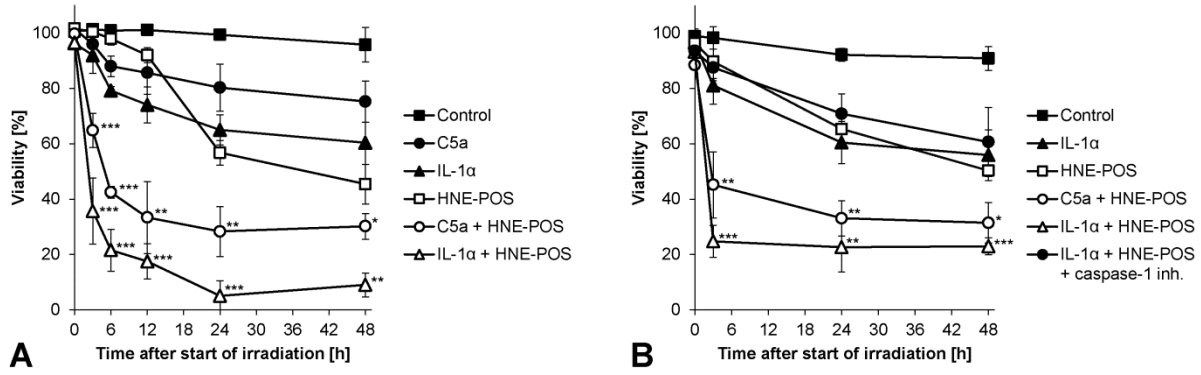

Figure VII.1: Inflammasome priming increases cell death by lipofuscin phototoxicity. Inflammasome priming increases cell death by lipofuscin phototoxicity. (A) ARPE-19 cells and (B) pRPE cells were incubated with HNE-POS to induce lipofuscin accumulation or were incubated without POS. In addition, cells were primed for inflammasome activation by C5a or IL-1α or left unprimed. Subsequently, all treatment groups were irradiated with blue light for 6 h (ARPE-19) or 3 h (pRPE). The time-course of cellular viability was assessed by means of LDH release over 48 h 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 $p < 0.001$ (***).

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.[254,302] 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.

7.4.2 Inflammasome priming changes 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 (Fig. VII.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 (Fig. VII.2A). Analysis of DNA fragmentation by TUNEL assay revealed a progressive increase in TUNEL staining over 24 h after irradiation in unprimed cells (Fig. VII.2B). Primed cells likewise exhibited positive TUNEL labeling that, however, was already detectable immediately after irradiation. In flow cytometric analysis of cells double-labelled with annexin

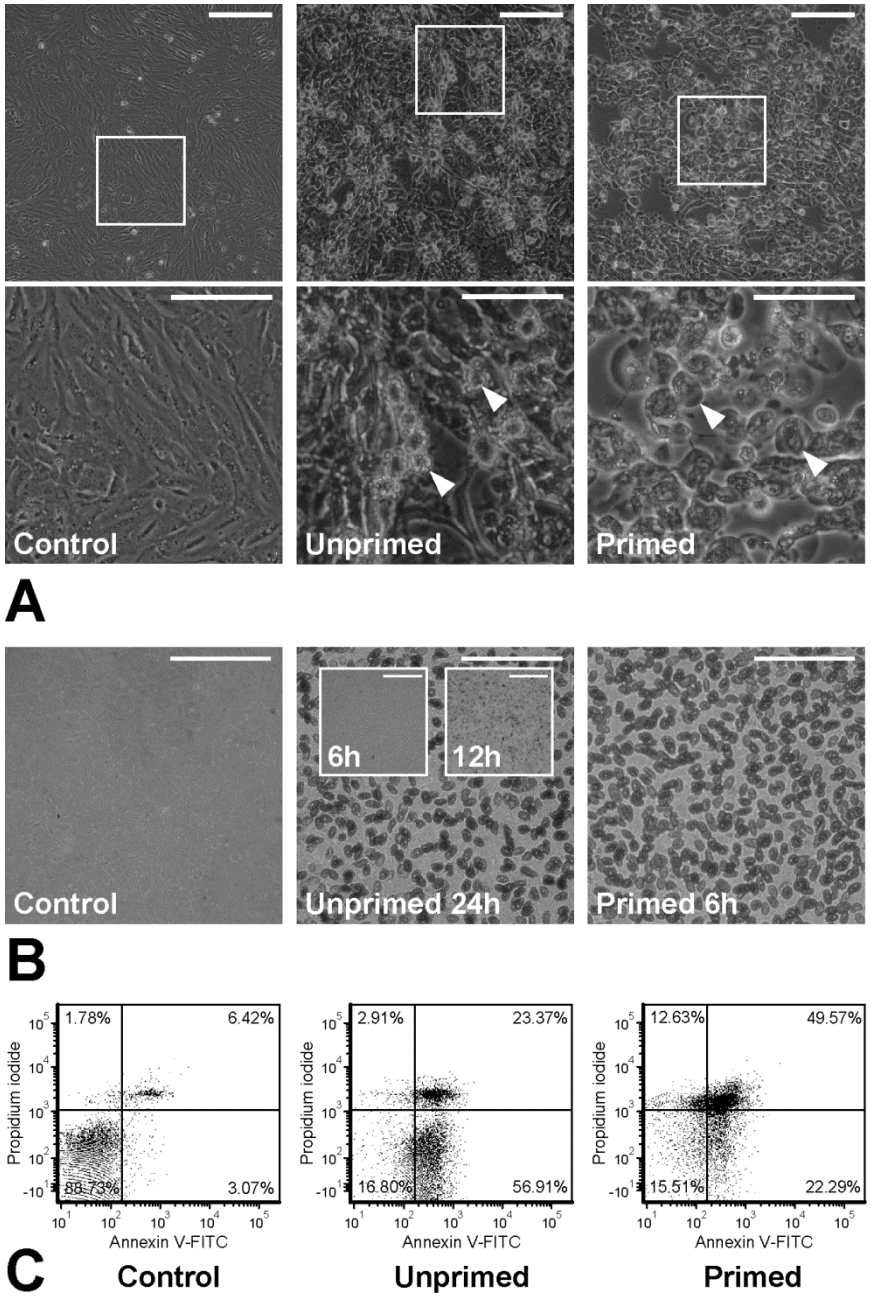

Fig. VII.2 Inflammasome priming changes the mechanism of lipofuscin phototoxicity-induced cell death from apoptosis to pyroptosis. (A) Differences in cell death morphology between unprimed and IL-1α-primed ARPE-19 cells following HNE-POS/light treatment were assessed by light microscopy. Images of unprimed 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 labeling 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.

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 (Fig. VII.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[254] and that the treatment induces release of IL-1β and IL-18 in both IL-1α primed and C5a primed but not in unprimed RPE cells.[301] 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.[303] Thus, our results reveal that inflammasome priming changes the predominant cell death mechanism induced by photooxidative damage in RPE cells from apoptosis to pyroptosis.

7.4.3 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.[304,305] 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-LeuOMe, or increasing concentrations of Leu-Leu-OMe (Fig. VII.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.

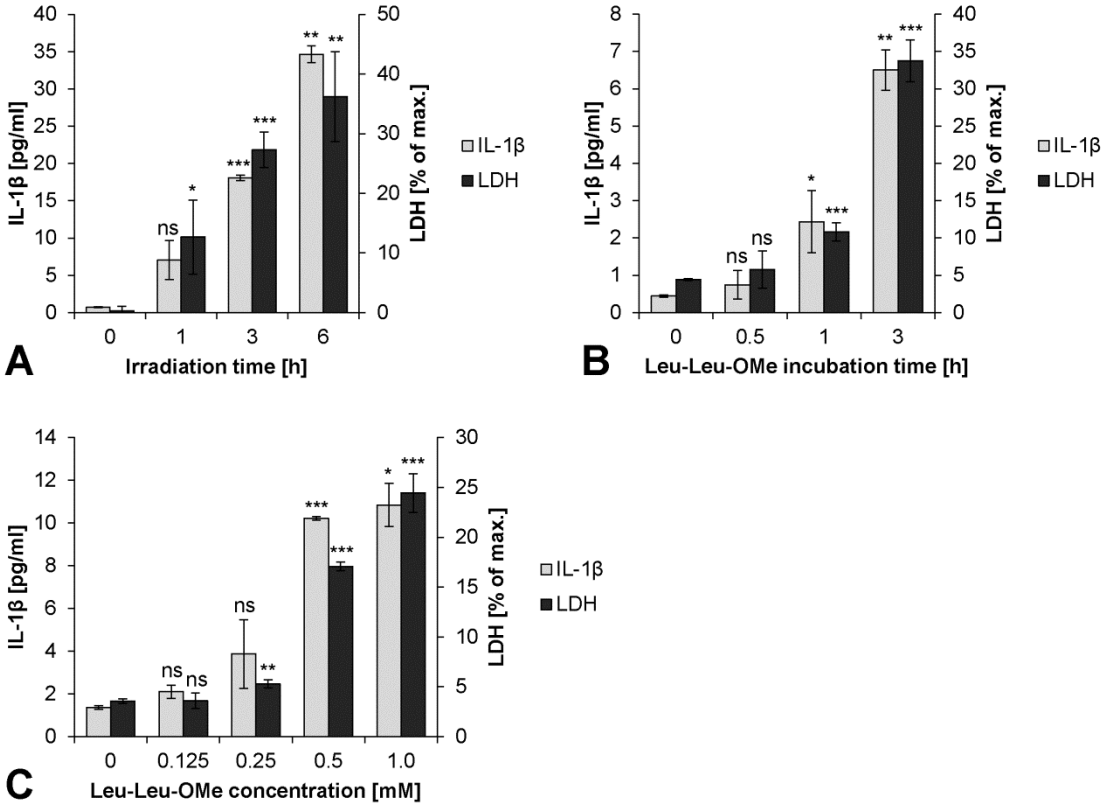

Fig. VII.3 Interleukin-1β release secondary to LMP is closely associated with pyroptotic cell lysis. (A) HNE-POS-treated ARPE-19 cells were primed with IL-1α and irradiated with blue light for increasing durations to induce LMP. Alternatively, LMP was induced by treatment of IL-1α-primed ARPE-19 cells with (B) increasing incubation times of Leu-Leu-OMe at a concentration of 1 mM or with (C) increasing concentrations of Leu-Leu-OMe for an incubation time of 3 h. 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 b 0.05 (*), p b 0.01 (**), and p b 0.001 (***).

7.4.4 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.[288,289,297] 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 (Fig. VII.4). Incubation with conditioned media for 48 h did not itself affect cell viability as indicated by the full viability of all treatment groups measured at the beginning

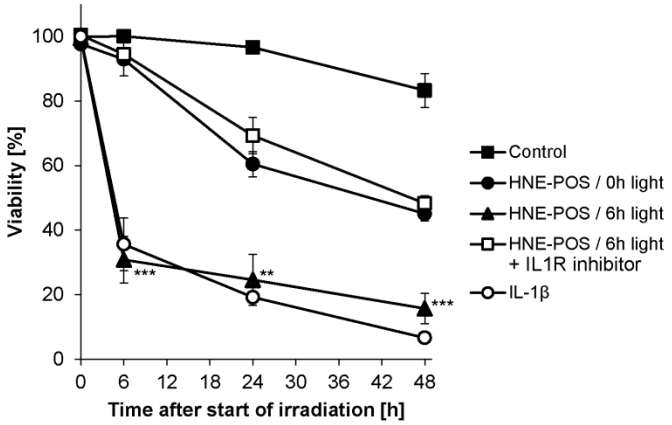

Fig. VII.4: Priming by conditioned media of inflammasome-activated RPE cells increases cell death by lipofuscin phototoxicity. ARPE-19 cells were incubated with HNE-POS and primed with conditioned media that had been collected from IL-1α-primed ARPE-19 cells treated by HNE-POS without irradiation (HNE-POS/0 h light) and by HNE-POS and 6 hour irradiation (HNE-POS/6 h light). In control experiments, the IL1R inhibitor anakinra was added during priming with conditioned media from HNE-POS/lighttreated cells (HNE-POS/6 h 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 h, and the time-course of cellular viability was analyzed by LDH release assay over 48 h after the start of irradiation. Significance levels of the HNEPOS/6 h light group as compared to the HNE-POS/0 h light group are indicated as $p < 0.01$ (**), and $p < 0.001$ (***).

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 h 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 45.0% vs. 15.7%, $p = 0.0006$). 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 a 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.[297]

7.4.5 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.[254] 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 (Fig. VII.5). As expected, apoptotic cell death of unprimed ARPE-19 cells was unaffected by cathepsin B or caspase-1 inhibition (Fig. VII.5A). In contrast, pyroptosis of primed ARPE-19 cells was partially prevented by inhibition of cathepsin B/L or caspase-1 (Fig. VII.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 yielded similar results (Fig. VII.5C and Fig. VII.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.

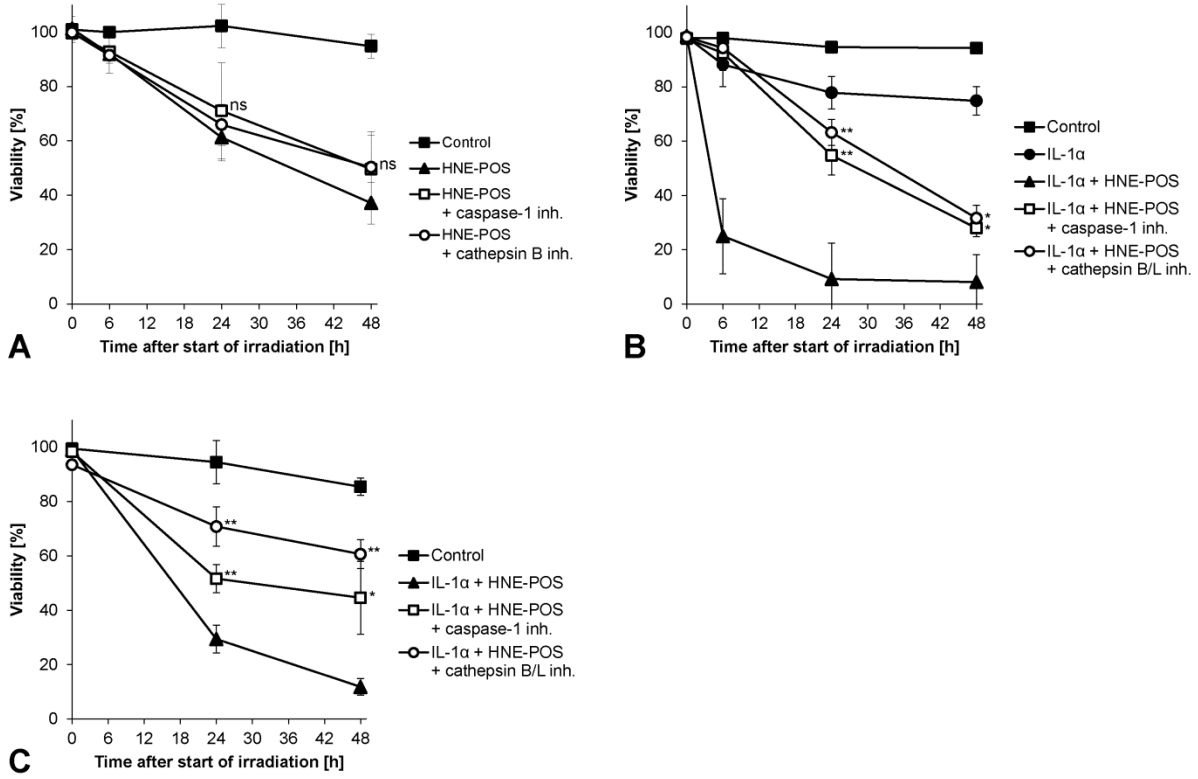

Fig. VII.5: Inhibition of inflammasome activation reduces cell death by lipofuscin phototoxicity. (A) The time-course of cell viability was measured in unprimed ARPE-19 cells over 48 h after the start of irradiation treatment without prior incubation with POS (Control) or after incubation with HNE-POS. For inhibition of caspase-1 and cathepsin B, cells were incubated with ZYVAD-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 b 0.05 (*), and p b 0.01 (**).

7.5 Discussion

Blue light irradiation of RPE cells in vitro results in lipofuscin-dependent generation of reactive oxygen species,[277] LMP by oxidative damage,[96,97] and cell death.[306] 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.[254,297] This mechanism may underlie the inflammasome activation observed in the RPE of AMD patients[185,232] 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.[185,297] 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.[307,308] Our combined results of 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,[301] annexin V-positive/PI-negative staining, and positive TUNEL labeling. 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,[254] IL-1β and IL-18 release,[301] lack of annexin V-positive/PI-negative staining, and positive TUNEL labeling. 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 and cathepsin B and L inhibitor Z-FFFMK. 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.[149,231] 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.[297] Here, we demonstrate that inflammasome priming by 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.[294] IL-1β induces expression of its own precursor pro-IL-1β via IL1R.[288,289] We previously reported that conditioned media of RPE cells collected following inflammasome-mediated IL-1β release induces IL1R-dependent inflammasome priming in other RPE cells, thus providing a paracrine amplification loop of inflammasome activation.[297] Consistently, our current results indicate that conditioned media of pyroptotic RPE 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 atrophic 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 e.g. secretory lysosomes, microvesicles, or exosomes or passively by cell lysis during cell death (terminal release).[304,305] 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 RPE 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.[185,280] 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 damagemediated 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.

VIII. General Discussion

A key pathogenic factor in AMD is the chronic activation of the innate immune system in the sub-RPE space that is detectable by both a local deposition and increased systemic levels of activated complement components.[149,231] A recent study examined AMD patients with the CFH risk genotype and demonstrated that patients showed increased systemic concentrations for the inflammasome-related cytokine IL-18, which represents a product of inflammasome activation.[261] Additionally, NLRP3 inflammasome activation in RPE cells was demonstrated in both neovascular and atrophic AMD.[185,232] These findings, among others, gave rise to the assumption that the inflammasome plays a role in the pathogenesis of AMD. The circumstances leading to NLRP3 inflammasome priming and activation in AMD pathogenesis are largely unknown. The NLRP3 inflammasome can be activated by a diverse range of substances.[309] Many of these substances that activate NLRP3 in AMD destabilize the lysosomal membrane with subsequent cytosolic leakage of lysosomal components.[126,199,201,310] This mechanism has also been detected to activate the inflammasome in RPE cells following chemical induction of LMP.[185] The fact that photooxidative damage mediated by intralysosomal accumulation of photoreactive lipofuscin results in LMP in RPE cells represent an interesting approach for the study of inflammasome activation in the context of AMD.[96,97] Thus, we were interested to examine the role of NLRP3 inflammasome activation by oxidative/ photooxidative damage in human RPE cells as a potential link between hallmark pathogenic features in age-dependent macular degeneration.

8.1 Cell culture model of lipofuscin induced photooxidative stress to study LMP

Experimental cell culture models are a valuable tool for understanding pathology of the cell in human disease and discovering novel therapeutic targets and drugs. Different cell culture models for the in vitro study of lipofuscin effects in RPE cells have been described.[246] As A2E has been considered as the major fluorophore of macular lipofuscin, A2E-loaded RPE cells were used as a model of lipofuscin phototoxicity in previous studies.[97] However, owing to an inadequacy in correlation between lipofuscin fluorescence and the spatial distribution of A2E in the human RPE, the pivotal role of A2E in lipofuscin phototoxicity has been questioned,[247] resulting in a need for a new in vitro model of lipofuscin-photoreactivity for basic and therapeutic research investigations. Therefore, we established a new model of lipofuscin phototoxicity based on a system of endogenous lipofuscin generation from phagocytosed POS in human RPE cells.[237] In our study, we employed the stable human RPE cell line ARPE-19, which arose spontaneously from a primary culture of RPE cells.[311] Altough ARPE-

19 cells are useful in a wide array of research, differences to parental cells may occur. Therefore, we confirmed the main findings by the use of human primary RPE cells. Primary cultures of RPE cells from various species have been shown to retain many normal physiological functions, including the ability to phagocytose rod outer segments.[312,313] RPE cells were incubated for 7 days daily with porcine POS. As human POS were not available in sufficent quantities we relied on porcine POS that are likewise recognized by human phagocytosis receptors. Cellular lipofuscinogenesis was reinforced by HNE- or MDA-modified POS, resulting in lysosomal dysfunction. Modification of retinal proteins with lipid peroxidation products such as HNE has been detected in vivo as a result of light irradiation and aging.[314,315] To allow for biologically meaningful studies of lipid peroxidation effects in vitro, it is crucial that the employed levels of lipid peroxidation reflect the in vivo situation. During oxidative stress, tissue HNE concentrations were reported in the range of 1-20mM,[68] similar to the concentrations used in our experiments. Within or near oxidized lipid membranes, HNE concentrations are estimated to reach 100mM or even higher levels.[68] The levels of POS protein modifications used in our cell culture were quantified previously[80] and were chosen to correspond to the range of carbonyl modifications detected in human cells in vivo.[248] Although our model means to simulate the in vivo situation in aged RPE cells, the composition of lipofuscin-like material that was generated in our model during 7 days of POS incubation probably differs from the composition of lipofuscin generated in RPE cells in vivo in a human lifetime. Nonetheless, blue light irradiation of RPE cells following lipofuscin loading induced photooxidative damage, LMP, and leakage of lysosomal enzymes into the cytosol. This is in accordance with RPE cells that were loaded with lipofuscin granules generated in human RPE in vivo.[96] Therefore, our model of lipofuscin generation was appropriate to study blue light-induced photooxidative stress in human RPE cells, intensified by accumulated lipofuscin, and subsequent lysosomal membrane permeabilization.

8.2 Inflammasome activation by blue light induced photooxidative damage

Initially we were interested whether lipofuscin-phototoxicity induced LMP plays a role in NLRP3 inflammasome activation in RPE cells. For this, we established an in vitro model of blue light-induced photooxidative damage. Using this model, we demonstrated inflammasome activation by means of caspase-1 activation and secretion of the inflammasome-related cytokines IL-1β and IL-18. In addition, inflammasome activation was amplified by the photosensitizer lipofuscin, that accumulates in the RPE in vivo with age and has the highest concentration in the macula (chapter II). Secondary to light-induced LMP, we

detected leakage of lysosomal enzymes into the cytosol of RPE cells. Inhibition of the lysosomal proteases cathepsin B and L suppressed inflammasome activation associated with light-induced LMP. Similarly, previous studies demonstrated cathepsin dependent NLRP3 inflammasome activation secondary to silica crystal induced LMP in macrophages.[126] These results suggest a cytosolic substrate of cathepsin proteolytic activity as a critical component of NLRP3 inflammasome activation. However, the molecular mechanism by which cytosolic activity of cathepsins or other lysosomal enzymes induce inflammasome activation has not yet been resolved. Furthermore, our results from a NLRP3 siRNA knockdown experiment revealed that inflammasome activation secondary to lipofuscin phototoxicity in RPE cells is mediated by NLRP3 (chapter II). The importance of NLRP3 in AMD pathogenesis has been underlined by the observation of activated NLRP3 inflammasome in the RPE of patients with atrophic and neovascular AMD.[185,232] Thus, photooxidative damage by visible light could be attributed as a new mechanism of NLRP3 inflammasome activation in human RPE cells in our model and therefore contribute to our understanding of light damage to the RPE. Moreover, this mechanism represents a novel molecular link between hallmark features of AMD pathogenesis such as photooxidative damage, innate immune response, and RPE cell dysfunction that may provide new therapeutic targets against for the blinding disease.

8.3 Inflammasome priming by activated complement component C5a

For the NLRP3 inflammasome to be activated in RPE cells, it must first be primed by activation of the transcription factor nuclear factor-κB (NF-κB) resulting in transcription of pro-IL-1β and NLRP3. In many studies, NF-κB activation is induced by LPS or IL-1α prior to inflammasome activation.[185,235,236,254,280] However, the relevance of these substances as priming agents of the RPE in vivo in the context of AMD is unclear. Observations of NLRP3 being expressed in the RPE of eyes affected by advanced AMD but not in non-AMD affected control eyes suggests that molecular or cellular changes associated with the development of AMD mediate the pathological priming of the RPE. For this study we considered reports that demonstrated an association between CFH risk genotype and significantly increased plasma levels of the inflammasome-regulated cytokine IL-18, suggesting a role for activated complement components like C3a and C5a in inflammasome activation in AMD.[261] Indeed, activated components of the complement system such as C3a and C5a are detectable both locally in the sub-RPE space and systemically in plasma of AMD patients.[149,231] Thus, RPE cells are in constant, direct contact with these bioactive substances, which therefore

represent candidates for the inflammasome priming signal in AMD via anaphylatoxin receptors such as C5aR that is expressed on the basolateral side of the RPE.[286] Inflammasome priming by complement activation products has already been proposed in other autoinflammatory diseases such as atherosclerosis and gout.[281,282] We therefore intended to investigate activated complement components as potential priming agents in RPE cells. The consecutive implementation of heat-inactivation of complement components, C5 depletion, and C5a receptor inhibition delineated C5a as the active priming agent in complement-activated human serum (chapter IV). Accordingly, inflammasome priming by C5a led to distinct expression of pro-IL-1β protein by ARPE-19 cells and enabled subsequent inflammasome activation by lipofuscin-mediated photooxidative damage. Again, inflammasome activation was dependent on activity of caspase-1 and cathepsin B. Our observations are in accordance with the results of a previous study that likewise reported a priming effect of C5a on RPE43 cells.[316] A group led by Prof. Joanne A. Matsubara described a study that shows the activation of the NF-κB pathway in RPE in vitro by C5a stimulation,[155] highlighting the relevance of our prior published results in terms of activated complement components as RPE cell priming agents. With the constitutive expression of the anaphylatoxin receptors C5aR, C3aR and C5aL on the human RPE cell line ARPE-19 and primary fetal human RPE cells (chapter IV) it is of importance to understand the effects of C5a on RPE function.

Considering our results, we concluded that activated comlement component C5a is capable of priming human RPE cells for NLRP3 inflammasome activation by stimuli such as lipofuscin-mediated photooxidative damage. This molecular pathway links hallmark events of AMD pathogenesis including complement activation, lipofuscin accumulation, oxidative damage, and RPE degeneration and may provide novel treatment targets.

8.4 Consequences of NLRP3 inflammasome activation

Following up on the investigation describing the mechanism of LMP-induced inflammasome activation induced by lipofuscin-mediated photooxidative damage in RPE cells, we were interested to elucidate the consequences of this mechanism, in particular with regard on pathologic processes in the outer retina.

Initially, we addressed this question by analyzing the profile of cytokines that are secreted by RPE cells following inflammasome activation. Using a model of LMP-induced inflammasome activation that employs Leu-Leu-OMe for chemical destabilization of lysosomes,[185] we revealed significant and caspase-1 dependent increase in inflammatory cytokines. In

particular, the secretion of the inflammasome-related cytokines IL-1β and IL-18 was predominantly directed towards the apical side of the RPE monolayer. This is in accordance with previous studies that likewise reported a predominantly apical secretion of inflammatory cytokines by RPE cells following induction by various stimuli.[269–271] The apical side of RPE cell layer corresponds to the neuroretinal side of the normal retina in vivo. This polarized secretion raised the question whether cells of the neuroretina may be affected by activation of the inflammasome. Retinal cell populations that are most likely affected by apical secretion of cytokines include resident microglia cells and infiltrating macrophages. Activation of these cell types and their migration into the subretinal space has been observed in AMD patients and animal models and has been discussed as a factor contributing to AMD pathogenesis.[35,272,273] Our results from a microglia chemotaxis experiment (chapter III) display a significant chemotactic effect of conditioned media from Leu-Leu-OMe–treated RPE cells on microglia cells, supporting a role of inflammasome-related cytokines in microglia recruitment during neovascular AMD. VEGF plays a crucial role in CNV formation in neovascular AMD, and inflammasome activation has been suggested to be involved in this process due to the effects of inflammasome-controlled cytokines on VEGF secretion and angiogenesis. However, the reports of the inflammasome-related effects in CNV formation are divergent. Doyle and coworkers[233] elucidated that incubation with inflammasome-regulated IL-18 reduces secretion of VEGF in RPE cells, suggesting a protective effect against CNV formation. In contrast, another inflammasome-regulated interleukin, IL-1β, has been shown to induce VEGF secretion in RPE cells[259] and to promote laser-induced CNV formation in mice.[255] In an attempt to elucidate the angiogenesis-related effects following LMP-induced inflammasome activation, we revealed reduced constitutive VEGF secretion by RPE cells following inflammasome activation. Conditioned media of RPE cells following inflammasome activation consistently reduced proliferation and migration of vascular endothelial cells compared to conditioned media of untreated control cells. In addition to the reduction in VEGF secretion, the released IL-18 may add to this effect due to its suggested antiangiogenic properties.[233] While our experiments demonstrate reduced VEGF secretion by RPE cells following inflammasome activation, inflammatory cytokines released by RPE cells after inflammasome activation in vivo may exert indirect angiogenic effects via other cell types such as recruited microglia cells.[35] There is now comprehensive evidence proving the involvement of immune cells in human neovascular AMD[317–319] as well as experimental choroidal neovascularization.[320–322] In contrast, histological studies demonstrate less immune cells in areas of geographic atrophy.[323] However, an increasing number of studies indicate a

contribution of immune-mediated tissue damage in geographic atrophy, by suggesting inflammasome-mediated RPE degeneration as a driving force in geographic atrophy.[185,232,236] To further elucidate the consequences of LMP-induced inflammasome activation, we focussed on the effects of inflammasome priming on mechanism and extend of photooxidative damage-induced cytotoxicity in RPE cells. Morphological investigations indicate apoptosis as the prevailing cell death mechanism secondary to photooxidative damage-mediated LMP in the absence of an inflammasome priming signal, that was likewise demonstrated in other studies.[307,308] However, inflammasome priming with IL-1α or C5a ahead of LMP-induced cell death in RPE cells changed the mechanism of cell death from apoptosis to pyroptosis (chapter V). Beside a change in cell death mechanism, an increased susceptibility to photooxidative cell damage was detected that was manifested by a significant higher extend of phototoxic cell death. This increase in susceptibility to photooxidative cell death was reversible by treatment of RPE cells with inflammasome inhibitors such as caspase-1 inhibitor Z-YVAD-FMK. Inflammasome activation leads to secretion of IL-1β, a proinflammatory cytokine that signals through its cognate receptor IL1R and is able to induce its own expression by an auto-/paracrine amplification loop.[288,289] Accordingly, the use of conditioned media, collected following inflammasome mediated IL-1β release was capable to prime cells for expression of pro-IL-1β protein (chapter IV) and enabled inflammasome activation via lipofuscin-induced photooxidative stress (chapter V). Inflammasome activation was significantly reduced by application of the IL1R-inhibitory drug anakinra, indicating a distinct amplification loop of inflammasome priming in our cell culture system that is mediated by an IL1R ligand such as IL-1β. Thus, cell priming by IL-1α or C5a not only changes the cell death mechanism from apoptosis to pyroptosis but also increases the susceptibility of the cells to photooxidative damage-mediated cytotoxicity (chapter V).

8.5 Therapeutic prospects

Elucidating the still unresolved pathogenesis of multifactorial, complex AMD will help to identify potential targets for therapeutic intervention. While patients with neovascular AMD can be effectively treated with VEGF blocking drugs, no effective therapeutic options are currently available for atrophic AMD. Incidence and prevalence of the disease are rising, due to demographic developments. Therefore, the unmed need for identification of potential pharmaceutical targets in atrophic and neovascular AMD is of crucial importance for clinical ophthalmology. My work outlined several pathways that appear to play a role in AMD pathogenesis and may be suitable for therapeutic interventions.

The inflammasome-related cytokines are likely to present therapeutic targets. The cytokine IL-18 has been reported to exert a direct cytotoxic effect on RPE cells in vivo,[232] but systemic or intravitreal administration of physiologically relevant doses of recombinant IL-18 did not correlate with pathological effects on mouse RPE cells in vivo.[292,325] Indeed, IL-18 concentrations of up to 10 µg/mL did not induce any cytotoxic effect on RPE cells in vitro,[292] a concentration that is about five orders of magnitude higher than IL-18 concentrations measured in conditioned media in our experiments. It is possible that these cytokines are not directly responsible for RPE cell death in geographic atrophy but act on cells in an autocrine or paracrine fashion. For instance, IL-1β is known to induce its own expression by an auto-/paracrine amplification loop.[288,289] Similarly, our experiments demonstrated that during lipofuscin-photoreactivity induced inflammasome activation in RPE cells in vitro, inflammasome-regulated cytokines such as IL-1β initiate a paracrine amplification loop of inflammasome priming (chapter IV and V). It is conceivable that in situations of localized RPE cell death such as in atrophic AMD, this mechanism could result in increased susceptibility of immediate bystander RPE cells to inflammasome-mediated cell death, thus contributing to the centrifugal progression pattern of RPE cell loss in AMD. Application of the IL1R-inhibitory drug anakinra reduced both inflammasome-related cytokine secretion and cell death, providing the rational for therapeutic IL-1β inhibition in atrophic AMD. Several biologic agents that target the IL-1 pathway are approved for use by the Food and Drug Administration. The recombinant IL-1 receptor antagonist Anakinra is approved for treatment of rheumatoid arthritis. The monoclonal anti-IL-1β antibody canakinumab and rilonacept, a “IL-1 trap” fusion protein, has been approved for the treatment of CAPS.[326,327] In contrast to the reported anti-angiogenic effects of IL-18 on laser-induced choroidal neovascularization,[233] IL-1β promotes neovascularization.[255] These opposing findings might imply that a single factor (IL-18) or pathway (NLRP3 inflammasome activation) can be anti-angiogenic and destructive to the RPE at the same time.[38] Therefore, the potential role of the NLRP3 inflammasome in the pathogenesis of AMD, as well as the downstream cytokines involved, must be elucidated in order to determine whether targeting the IL-1 pathway will be effective. Further investigations demonstrated C5 activation product C5a is capable to prime RPE cells for inflammasome activation (chapter IV) demonstrating C5a increase the susceptibility of RPE cells to oxidative damage-mediated cell death. Activated complement component C5a is detectable both locally in the sub-RPE space and systemically in plasma of AMD patients.[149,231] RPE cells are in constant, direct contact with these bioactive substances that, therefore, represent candidates for the inflammasome priming signal in

AMD via anaphylatoxin receptors such as C5aR that is expressed on the basolateral side of the RPE.[286] Inhibition of the C5a/C5aR axis reduced inflammasome activation by lipofuscin phototoxicity in RPE cells in our experiments. This demonstrates that inflammasome priming agents such as C5a increase the susceptibility of RPE cells to oxidative damage-mediated cell death. Thereby, our results support therapeutic strategies for treatment of atrophic AMD by complement inhibition. Inhibitors of C5 and C5a as well as of complement components upstream of C5a generation such as CFD and C3 are currently evaluated in clinical studies in patients with AMD. These include various protein-binding and inhibiting substances, such as antibodies (LFG316, FCFD4514S, eculizumab), peptides (POT-4) or aptamers (ARC1905), which target specifically the complement system (for example, C3, C5, CFD). The recombinant mAb antibody fragment Lampalizumab that blocks complement factor D demonstrated a promising effects in a phase II trial to slow down atrophy progression. In addition, agents with direct inhibitory effects on inflammasome activation such as small molecule NLRP3 inhibitors provide specific inhibition of NLRP3, representing interesting candidates for future interventional approaches in atrophic AMD.[294] Further investigation of a pathogenic versus a protective role for the inflammasome is required to determine whether targeting the inflammasome pathway will be effective in AMD.

8.6 Future prospects

The molecular pathogenesis of AMD is still not completely resolved which hinders the development of effective therapies, in particular for the atrophic subtype of the disease. Progressive death of retinal pigment epithelium cells is a hallmark of AMD, the leading cause of blindness in all developed countries. Several lines of clinical and experimental evidence indicate oxidative and lipofuscin-mediated photooxidative stress as well as activation of the NLRP3 inflammasome as contributing factors to this process.

My findings demonstrate 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.

To demonstrate that the NLRP3 inflammasome mediates AMD pathogenesis in response to RPE lysosomal destabilization via lipofuscin photoreactivity, it will be necessary to investigate whether the identified mechanism can be demonstrated in vivo. In accordance to our established cell culture model of blue light-induced photooxidative damage, a photoreactive effect in the lipofuscin-containing RPE could be induced by illumination of mice. Subsequently, RPE cells may be examined for evidence of inflammasome activation by measurement of IL-1β secretion, caspase-1 activation or signs of pyroptotic cell death. The lipofuscin-mediated effect can be enhanced by the use of ABCA4 knockout mice, an animal model for AMD and Stargardt disease that is characterized by the accelerated accumulation of lipofuscin in the RPE.[328–330] Influencing inflammasome activation may offer new therapeutic stragegies. The use of anakinra (Kineret) or drugs that directly inhibit the inflammasome may provide a base for clinical trials in patients with AMD.

8.7 Conclusion

In AMD, the RPE becomes progressively dysfunctional and eventually degenerates, resulting in photoreceptor death and visual function loss.

Evidence that the inflammasome is functional in RPE cells and can mediate IL-1β release in response to activation is a key finding that may provide new therapeutic targets for AMD. However, research on inflammasomes in AMD remains nascent, and many questions regarding the role that the NLRP3 inflammasome play in AMD are still unresolved.

This study aimed to identify a novel mechanism of inflammasome activation by light damage. The finding that photooxidative damage to human RPE cells, intensified by accumulated lipofuscin, causes NLRP3 inflamamsome activation via LMP confirms blue light damage as a new mechanism of inflammasome activation and contributes to our understanding of light damage to the RPE. In contrast to inflammasome activation, the mechanism of inflammasome priming in AMD has been little investigated so far. Recent studies demonstrated the CFH risk genotype to be associated with significantly increased plasma levels of the inflammasome-regulated cytokine IL-18, suggesting a role for activated complement components like C3a and C5a in inflammasome activation in AMD. We investigated whether products of complement activation are capable of providing the priming signal for inflammasome activation in RPE cells. Our study identified complement component C5a as a priming agent for the inflammasome in RPE cells that enables subsequent NLRP3 inflammasome activation by stimuli such as lipofuscin-mediated photooxidative damage. Furthermore, inhibition of the C5a/C5aR axis reduced inflammasome activation by lipofuscin phototoxicity in RPE cells and supports the rational for therapeutic complement inhibition in atrophic AMD. Examining the mechanisms of photooxidative damage-induced cell death in cultured human RPE cells demonstrated that LMP-induced NLRP3 inflamamsome activation in RPE cells results in apical secretion of inflammatory cytokines with chemotactic effects on microglia cells and reduced constitutive secretion of VEGF. Via these mechanisms, lipofuscin phototoxicity may contribute to local immune processes in the outer retina as observed in AMD. In addition, we demonstrated that inflammasome priming with IL-1α or C5a not only changes the cell death mechanism from apoptosis to pyroptosis but also increases the susceptibility of the cells to photooxidative damage-mediated cytotoxicity. In summary, our results provide new insights into the complex interplay of complement system, inflammasome activation, oxidative damage, and RPE cell pathology in AMD pathology and suggest inhibition of inflammasome priming or activation as potential treatment strategies for atrophic AMD.

IX. Appendix

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List of Figures and Table

List of Abbreviations

A2E	N-retinylidene-N-retinyl-ethanolamine
AIM2	Absent in melanoma 2
ALR	AIM2 like receptor family
AMD	Age-related macular degeneration
AO	Acridine Orange
AP	Alternative pathway of the complement system
AREDS	Age-related eye disease study
AREDS 2	Age-related eye disease study 2
ARPE-19	Humane Zelllinie des retinalen Pigmentepithliums
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BRB	Blood-retinal barrier
C3	Complement component 3
C3a	Complement component 3a
C3aR	Complement component 3a receptor
C5	Complement component 5
C5a	Complement component 5a
C5aR	Complement component 5a receptor
CA-074	Cathepsin B inhibitor
CAD	Caspase-activated DNase
CAPS	Cryopyrin-associated periodic syndromes
CARD	Caspase activation and recruitment domain
CEP	Carboxyethylpyrrole
CFD	Complement factor D
CFH	Complement factor H
CFI	Complement factor I
CNV	Choroidal neovascularization
CO_2	Carbon Dioxide
CRP	C-reactive protein
DABCO	1,4-Diazabicyclooctane
DAMP	Danger-associated molecular structures
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
e.g.	*exempli gratia*
FACS	Fluorescence-activated cell sorting

FAM-YVAD-FMK	Carboxyfluorescein-Tyr-Val-Ala-Aspfluoromethylketone
FBS	Fetal-bovine-serum
FIIND	Function-to-find domain
FITC	Fluorescein isothiocyanate
FLICA	Fluorochrome-labeled inhibitor of caspases
fMLP	N-formyl-methionine-leucine-phenylalanine
GA	Geographic atrophy
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HIN	Hematopoietic interferon-inducible nuclear protein domain 200
HI-NHS	Heat-inactivated normal human serum
HNE	4-hydroxy-2-nonenal
H-RPE	Human retinal pigment epithelial cells
HUVEC	Human umbilical vein endothelial cells
i.e.	*id est*
ICAD	Inhibitor of caspase-activated DNase
IL-18	Interleukin-18
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
K+	Potassium Kation
kDa	Kilodalton
LAMPs	Lysosome-associated membrane proteins
LDH	Lactate dehydrogenase;
LED	light-emitting diode
Leu-Leu-OMe	L-Leucyl-L-leucine methyl ester
LIMPs	Lysosomal integral membrane proteins
LMP	Lysosomal membrane permeabilisation
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat domain
MDA	Malondialdehyde
mM	Millimolar
mRNA	Messenger RNA
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH_4Cl	Ammonium chloride
NHS	Normal human serum

NLR	Nucleotide-binding oligomerization domain (Nod)-like receptors
NLRC	NLR family, CARD domain containing
NLRC4	NOD-, LRR- and CARD-containing 4
NLRP	NLR family, pyrin domain containing
NLRP3	NLR family, pyrin domain containing 3
Nm	Nanometer
NOD	Nucleotide-binding and oligomerization domain
P	Properdin
PAMP	Pathogen-associated molecular pattern
PEDF	Pigment epithelial-derived factor
pH	Potential hydrogen
PI	Propidium iodide
POS	Photoreceptor outer segments
pRPE	Human fetal primary RPE cells
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PTX	Pertussis toxin
PUFA	Polyunsaturated fatty acids
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
sec	Second
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
TdT	Terminal desoxynucleotidyl transferase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TUNEL	TdT-mediated dUTP-biotin nick end labeling
UV	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
Z-FF-FMK	Z-Phe-Phe-fluoromethylketone
Z-YVAD-FMK	Z-Tyr-Val-Ala-Asp-fluoromethylketone

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