SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

In vitro investigation of the clearance of anoikic and autophagy-associated dying retinal pigment epithelial cells – implications for age-related macular degeneration

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The Examination took place at the library of Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen at 1 p.m., on 26th of November, 2015.

Head of the **Defense Committee**:	Prof. Gábor Szabó MD, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 10:30 a.m., on 28th of October, 2016.

1. INTRODUCTION

1.1. The retina

The wall of the human eye is composed of three main layers: the outermost layer (the opaque white sclera and the transparent cornea), the middle vascularized layer (the iris anteriorly, an intermediate ciliary body and the choroid posteriorly) and the innermost layer (the retina). The retina can be found in the posterior chamber of the eye, which comprises multiple layers of different cell types. The neuroretina and the outermost retinal pigment epithelium (RPE) form the retina. Two types of photoreceptors can be distinguished: rods for vision in dim light, and cones for daylight and color vision. The macula is the small, central area of the retina that is responsible for central and fine detail vision needed to do daily tasks such as reading or driving.

1.2. The retinal pigment epithelium (RPE)

The RPE is composed of a single cell layer of highly pigmented, polarized, hexagonal cells. It is attached to the underlying Bruch's membrane - an extracellular matrix (ECM) structure, which forms an important physiological barrier - the outer blood-retinal barrier (BRB). The BRB has an essential role in maintaining the eye as a privileged site, and required for retinal homeostasis and normal visual function.

Moreover, the RPE has a crucial and multifunctional role in supporting the overall health of the retina and the homeostasis of overlaying photoreceptors by regulating the rate of phagocytosis of photoreceptor discs, as well as transporting nutrients, such as glucose, retinol and fatty acids from the blood to the photoreceptors. The RPEs are responsible for absorbing scattered light to improve the optical quality by forming a pigmented cell layer to cover of the inner wall of the bulbus, and accumulate high amount of antioxidants in order to protect against oxidative stress. Another function is their secretion of growth factors, as well as factors that are critical for the structural integrity of the retina and choriocapillaris, for example pigment epithelium- or vascular endothelium- derived growth factors (PEDF or VEGF, respectively); in addition, secretion of immunosuppressive factors that contribute to the maintenance of immune privilege of the eye.

1.3. *In vitro* RPE cell models

During the last decade, adult retinal pigment epithelium-19 (ARPE-19) cells became the most commonly used immortalized cell line to evaluate RPE function. Originally, they were extracted from the eye of a 19-year old male donor. The typical cobblestone morphology of the uniform epithelial monolayer can be detectable up to passages 15 to 20 of original ARPE-19 cells in tissue culture flasks. Further passages of these cells result in a heterogeneous mixture of elongated and polygonal cells. The immortalized cell lines can be easily obtained, but may lose some of their important features of the original RPE cells.

The primary hRPE cells can be regularly isolated from adult cadaver human eyes. Moreover, primary hRPE cells are commercially available as well. Researchers in different laboratories use essentially the same protocol to isolate RPE cells from an adult human eye. It has been demonstrated that the primary cells polarize and form a cell layer with barrier properties. Primary hRPE may preserve the typical properties of the original tissue. They show relatively heterogeneous morphology and exhibit donor-to-donor variability, in contrast to the immortalized cell lines. Generally, primary cells can be expanded only for a few passages.

In the recent years, developments in embryonic stem cell technologies have generated novel cell sources to establish RPE cells. Skottman et al. previously created a xeno-free culture and differentiation protocol, without any animal-derived components, to develop functional RPE-like cells from pluripotent human embryonic stem cell (hESC) lines. *In vitro,* it was shown that these cells have hexagonal, cobblestone-like morphology with high pigmentation rate, and form a highly polarized epithelial cell layer. It was observed that the functional properties of the hESC-derived RPE-like cells are very similar to the native RPE.

1.4. The age-related macular degeneration (AMD)

AMD is one of the most common irreversible causes of legal blindness, manifesting in the elderly people. Approximately 50 million people suffer from symptoms of AMD worldwide. Typical symptoms include drastically decreasing visual acuity, contrast sensitivity defects, distortion of straight lines, abnormal dark adaptation, scotomas and color vision impairment. The loss of central vision in AMD can interfere with reading, driving, or recognizing faces. The internal and environmental risk factors together contribute to the clinical manifestation of this disease, including aging, genetic

background, smoking, low antioxidant content, unhealthy diet, obesity, high blood pressure, hypercholesterolemia and arteriosclerosis.

AMD can be classified as either dry or wet type; these two major forms can be subdivided into early and late stage. Dry AMD is the most common, slowly progressing, chronic form of this disease, which is characterized by some degree of visual impairment. In contrast, the wet type of AMD occurs only about 10-15% of the total AMD patients, emerges abruptly and its progression is relatively rapid, with acute loss of vision.

1.5. Cell death types in AMD pathogenesis

1.5.1. Anoikic cell death

Induction of anoikic cell death occurs when cells lose their attachment to the ECM, or due to inappropriate cell adhesion. Anoikis is essential for ensuring development and tissue homeostasis and functions as a major defense mechanism for the organism by preventing adherence-independent cell growth and attachment to an inappropriate matrix. In accordance with classical apoptosis, the anoikis program can be mediated by both, the extrinsic pathway triggered by cell surface death receptors and the intrinsic pathway driven by mitochondria.

Anoikic cell death can be induced in photoreceptors and RPE cells by accumulation of drusen in the space between the Bruch's membrane and the RPE layer, thus resulting in decreased cell adhesion and elevation of RPE cells from the membrane. Recently, our research group reported an *in vitro* model for triggering anoikic cell death in ARPE-19 cell line upon culturing on poly-(2-hydroxyethylmethacrylate) (poly-HEMA) covered culture dishes for 24h to block cell attachment.

1.5.2. Autophagy and autophagy-associated cell death

Autophagy is an evolutionary conserved mechanism in eukaryotic cells for the lysosomal degradation of cytosolic constituents and the recycling of breakdown products. In mammals, three different types of autophagy have been described: macroautophagy, chaperon-mediated autophagy and microautophagy. The main topic here will be the macroautophagy pathway (referred further hereby as autophagy).

The mammalian target of rapamycin (mTOR) negatively regulates the autophagy process. Under nutrient deprivation or rapamycin treatment, the mTORC1 is

inactivated, the phosphorylation status of ULK1 and Atg13 is changed, which finally leads to the enhancement of ULK1 kinase activity and triggering of autophagy. During autophagy, the next step is formation of isolation membrane called phagophore, which expands and encloses its cargo to form double-membraned vesicles termed autophagosomes. The Atg12-Atg5-Atg16 complex and the microtubule-associated protein 1 light chain 3 (LC3) conjugation system are required for the expansion of the phagophore and the maturation of the autophagosomes. The cytosolic form of LC3, named LC3-I, covalently conjugates to the lipid phosphatidyl-ethanolamine (PE), which leads to LC3-II form with an insertion into the phagophore membrane. p62, also known as sequestosome 1 (SQSTM1) protein, functions as a link between LC3 and ubiquitilated proteins and facilitates the degradation of ubiquitilated substrates. Finally, the outer membrane of the autophagosome fuses with primary lysosomes, forming autophagolysosomes where their sequestered content gets degraded. After degradation, the resulting molecules can be reused to synthesize proteins.

A low basal rate of autophagy occurs under normal physiological conditions in most eukaryotic cells and can be induced by mode of starvation, endoplasmic reticulum stress inducers (brefeldin A) and rapamycin. To date, many chemical inhibitors of autophagy have been described, such as 3-methyladenine (3-MA), wortmannin, bafilomycin A1, chloroquine (CQ), NH4Cl or pepstatin A. The most commonly used methods to monitor activation of autophagy is the detection of conversion from endogenous LC3-I to LC3-II by immunoblotting with antibodies against LC3. Autophagic flux indicates the whole dynamic process of autophagy. It can be monitored based on the turnover of LC3-II using Western blot analysis in the presence and absence of an autophagy inhibitor, such as CQ.

If autophagy occurs at excessive levels, it can promote cell death. Inconsistencies can be found in the literature about the correlation between the cell death and the autophagy. In our study the investigated cell death type was classified into the autophagy-associated cell death based on the literature category similarity.

1.6. Inflammation in AMD pathogenesis

The retina is considered to be an immune privileged tissue. Multiple factors and mechanisms are responsible for maintaining the immune privilege, such as the presence of BRB and the absence of lymphatic drainage, the inhibitory ocular microenvironment

with cell-bound and soluble immunosuppressive factors, as well as the regulation of systemic immune responses within the eye.

The wet type of AMD can be characterized by breakdown of BRB, which results in choroidal neovascularization (CNV), infiltration of macrophages from the peripheral circulation, accumulation of immune cells and highly immunogenic environment. Nowadays, an increasing number of studies focus on identification of reliable biomarkers of AMD. These selective markers would help in the diagnosis of early AMD. To date, there is no accepted, specific marker that meets the requirements of initial AMD detection.

1.7. Clearance of dying cells by professional and non-professional phagocytes

In human adults, billions of cells die each day in the body. The rapid elimination of these dying cells is essential for maintaining tissue homeostasis and innate immune balance. The removal of apoptotic cells can be generally divided into four major steps: 1) Release of soluble chemoattractants, so called "find-me" signals by dying cells, such as ATP, UTP, lysophosphatidylcholine, to recruit phagocytes to the site of death; 2) Specific recognition of "eat-me" signals, such as phosphatidylserine (PS), on the dying cell surface by engulfment receptors on the phagocytes. During apoptosis PS rapidly gets exposed on the outer leaflet of the plasma membrane. The members of the TAM family of receptor tyrosine-kinases, such as Tyro3, Axl, Mer, cannot engage PS on apoptotic cells directly, but through use of bridging molecules that bind PS on apoptotic cells. Gas6 is PS binding bridging molecule, which contributes to the enhancement of the clearance of apoptotic cells; 3) Engulfment of cellular corpses by phagocytes. The reorganization of actin filaments, the formation of phagocytic cup around the dying cell corpses and subsequent internalization, as well as the series of phagosome maturation steps are required for the uptake of apoptotic cells. The phagosomal lumen becomes increasingly acidic, leading to the fusion with an acidic lysosome structure and efficient degradation of dying cells; 4) Immune response to the engulfed corpses by phagocytes. The phagocytosis of apoptotic cells is an immunologically silent process.

In the wet type of AMD, the proliferation of new blood vessels and their penetration through the Bruch's membrane contribute to the infiltration of macrophages and dendritic cells (DCs) into the subretinal space, and these professional phagocytes are able to eliminate the apoptotic cells from there. However, in the dry form of AMD, the BRB is intact, therefore the professional phagocytes cannot reach the dying cells, and in

that case the non-professional phagocytes are responsible for the uptake of dying RPE cells.

1.8. Treatment of AMD

There is currently no approved treatment for dry type of AMD. However, the beneficial effect of antioxidants and omega-fatty acids on the pathogenesis of this disease has been demonstrated. The dietary supplement-based treatment, including antioxidant vitamin C and E, trace element zinc and carotenoids, lutein and zeaxanthin, as well as omega-3 fatty acids, has been associated with the protection of the retinal tissues and the prevention or the delay of progression to the late wet form of AMD.

In case of wet type of AMD, laser photocoagulation or photodynamic therapy (PDT), may help prevent the further proliferation of CNV. Furthermore, the intravitreal injection of anti- VEGF agents is another way to block the growth of new blood vessels. Triamcinolone (TC) is a conventional corticosteroid (CS), which has been used as an intravitreal injection for the wet form of AMD. Combined therapies are currently used in the ophthalmology practice, to increase the efficiency of primary treatments. On the other hand, tissue engineering strategies have been developed based on the transplantation of scaffolds, which mimic the Bruch's membrane and serve for cell engraftment. Recent studies demonstrate that several miRNAs play a crucial role in the regulation of AMD-associated processes, suggesting that miRNAs may represent novel therapeutic targets for AMD. Moreover, the cell-based transplantation therapy focuses on the replacement or regenerating of the damaged RPE cell layer *in vivo*, using retinal and iris pigment epithelium cells as well as stem cells.

2. AIMS OF THE STUDIES

- To induce autophagy-associated cell death in ARPE-19, hRPE and hESC-RPE cells by serum deprivation and oxidative stress using hydrogen-peroxide (H_2O_2).

- To establish an *in vitro* detection model for studying autophagy in H_2O_2-treated ARPE-19, hRPE and hESC-RPE cells.

- To study and quantify the clearance of anoikic and autophagy-associated dying RPE cells by non-professional phagocytes, macrophages or dendritic cells using FACS analysis as a model for AMD pathogenesis.

- To examine the effect of TC-treatment on the phagocytic capacity of professional and non-professional phagocytes during the engulfment of autophagy-associated dying RPEs.

- To investigate the role of MerTK receptor in the TC-enhanced phagocytosis of anoikic dying RPE cell by professional and non-professional phagocytes.

- To determine the released pro-inflammatory cytokines during phagocytosis of anoikic and autophagy-associated dying RPE cells by macrophages.

3. MATERIALS AND METHODS

3.1. Ethics statements

All protocols for the human cell isolation experiments complied with the Guidelines of the Declaration of Helsinki and was approved by the National Medical Ethics Committee of the University of Debrecen, Hungary (DEOEC RKEB/IKEB Prot. No. 2745 -2008 and 3093 - 2010) and the National Medical Ethics Committee of Kuopio University Hospital, Finland (42/2014), the National Authority for Medicolegal Affairs Finland (Dnro 1426/32/300/05) and the Ethics Committee of Pirkanmaa Hospital District, Finland (Skottman/R05116).

3.2. Cell culturing

ARPE-19 cell line was kindly provided by Prof. Stephen Moss, (UCL, London, UK). It was cultured in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Gibco), 200 mM L-glutamine (Sigma-Aldrich) and 1% Antibiotic/antimycotic solution (HyClone) at 37^{o}C, 5% $CO_{2.}$ ARPE-19 cells were used up to passages 10-15 for all experiments. The primary hRPE cells were obtained from adult cadaver human eyes (age range: 64-92 yrs) without any known ocular diseases. The hRPE cells were isolated as described by Johnen et. al and cultured in DMEM Nutrient mixture F12 medium (Sigma-Aldrich) supplemented with 10% FCS (Gibco), 200 mM L-glutamine (Sigma-Aldrich) and 1% Antibiotic/antimycotic solution (HyClone). All experiments were performed up to passage 2-5 in hRPE cells. The RPE differentiation and the enrichment process from hESCs was performed as previously described. The confluent hESC-RPE cells were seeded onto collagen IV-coated (Sigma-Aldrich) well plates in RPE basic medium.

Human monocytes from ‘buffy coats’ of healthy blood donors were isolated by density gradient centrifugation using Ficoll–Paque Plus (Amersham Biosciences), then CD14 human MicroBeads (Miltenyi Biotec) were used for magnetic separation. Human macrophages were obtained through a 5-day differentiation using 5 ng/ml M-CSF (Pepro-Tech) at 37°C in IMDM (Gibco) supplemented with 10% human AB serum (Sigma-Aldrich) and 10000 U/mL penicillin- 10 mg/mL streptomycin in a 5 % CO_2 atmosphere (Sigma-Aldrich). To generate iDCs, freshly isolated monocytes were seeded into 6-well plates at a density of $2x10^6$ cells/mL and cultured for 5 days in serum-free AIM V medium (Gibco) containing 80 ng/ml GMCSF (Pepro-Tech) and 100 ng/ml IL-4 (Pepro-Tech). Medium was supplemented with the same amounts of GMCSF and IL-4

on day 0 and 3. Inflammatory cytokine mixture was used for the activation of resting DCs on day 5, which containing 10 ng/ml TNF-α (Pepro-Tech), 5 ng/ml IL-1β (Pepro-Tech), 20 ng/ml IL-6 (Pepro-Tech), 75 ng/ml GMCSF (Pepro-Tech), and 1 mg/ml PGE_2 (Sigma-Aldrich) and the cells were harvested on Day 6.

3.3. Induction of anoikic and autophagy-associated cell death

ARPE-19, primary hRPE or hESC-RPE cells were plated on poly-HEMA (Sigma-Aldrich) coated culture dishes for 24h to induce anokic cell death through ECM detachment. For triggering of autophagy-associated cell death, ARPE-19 and hRPE cells were plated over a 24h period and cultured until they formed confluent (80-90%) monolayers, then co-treated by serum deprivation and 0.4-1 mM H_2O_2 (Sigma-Aldrich) for 2-4h before harvesting. In the case of hESC-RPE cells, the autophagy-associated cell death was induced by serum deprivation and 2h of 1mM H_2O_2 co-treatment.

3.4. Assay for cell death analysis

Anoikic and autophagic-associated cell death in ARPE-19, primary hRPE and hESC-RPE cells was determined by the Annexin-V-FITC Apoptosis Detection Kit (MBL) according to the manufacturer's recommendations. The percentage of Annexin-V (AnxV) and/or Propidium Iodide (PI) positive cells was determined by BD FACS Calibur or BD Accuri C6 flow cytometer (BD Biosciences) and data was analyzed using WinMDI 2.8. Software.

3.5. Antibodies and immunoblotting

Cells were collected and washed with PBS, homogenized either in ice-cold lysis buffer containing 50 mM Tris–HCl; 0.1% Triton X-100 (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich), 15 mM 2-mercaptoethanol (2-MEA) (Sigma-Aldrich) and protease inhibitor (Sigma-Aldrich) or in M-PER cell lysis reagent (Thermo Scientific). Insoluble cellular material was removed by centrifugation and the protein concentration was determined by using Bradford reagent (Sigma-Aldrich), and then the lysates were mixed with 5x Laemmli loading buffer, boiled for 10min. Equal amounts of protein (20 μg) were separated on SDS- polyacrylamide gel. Proteins were transferred onto a PVDF membrane (Merck-Millipore) and followed by blocking with 5% skimmed milk (AppliChem) for 1h. Then the membranes were probed overnight at 4ºC by anti–LC3 (1:2000) rat polyclonal antibody (Novus Biologicals), anti–p62 (1:2000) mouse

monoclonal antibody (Santa Cruz Biotechnology) or anti-MerTk (1:1000) mouse monoclonal antibody (R&D Systems), followed by incubation with HRP-conjugated species-corresponding secondary antibodies (Sigma-Aldrich) for 1h at room temperature. For loading control, mouse monoclonal antibodies against GAPDH (1:5000) (Covalab), tubulin (1:5000) (Sigma-Aldrich) or β-actin (1:5000) (Sigma-Aldrich) were used overnight at 4°C. Immunoreactive products were visualized using Immobilon Western chemiluminescent substrate (Merck-Millipore). Densitometry analysis of Western blots was performed using Image J software.

3.6. Electron microscopy

For transmission electron microscopy (TEM) analysis, H_2O_2-treated (1mM, 2h) ARPE-19 cells were fixed in 0.1 M sodium cacodylate-buffered, pH 7.4 and 2.5% glutaraldehyde solution for 2h and then rinsed (3 times, 10min) in 0.1 M sodium cacodylate buffer, pH 7.4 and 7.5% saccharose and followed by post-fixation with 1% OsO_4 solution for 1h. Then the samples were dehydrated through an ethanol gradient (70% ethanol (20min), 96% ethanol (20min), 100% ethanol (2 times, 20min), and embedded in Durcupan ACM. To stain ultrathin sections, uranyl acetate and lead citrate were used. Sections were examined in a Philips CM-10 TEM (Philips Electronic Instruments) at 80 kV.

3.7. Quantification of LC3 positive cells

The GFP-LC3 expression plasmid was kindly provided by Prof. Noboru Mizushima (Tokyo Medical and Dental University). ARPE-19 cells were seeded on glass coverslips and the confluent cells were transiently transfected with the GFP-LC3 expression plasmid using PEI reagent (Sigma-Aldrich) (PEI:DNA ratio=4:1, 1μg DNA/well). Transfected ARPE-19 cells were pre-treated with CQ (25 μM, 1h) (Sigma-Aldrich) and then incubated with H_2O_2 (1mM, 2h) in serum-free condition. After treatments, cells were fixed with 4% PFA (Sigma-Aldrich) for 10min and stained with DAPI (0.3 μg/ml) (Sigma-Aldrich) to visualize cell nuclei. Fluorescent images were taken by Axiovert-200 Zeiss microscope (Carl Zeiss MicroImaging GmbH). The number of GFP-LC3 positive cells were counted manually based on the fluorescent images. In parallel, the percentage of GFP-LC3 positive cells was quantified by flow cytometry using a BD FACS Aria III (BD Biosciences). Moreover, the GFP-LC3-positive, AV-containing and the GFP-LC3 negative ARPE-19 cells were sorted out on the basis of

their GFP fluorescence using a BD FACS Aria III. Data acquisition and analysis were performed using BD FACS Diva 6.2 software.

To detect AVs and cell death simultaneously, ARPE-19 cells were transiently transfected with mCherry-LC3 plasmid, which was kindly provided by Dr. Gian Maria Fimia (University of Salento), treated with H_2O_2 (1mM, 2h) and then Annexin V-FITC labelling was used. Cells containing LC3-positive AVs and/or cells exposing PS on their surface were quantified using a BD FACS Calibur flow cytometer.

3.8. Phagocytosis assay

Pre-treatment of phagocytes with 1μM TC (Sigma-Aldrich) was performed 48h prior to the phagocytosis assay. Living RPE cells acting as phagocytes were plated in serum-free medium 24h before phagocytosis. The phagocytes were stained with 7.5μM CMTMR (Molecular Probes) for 16h. Anoikic and autophagy-associated dying RPE cells were stained for 2h with 12.5mM CFDA (Molecular Probes). Phagocytes were co-cultured with anoikic or autophagy-associated dying RPE cells for 4, 8, 12 or 24h at a ratio of 1:3, 1:2 or 1:5 at 37°C, 5% CO_2 atmosphere in IMDM medium the absence of human 10% AB serum. After co-culture, the phagocytic cell mixture was collected by trypsinization, centrifuged, washed twice with PBS and fixed with 1% PFA. The phagocytosis rate was determined by FACS Calibur BD flow cytometer and WinMDI 2.8. Software was used for data analysis.

To investigate the impact of 3-MA (10mM, 24h) treatment on phagocytosis, pre-treatment with 3-MA and with H_2O_2 (1mM, 2h) was carried out on ARPE-19 cells, which were then co-incubated with macrophages. In addition, the GFP-LC3-positive or the negative sorted ARPE-19 cells were resuspended in IMDM medium and added to the macrophages, to analyse the rate of phagocytosis. For the blocking experiments, macrophages were pre-incubated with 10 μg/mL anti-MerTk antibody (R&D systems) for 10min before co-incubation with dying cells. For testing the effect of Gas6 on phagocytosis, macrophages were treated with 200ng/mL Gas6 (R&D systems) for 24h before adding the dying cells.

3.9. Time-lapse imaging microscopy

For *in vitro* phagocytosis assay, the macrophages were seeded on 24 well cell culture plates, stained with CMTMR (7.5μM, 16 h) and co-incubated with the CFDA-stained (12.5mM, 2h) or GFP-LC3 transfected dying RPE cells in a ratio of 1:2. For the time-

lapse microscopy of the co-cultures, an incubation chamber system (Solent Scientific) attached to a motorized Olympus IX-81 inverted microscope (Olympus Europa Holding) equipped with a cooled high-speed Hamamatsu ORCA-R2 camera (Hamamatsu Photonics) was used. Cells were monitored for 24h and, in each 5min, an image was taken per channel and per well automatically. To generate time-lapse videos from the images, the XCE-RT xCellence Real Time software (Olympus) was used.

3.10. siRNAs and electroporation of macrophages

siRNA constructs were obtained from Ambion (Life Technologies), targeting adenosine A3 receptor (ADORA3) [3′-CGUCUAUGCCUAUAAAAUAtt (sense) and 5′-UAUUUUAUAGGCAUAGACGtt (antisense)], AXL receptor tyrosine kinase [3′-GGAACUGCAUGCUGAAUGAtt (sense) and 5′-UCAUUCAGCAUGCAGUUCCtg (antisense)], c-mer proto-oncogene tyrosine kinase (MERTK) [3′-GAACUUACCUUACAUAGCUtt (sense) and 5′-AGCUAUGUAAGGUAAGUUCaa (antisense)] and thrombospondin-1 (THBS1) [3′-GGACUGCGUUGGUGAUGUAtt (sense) and 5′- UACAUCACCAACGCAGUCCtt (antisense)]. Living ARPE-19 cells and macrophages, three days after isolation, were harvested from the 24 well plates and washed once with DMEM or IMDM accordingly and once with PBS. The cells were resuspended in OptiMEM without phenol red (Invitrogen Life Technologies) at a concentration of 4×107/mL. siRNA was transferred to a cuvette (3 μM). A volume of 100μl of cell suspension was added and incubated for 3min before being pulsed in a Genepulser Xcell (Bio-Rad). Pulse conditions were square-wave pulse, 500V, 0.5ms for macrophages and 250V, 10ms for non-dying ARPE-19 cells. Immediately after electroporation, the cells were transferred to human AB serum supplemented IMDM with M-CSF and TC or FBS supplemented DMEM, accordingly.

3.11. Immunofluorescent staining and confocal microscopy

Macrophages were seeded on glass coverslips and cultured as previously described. After co-culturing of the macrophages with the anoikic dying cells for 4h, the cells were washed and incubated with anti-MerTk primary antibody (R&D systems) for 1h on ice. As secondary antibody, anti-mouse NL-493 (R&D systems) was used before being washed and fixed in 4% PFA for 10min on ice. Confocal fluorescent images were taken by a Zeiss LSM 510 or Olympus FV1000 confocal laser scanning microscope. Images were processed and protein accumulation was analyzed by Fiji software.

3.12. Quantification of IL-6 and IL-8 release by ELISA

The released pro-inflammatory cytokines by the macrophages was investigated during phagocytosis of anoikic or autophagy associated dying RPE cells. The concentration of released IL-6 (pg/ml) and IL-8 (pg/ml) cytokines was measured from the collected cell culture media using Human IL-6 ELISA OptEIA^{TM} and Human IL-8 ELISA OptEIA^{TM} kits (BD Biosciences) according to the manufacturer's specifications.

3.13. Statistical analysis

All the data are representative of at least 3 independent experiments and each sample was tested in triplicate. Results are expressed as the mean ± SD or mean ± SEM. For multiple comparisons of groups, statistical significance was calculated and evaluated by one-way ANOVA followed by Tukey post-hoc test. Statistically significant difference between the two groups was determined with paired Student's t-test (two tailed) and values of $p<0.05$ were considered significant.

4. RESULTS

4.1. ARPE-19 and primary hRPE cells die in a time- and concentration-dependent manner due to serum deprivation and H_2O_2 co-treatment

Time- and concentration-dependent cell death was observed with a mixture of viable, early or late apoptotic and necrotic cell populations upon increasing time intervals (2h, 4h) and concentrations (0.4mM, 0.8mM, 1mM) of H_2O_2 co-treatment in ARPE-19 and hRPE cells in the absence of serum by Annexin V-FITC/PI double staining. We could determine that most of the cells die in parallel with PS externalization, especially after 2h, 1mM H_2O_2 treatment. The ratio of viable ARPE-19 cells significantly decreased from 91.4±1.7% to 28.6±14.2% and, parallely, the percentage of only $AnxV^+$ ARPE-19 cells significantly increased from 2.1±2% to 41±10.8% upon 2h of 1mM H_2O_2 treatment, compared to the untreated control ones. In the case of primary hRPE cells, similar trend was observed: the percentage of viable cells also changed from 87.1±4.9% to 51.6±3.6% as a result of 2h 1mM H_2O_2 treatment, and 17.7±12.7% of the hRPE cells became $AnxV^+$, while the untreated control contained only 3.2±2.8% of these early apoptotic cells. Sodium-azide administration (4h, 1mM) was used as positive control for the induction of necrotic cell death. This treatment mostly resulted in PI single positive cells, e.g. 67.92±3.32% in ARPE-19 and 42.59±12.80% in hRPE cells, the pattern markedly being different compared to that in H_2O_2 administration, in which case only (10-30%) of the cells were PI positive. These results suggest that most of the cells die with PS externalization. Altogether, the H_2O_2 administration causes RPE cells to undergo a mixture of different cell death modalities over the tested time.

4.2. The impact of serum deprivation and H_2O_2 co-treatment on the morphology and cell viability of hESC-RPE cells

The typical RPE cell morphology, such as the high degree of pigmentation and the cobblestone morphology have been observed in untreated hESC-RPE cells by phase-contrast microscopy, but the serum deprivation (2h) led to the disruption of the monolayer structure. Swelling and detachment of hESC-RPE cells were detected after H_2O_2 treatment (2h, 1mM) in the presence of serum. In addition, H_2O_2 (2h, 1mM) and serum deprivation co-treatment further enhanced the amount of detached, dead cell aggregates. The percentage of viable cells significantly decreased from 88.35±0.88% to

55.22±13.12%, as a result of serum deprivation and H_2O_2 co-treatment in hESC-RPE cells, compared to the untreated controls. Simultaneously, we detected a significantly increased rate of AnxV single positive hESC-RPE cells upon co-treatment, compared to the untreated controls: an increase from 9.85 ±1.13% to 39.86±11.73% was observed. The response to the co-treatment was similar in hESC-RPE and ARPE-19 cells, approximately 40% of single positive AnxV or PS externalizing cells were observed in both cases.

4.3. ECM-detachment induced anoikis cell death in hESC-RPE cells

We used the protocol of Petrovski et al. for anoikis induction in hESC-RPE cells. The representative phase contrast image of untreated hESC-RPE cells shows a confluent, highly pigmented cobblestone monolayer. In contrast, we determined floating aggregate formations in anoikic hESC-RPE cells. ECM detachment resulted in significantly decreased proportion of viable hESC-RPE cells, compared to the control; it changed from 88.35±0.88% to 64.36±1.84%. Simultaneously, we determined significantly increased rate of only AnxV single positive cells in anoikis-induced hESC-RPE (27.86±3.58%) than in the case of untreated controls (9.85±1.13%). Moreover, the anoikic cells contained 7.10±3.30% of double positive, late apoptotic cells; in contrast, only 1.14±0.11% of the control cells were double positive.

4.4. Time- and concentration-dependent induction of autophagy in ARPE-19 and hRPE cells

Increasing LC3-II/LC3-I ratio was found in a time-and concentration-dependent manner in ARPE-19 and hRPE cells as a result of increasing concentrations (0.4mM, 0.8mM, 1mM) and time intervals (2h, 4h) of H_2O_2 exposure in the absence of serum, and compared to the untreated controls by Western blot analysis. In hRPE cells, the highest autophagic activity was observed at 2h of 1mM H_2O_2 treatment in serum-free condition, and in the case of ARPE-19 cells also an increased conversion of LC3-I into LC3-II was detected under the same treatment. Parallelly, we monitored the expression level of SQSTM1/p62. Decreasing trend of p62 expression was detected in response to serum deprivation and H_2O_2 co-treatment in ARPE-19 cells, compared to the untreated controls. Moreover, the double-membraned autophagic vacuoles (AVs) with cytosolic components could be determined as a result of H_2O_2 treatment (2h, 1mM) by TEM.

Taken together, these data suggest that 1mM H_2O_2 treatment for 2h can lead to a significant induction of autophagy in ARPE-19 and hRPE cells.

4.5. Serum deprivation and H_2O_2 co-treatment result in induced autophagy in hESC-RPE cells

We investigated the autophagic activity as a result of serum deprivation and H_2O_2 (2h, 1mM) co-treatment in hESC-RPE cells. In the case of untreated control cells, basal autophagic activity was observed, which was increased as a result of serum deprivation over 2h and H_2O_2 (2h, 1mM) treatment in the presence of serum, separately. The LC3-II/LC3-I ratio was further enhanced in response to H_2O_2 (2h, 1mM) treatment in the absence of serum. These results suggest the autophagy process can be upregulated by serum deprivation and H_2O_2 (2h, 1mM) co-treatment in hESC-RPE cells.

4.6. Increased autophagic flux in ARPE-19 and hRPE cells

In ARPE-19 cells, we could determine larger difference in the ratio of LC3-II/LC3-I in the absence and presence of CQ (0,5h, 12.5μM) upon H_2O_2 treatment (2h, 1mM), both in serum-free or in serum-containing condition, which indicates that autophagic flux was increased during H_2O_2-exposure. In case of hRPE cells, CQ pre-treatment also led to the accumulation of LC3-II, the ratio of LC3-II/LC3-I was significantly increased in the presence of serum, which confirmed the increased autophagic flux.

4.7. Induction of autophagy-associated cell death in RPE cells

Due to serum-deprivation and H_2O_2 (2h, 1mM) co-treatment, accumulation of perinuclear GFP-LC3 positive aggregates or ring-shaped AVs were observed. The CQ-pre-treated (1h, 25μM) cells and then H_2O_2-exposed (2h, 1mM) cells showed more intense fluorescent punctuates and bigger GFP-LC3 positive AVs; 40.9±8.4% of the cells contained GFP-LC3-positive vacuoles upon H_2O_2 (2h, 1mM) treatment in serum-free medium, when counting manually the fluorescent images. Parallelly, 23.9±1.2% of the cells were quantified as GFP-LC3-positive, based on their fluorescence using flow cytometry analysis. These data also indicate that autophagy process can be triggered in ARPE-19 cells by H_2O_2-treatment in serum-deprived condition.

We investigated the LC3-positive AVs and PS-externalization simultaneously in H_2O_2-treated (2h, 1mM), mCherry-LC3 transiently transfected ARPE-19 cells. We found that 15.02% of the untreated cells were $LC3^+$ by FACS analysis, and it was increased to

28.76% as a result of H_2O_2 treatment; meanwhile, the percentage of $AnxV^+$ cells increased from 3.4% to 17.5%. We observed 52.3% of $LC3^+$ cells were $AnxV^+$; meanwhile 83.88% of $AnxV^+$ cells were $LC3^+$ as well. These findings suggest that autophagy-associated processes are being activated in most of the dying ARPE-19 cells upon H_2O_2 treatment.

4.8. Anoikic dying RPE cells are efficiently phagocytosed by macrophages and non-dying RPE cells

We found that living RPE cells and macrophages were able to efficiently and increasingly engulf the anoikic dying RPE cells during the 24h period. The average of phagocytosis percentage of anoikic dying ARPE-19 cells by non-professional living ARPE-19 cells was 3.16±0.91% at 4h of co-incubation. We observed higher phagocytosis rate during the engulfment of anoikic dying ARPE-19 cells by macrophages after 4h of co-incubation (5.07±0.27%). The primary hRPE cells dying through anoikis were taken up by macrophages even more efficiently, the phagocytic capacity being 12.56±2.38% after 4h of co-incubation. These results show that TC treatment further enhanced the clearance process under most conditions.

4.9. Macrophages, and non-dying RPE cells are able to efficiently engulf autophagy-associated dying RPE cells

In our study, ARPE-19 cells and macrophages could engulf autophagy-associated dying RPE cells with increasing capacity over 24h. ARPE-19 cells took up the autophagy-associated dying ARPE-19 cells in an efficient manner, the average phagocytosis percentage being 3.8±1.1% at 8h of co-incubation. We found similar phagocytosis rate during the engulfment of autophagy-associated dying ARPE-19 cells by macrophages after 8h of co- incubation (e.g. 6.9±1.7%). The autophagy-associated dying primary hRPE cells were removed by macrophages even more efficiently, the phagocytic capacity being 21.2±3.3% after 8h of co-culturing. TC treatment of the phagocytes 48h prior the phagocytic assay, enhanced the engulfment capacity under most conditions.

4.10. The inhibitory effect of 3-MA on autophagy-associated cell death of ARPE-19 cells and subsequent impact on phagocytosis

To confirm the inhibition of the autophagy process as a result of 3-MA (24h, 10mM) in H_2O_2-treated (2h, 1mM) ARPE-19 cells, we determined the partially blocked

conversion of LC3-I to LC3-II using Western blot analysis. Then we found that the number of living cells was significantly increased, while the number of $AnxV^+$ and PI^+ as well as the double positive H_2O_2-treated ARPE-19 cells were significantly decreased upon treatment with the inhibitor. Therefore, 3-MA pre-treatment inhibited the autophagy-associated cell death of ARPE-19 cells. The inhibition of autophagy in ARPE-19 cells by 3-MA led to a significant change in the phagocytosis rate of autophagy-associated dying cells - a significant decrease from 8.45±0.56% to 3.24±0.52% was observed by the untreated macrophages, and from 10.49±1.01% to 5.90±2.89% by the TC-treated macrophages after 8h co-incubation.

4.11. The clearance of GFP-LC3 positive autophagy-associated dying ARPE-19 cells by macrophages

To study whether the phagocytosed cells were actually the autophagy-associated dying cells and not any other type of dying cells, the GFP-LC3 transfected, H_2O_2-treated ARPE-19 cells were co-cultured with macrophages for 8h and the phagocytic rate was quantified by flow cytometry. The macrophages were able to engulf the H_2O_2-treated, GFP-LC3 transfected ARPE-19 cells, the average of phagocytosis percentage being 9.3±3.7%, which was comparable to the clearance of vitally stained, H_2O_2-treated ARPE-19 cells by macrophages. Furthermore, the macrophages were co-cultured with the GFP-LC3 positive and negative ARPE-19 cells for 8h, which were sorted out by FACS Aria III. We observed that, negligible amount of GFP-LC3 negative, non-autophagic ARPE-19 cells were engulfed (0.6±0.2%). In contrast, the phagocytic rate of the GFP-LC3 positive RPEs was kept the same (13.7±3.3%) as in the case of non-sorted autophagy-associated dying cells, therefore we could prove their fate in the engulfment process. Moreover, significantly enhanced clearance of GFP-LC3 transfected, H_2O_2-treated ARPE-19 cells was found as a result of TC pre-treatment.

4.12. The engulfment of anokic and autophagy-associated dying hESC-RPE cells by macrophages

The macrophages efficiently engulfed the anoikic dying hESC-RPE cells: the phagocytosis rate was 32.40±3.45% after 4h co-culturing. On the other hand, the uptake of autophagy-associated dying hESC-RPEs by macrophages after 8h of co-incubation, was more efficient: 50.72±2.98%. TC treatment resulted in moderately enhanced

phagocytic capacity of macrophages during the engulfment of anoikic dying hESC-RPE cells (35.15±3.85%) and autophagy-associated dying hESC-RPEs (58.70±8.68%).

4.13. DCs are able to efficiently take up the dying cells *in vitro*

We found a low engulfment rate of anoikic dying ARPE-19 cells by the immature (3.26±0.58%) as well as the mature DCs (2.88±1.14%), after 4h co-culturing. In contrast, the phagocytic capacity of DCs during the engulfment of autophagy-associated dying ARPE-19 cells was much higher, it was 26.7±10.8% in the case of iDCs after 8h of co-incubation and 21.4±6.4% when a cocktail of inflammatory cytokines was used to stimulate DC maturation.

4.14. The impact of knocking-down of TC upregulated genes on phagocytosis

We could determine significantly altered expression level of 55 out of the analysed 95 genes upon TC treatment during the phagocytosis of anoikic ARPE-19 cells as compared to TC-treated macrophages in the absence of dying cells using a TaqMan Low Density Array. Next, we intended to show whether TC-induced upregulation of apopto-phagocytic genes had a direct impact on the enhanced phagocytic capacity of macrophages. siRNA transfection method was used to silence a selection of the upregulated genes: ADORA3, THBS1, AXL, MERTK or both AXL and MERTK simultaneously. We observed the silencing efficiency of the target genes show biological variance among different macrophage donors and varied among replicates of living ARPE-19 cells. The members of the TAM family represented the weakest silencing effect in macrophages and significantly decreased phagocytosis was determined only in the TC-treated MERTK and AXL knock-down macrophages. The combination of MERTK silencing with siRNAs for AXL did not show a significant synergistic effect. A significant decrease in non-professional engulfment of anoikic ARPE-19 cells was found as a result of silencing of MERTK and AXL in living TC-treated ARPE-19 cells.

4.15. MerTk receptor has a pivotal role in the TC enhanced clearance of anoikic dying ARPE-19 cells by macrophages

We observed that the phagocytic capacity of macrophages in the absence of TC could not be inhibited as a result of MerTK blocking antibody, meanwhile, the TC-enhanced engulfment of anoikic dying ARPE-19 by macrophages could be prevented using this

blocking antibody. The distribution of the MerTk receptor at the engulfment portals of macrophages during the uptake of anoikic dying ARPE-19 cells in the presence or absence of TC was determined using immunofluorescent staining. We could detect MerTK in punctuated structures at the cell borders of macrophages and anoikic ARPE-19 cells, which was significantly accumulated at the connecting points between dying ARPE-19 cells and TC treated macrophages. Moreover, the MerTk protein expression was evaluated by Western blot analysis; both untreated cell types appeared to have lower expression levels, while TC treatment resulted in much more elevated expression of MerTk in macrophages even during the engulfment of anoikic dying ARPE-19 cells.

4.16. The effect of MerTk blocking on the TC-enhanced clearance of dying RPE cells by non-professional phagocytes

MerTk blocking antibody could not inhibit the phagocytic capacity of living ARPE-19 cells in the absence of TC. Contrarily, the TC-enhanced phagocytosis rate could be significantly decreased as a result of treatment with this blocking antibody either on the side of the phagocytes or the anoikic ARPE-19 cells. We could also determine MerTk in punctuated structures at the cell borders of non-professional phagocytes and anoikic dying ARPE-19 cells by immunofluorescent staining, which was significantly accumulated at the engulfment portals between TC-treated phagocytes and dying cells. Furthermore, the MerTk protein expression was investigated by Western blot analysis: lower expression was detected in TC untreated cells, and much more elevated expression during the engulfment process even as a result of TC treatment of the phagocytosing ARPE-19 cells.

4.17. The effect of blocking and augmentation of MerTk receptor on the phagocytosis of dying primary hRPE cells by macrophages

TC treatment (48h, 1μM) significantly enhanced the phagocytic capacity of macrophages during the clearance of dying hRPE cells, about 1.5-2 times increase of phagocytic rate was observed at 4h of co-incubation. Significantly reduced engulfment of dying hRPE cells was determined by TC-treated macrophages upon administration of MerTk blocking antibody. Finally, we examined whether the augmentation of MerTk by its ligand, the bridging molecule Gas6, can affect the phagocytosis of anoikic ARPE-19 and hRPE cells. In the absence of TC, the phagocytic capacity of macrophages moderately increased upon Gas6 treatment; parallelly, a remarkable clearance

enhancing effect was found in a concentration-dependent manner in the case of TC-treated macrophages in which MerTk was significantly overexpressed both at mRNA and protein level.

4.18. Release of IL-6 and IL-8 during the clearance of autophagy-associated dying RPE cells by professional phagocytes

We observed a negligible amount of IL-6 and IL-8 secretion by TC-treated and untreated MΦs. Autophagy-associated dying ARPE-19 and hRPE cells themselves released significantly higher amount of IL-8 compared to the amount of secreted IL-6. Co-culturing of macrophages with the autophagy-associated dying ARPE-19 or hRPE cells resulted in the increased concentration of IL-6 and IL-8. Larger amount of IL-8 was detected upon macrophage-dying cell co-incubation as compared to the secreted amount of IL-6. We determined the decreased production of IL-6 and IL-8 cytokines during the phagocytosis of ARPE-19 and hRPE cells by TC-treated macrophages. These data indicate that the clearance of autophagy-associated dying RPE cells by macrophages leads to a pro-inflammatory response *in vitro*.

4.19. The engulfment of anoikic and autophagy-associated dying hESC-RPE cells by macrophages led to the release of IL-6 and IL-8

We detected that the macrophages by themselves did not produce IL-6. The phagocytosis of anoikic hESC-RPE cells by macrophages led to a significantly increased IL-6 secretion (836.33±252.27 pg/ml), which decreased as a result of TC treatment (780.87± 279.18 pg/ml). In the case of autophagy-associated dying cells' uptake, significantly lower levels of IL-6 release (324.37±67.43 pg/ml) were observed compared to the clearance of anoikic dying cells. We found low amount of IL-8 secretion by TC-treated (120.92±1.90 pg/ml) and untreated (84.40±2.48 pg/ml) macrophages by themselves. Interestingly, macrophages responded to the uptake of anoikic cells with increased IL-8 production (1057.33±416.56 pg/ml), which significantly decreased upon TC-treatment (892.11±442.08 pg/ml). Lower amount of IL-8 was released during the engulfment of autophagy-associated dying cells (318.13±67.99 pg/ml) as compared to anoikic ones.

5. DISCUSSION

AMD is a complex, neurodegenerative disease; its prevalence is increasing worldwide, which will likely make this disease a significant global public health problem. Dry AMD is the most common form of the disease; no specific cure for it exists so far. In the wet form of AMD, detrimental new blood vessels grow through the Bruch's membrane into the subretinal pigment epithelial space, where they can cause membrane disruption and blood or fluid leakage. To date, only some treatment options are available for wet AMD. Therefore, the establishment of novel models for studying the pathogenesis of AMD, which can help to generate new therapeutic approaches are much needed.

Many pathways have been linked with the progression of AMD, including cell death, phagocytosis and inflammation pathways. Different types of cell death may contribute to the development of AMD, including apoptosis, anoikis or autophagy. In the present study, we aimed to establish an *in vitro* detection model for investigating the anoikic and autophagy-associated cell death in ARPE-19, primary hRPE and hESC-RPE cells.

In hereby investigated whether anoikic cell death would be induced in hESC-RPE cells using poly-HEMA-covered culture plates. We found that these cells could not attach to the poly-HEMA covered surface using phase contrast microscope; in addition, they formed floating aggregates. We observed high rate of AnxV positivity (27%) in the anoikic hESC-RPE cells.

It has been shown that the increased oxidative damage of RPE cells is implicated in the progression of AMD. The most frequently used *in vitro* oxidative stress inducer is the H_2O_2. Moreover, it has been shown that oxidative stress is related to high autophagic activity. We applied H_2O_2-treatment as an oxidative stress model to induce and study autophagy and autophagy-associated cell death in RPE cells. We used increasing concentrations (0.4-1mM) and time-intervals (2-4h) of H_2O_2-treatment in ARPE-19 and hRPE cells to provide the most effective treatment for autophagy induction. Moreover, we combined the H_2O_2-treatment with serum deprivation, which is a well-known autophagy inducer, and tested the effect of this co-treatment on the cell death rate and the level of autophagy in RPEs. The co-treatment resulted in heterogeneous dying RPE cell populations to be present at the same time. We found that the ratio of PS^+ or dying RPE cells was increased in a time-and concentration dependent manner upon H_2O_2-treatment. The highest percentage of single positive AnxV cells was found upon 2h of

1mM H_2O_2 treatment, 41±10.8% of the ARPE-19 cells, 17.7±12.7% of the hRPE and approximately 40% of hESC-RPE cells became $AnxV^+$.

In the current study, we used different methods to confirm the induction of autophagy in RPE cells as a result of H_2O_2-treatment. The results of the Western blot analysis showed the LC3-II/LC3-I ratio increases in a time- and concentration-dependent manner, especially upon 2h, 1mM H_2O_2 treatment in the three RPE cell types applied here. Moreover, in ARPE-19 cells we could determine a significantly decreased level of p62 by Western blot analysis, accumulation of autophagosomes by TEM, increased number of GFP-LC3-positive cells using fluorescent microscopy and higher GFP fluorescence intensity in GFP-LC3 transfected cells by flow cytometry after H_2O_2 treatment (2h, 1mM) in absence of serum, and as compared to the untreated control cells.

We assessed the whole dynamic of autophagy during H_2O_2-expoure (2h, 1mM) in ARPE-19 and hRPE cells. Therefore, we checked the turnover of LC3-II by Western blot analysis in the presence of CQ to evaluate the autophagic flux. We found accumulation of LC3-II upon CQ treatment, which significantly increased the ratio of LC3-II/LC3-I, thus proving an increased autophagic flux upon H_2O_2-administration. We can conclude that autophagy could be activated by H_2O_2-treatment (2h, 1mM) in RPE cells; in addition, the combination of these approaches resulted in the establishment of *in vitro* detection model for autophagy in RPE cells.

We further studied what percentage of the $LC3^+$ RPE cells is also $AnxV^+$, and how much of the $AnxV^+$ dying RPE cells show LC3-positivity upon H_2O_2-exposure. Therefore, we simultaneously, analysed the LC3-positive AVs using mCherry-LC3 plasmid transfection and study of the PS-externalization using Annexin V-FITC labelling in H_2O_2-treated (2h, 1mM) ARPE-19 cells in absence of serum by flow cytometry. Our results confirm that the autophagy-associated process was triggered in most of the dying ARPE-19 cells as a result of serum deprivation and oxidative stress caused by H_2O_2 (2h, 1mM) treatment.

In the current study, we observed that ARPE-19 cells and macrophages can efficiently and increasingly over time autophagy-associated dying ARPE-19, primary hRPE and hESC-RPE cells *in vitro*; moreover, the enhancing effect of TC pre-treatment in the clearance capacity could be demonstrated. In parallel with the FACS analysis, we could follow up the clearance process of autophagy-associated dying RPEs using time-lapse microscopy. In line with these results, we could show that macrophages were able to

efficiently remove the anoikic dying primary hRPE and hESC-RPE cells; in addition, the phagocytic percentage was increased as a result of TC-pre-treatment in both cases.

Interestingly, our *in vitro* data indicate that DCs can engulf much more autophagic-associated dying ARPE-19 cells than the non-professional ARPE-19 or the macrophages. However, the anoikic dying ARPE-19 cells were also taken up with low efficiency by the DCs.

Next, we checked how the cell death rate and the clearance of H_2O_2-treated RPE cells are changed by macrophages, if we block the formation of autophagosome using PI3K inhibitor, namely 3-MA. First of all, we confirmed that the autophagic process was partially blocked as a result of 3-MA-treatment by Western blot analysis. Parallelly, we observed significantly increased percentage of viable and significantly decreased percentage of $AnxV^+$ and PI^+ H_2O_2-treated ARPE-19 cells upon this inhibitor, which indicated the blocking of autophagy-associated cell death in ARPE-19 cells. Finally, when we co-incubated the 3-MA pre-treated and then H_2O_2-treated APRE-19 cells with the macrophages, a significantly decreased rate of phagocytosis of these RPE cells was found. These findings suggest that autophagy contributes to the specific changes of the cell surface, which are linked with the recognition and engulfment of these dying cells by macrophages.

We next determined the clearance of autophagy-associated dying ARPE-19 cells as a mixed dying cells' population to be engulfed by macrophages. Therefore, we applied a well-established, sensitive, high-throughput flow cytometry technique to sort out the GFP-LC3 positive and negative sub-populations of H_2O_2-treated transfected ARPE-19 cells based on their size, granularity and fluorescence signal; consequently, these sub-populations were co-incubated with the macrophages. A similar phagocytic rate was found during engulfment of GFP-LC3 positive RPEs and non-sorted autophagic dying cells by macrophages, thus verifying their fate in the clearance process. As far as we are aware, this is the first report demonstrating the phagocytic rate of H_2O_2-treated, GFP-LC3 positive sorted ARPE-19 cells by macrophages using a high-performance cell sorter, as well as the clearance process being visualized by time-lapse microscopy.

To investigate the gene expression pattern changes in TC-treated and untreated macrophages before and during engulfment of anoikic ARPE-19 cells, TLDA was used. In the current study, we applied siRNA transfection method for silencing the following genes: ADORA3, THBS1, AXL, MERTK (all being upregulated by the TC treatment), to determine, whether this upregulation of apopto-phagocytic genes had a direct impact

on the increased clearance efficiency of professional phagocytes. Only silencing of MERTK and AXL could prevent TC-induced enhancement in engulfment of anoikic ARPE-19 cells by macrophages. Parallelly, the pivotal role of MerTk receptor in this clearance process was further confirmed using blocking antibodies against MerTk. The role of MerTk receptor could be confirmed in case of TC-mediated non-professional phagocytosis of anoikic ARPE-19 cells, due to the TC-enhanced phagocytosis rate being significantly reduced upon MerTk blocking antibody treatment either on the side of the phagocytes or to the anoikic ARPE-19 cells. In addition, the clearance of anoikic dying primary hRPE cells by TC-treated macrophages was significantly decreased as a result of blocking MerTk receptor. Finally, we found that Gas6 treatment led to considerable increased phagocytic capacity of TC-treated macrophages, which showed concentration dependence. To our present knowledge, this is the first study demonstrating the major role of MerTk in the regulation of TC-enhanced clearance of anoikic dying RPE cells not only by non-professional phagocytes, but also by macrophages. The upregulation of MFG-E8, ITGAV, TYRO3, AXL and THSB-1 genes in the TC-treated macrophages confirms the role of TC in the different stages of phagocytosis.

Growing evidence suggests that the pathogenesis of AMD is strongly associated with immunological processes. In this study, we could demonstrate that the concentration of released IL-6 and IL-8 cytokines was increased during the clearance of autophagy-associated dying ARPE-19 or hRPE cells by macrophages; in addition, a significantly decreased release of these interleukins by TC-treated macrophages was found, as a result of the anti-inflammatory effect of the glucocorticoid TC. We detected a similar impact when the macrophages engulfed the autophagy-associated dying hESC-RPE cells. These results suggest that the clearance of autophagy-associated dying RPE cells can trigger a pro-inflammatory response in human macrophages *in vitro*.

Surprisingly, in the case of phagocytosis of anoikic dying hESC-RPE cells by macrophages, high amount of IL-6 and IL-8 production was determined, which was reduced in response to TC treatment. The induction of the secretion of these pro-inflammatory mediators might be related to the improper clearance of anoikic dying cells. Moreover, the possibility cannot be excluded that a small ratio of secondary necrotic population, as a result of cell culturing on poly-HEMA-covered dishes over a 24h period, could also contribute to the release of these pro-inflammatory cytokines.

6. KEYWORDS

age-related macular degeneration, anoikis, autophagy, AXL, inflammation, macrophage, MERTK, phagocytosis, RPE, triamcinolone

7. SUMMARY

The clearance of dying RPE cells is crucial for maintaining retinal homeostasis. Inefficient removal of these cells by phagocytes may result in accumulation of debris and progression of AMD. We hypothesize that not only intracellular protein clearance in RPE cells, but also clearance of autophagy-associated dying cells by phagocytes are essential in the pathogenesis of AMD. In this work, the engulfment of autophagy-associated and anoikic dying RPE cells by phagocytes is being investigated.

An increasing percentage of PS^+ or dying RPE cells due to serum deprivation and H_2O_2 co-treatment could be observed. The induction of autophagy could be detected within 2h of treatment with 1mM H_2O_2 using TEM, LC3/p62 expression and GFP-LC3 transfection assays, as well as increased autophagic flux could be measured during H_2O_2-exposure under CQ treatment. Autophagy-associated cell death could be induced in ARPE-19, hRPE or hESC-RPE cells. Moreover, the anoikic cell death could be triggered in hESC-RPE cells by ECM detachment.

The *in vitro* phagocytosis assays found that autophagy-associated and anoikic dying RPE cells can be efficiently and increasingly engulfed by macrophages, DCs and living RPE cells in a time-dependent manner. Inhibition of autophagy by 3-MA led to decreased phagocytic capacity of macrophages during the engulfment of autophagy-associated dying cells, while sorting out the GFP-LC3 positive cell population or TC treatment enhanced it. We could demonstrate a phagocytosis-enhancing effect of TC treatment in non-professional and professional phagocytes during the clearance of anoikic dying RPE cells via the MerTk signalling pathway. The key role of MerTK in the macrophages engulfing dying cells could be confirmed using siRNA-based gene silencing or blocking antibodies. The phagocytic efficiency of TC-treated macrophages was remarkably increased upon Gas6 treatment via the MerTK receptor. The clearance of autophagy-associated dying RPE cells by macrophages resulted in a pro-inflammatory response.

Our findings suggest that the phagocytosis of anoikic and autophagy-associated dying RPE cells can be used as models for studying AMD *in vitro*, and for investigating future pharmacological agents for treating AMD. The hESC-RPE cell death models might be appropriate for examining the *in vivo* potential or immunological consequence of using such cells for stem cell therapy. Specific agonists of the MerTK receptors may have a potential role as phagocytosis enhancers in the retina and might serve as future targets for AMD therapy.

8. PUBLICATIONS

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Candidate: Mária Szatmári-Tóth
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List of publications related to the dissertation

1. **Szatmári-Tóth, M.**, Kristóf, E., Veréb, Z., Akhtar, S., Facskó, A., Fésüs, L., Kauppinen, A., Kaarniranta, K., Petrovski, G.: Clearance of autophagy-associated dying retinal pigment epithelial cells - a possible source for inflammation in age-related macular degeneration. *Cell Death Dis. [Accepted by Publisher]*, 2016.
   IF: 5.378 (2015)
2. Albert, R., Kristóf, E., Zahuczky, G., **Szatmári-Tóth, M.**, Veréb, Z., Oláh, B., Moe, M. C., Facskó, A., Fésüs, L., Petrovski, G.: Triamcinolone regulated apopto-phagocytic gene expression patterns in the clearance of dying retinal pigment epithelial cells. A key role of Mertk in the enhanced phagocytosis.
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List of other publications

3. Szabó, D. J., **Szatmári-Tóth, M.**, Doró, Z., Nagymihály, R., Josifovska, N., Facskó, A., Petrovski, G.: Cell death, clearance and inflammation: molecular crossroads and gene polymorphisms in the pathogenesis of age-related macular degeneration.
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