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Molecular mechanisms of the clearance of retinal dying cells by different phagocytes - implications to age-related macular degeneration

Age-related macular degeneration (AMD) is the leading cause of blindness in the 50 years and older age group in the developed world. The disease affects the central part of the retina - the macula, which is responsible for maintaining sharp vision. About 50 million people suffer from AMD worldwide. A hallmark of AMD is deposition of drusen between the retinal pigment epithelium (RPE) and the Bruch's membrane (part of the blood-retina barrier). This can lead to cell death due to detachment from the extracellular matrix (ECM) - known as anoikis and apoptosis; alternatively, autophagy and some form of necrosis have also been detected in post-mortem eyes of patients with AMD. Dry type of AMD occurs under such conditions as long as the Bruch's membrane is intact. When this membrane breaks and penetrating choroidal neovascularizations (CNV) develop, wet type of AMD fulminates. Failure of RPE cells (under intact Bruch's membrane/dry AMD), and macrophages or dendritic cells (DCs) (under CNV penetrated Bruch's membrane/wet AMD) to engulf different dying cells in the retina may result in accumulation of debris and disease development.

ARPE-19 cells are the most widely used human immortalized cell line resembling the human RPE cells. These cells and primary human RPE (hRPE) can undergo anoikis when cultured on teflon-like (polyHEMA) surface in a time-dependent manner as determined by Annexin V-FITC/Propidium Iodide (PI) FACS analysis. Similarly, necrosis induction by heat-shock and autophagic cell death induction by serum depletion and oxidative stress from hydrogen peroxide can be modelled *in vitro*. During the first year of the project, such *in vitro* cell death-inducing assays and quantification of the different forms of cells death (early apoptosis, late apoptosis/secondary necrosis and primary necrosis) were established, as well as basic phagocytic assays carried our.

During the second year of the project, an in-depth apopto-phagocytic gene expression pattern analysis during retinal clearance processes and the effect of triamcinolone (TC) were investigated. ARPE-19 cells and hRPE were induced to undergo cell death by anoikis. Phagocytic clearance assays of the engulfment of anoikic cells by living hRPE and ARPE-19 cells, and human monocyte-derived macrophages (HMDMs) were performed in the presence or absence of TC. Phagocytosis was quantified using a standard double-staining method on flow cytometry. TaqMan low-density array determining the gene expression of known markers of phagocytosis was carried out in HMDMs engulfing anoikic cells. HMDMs expressed all the necessary genes needed for carrying out efficient phagocytosis. Using a self-designed apopto-phagocyte gene-array, gene expression changes were examined in control and TC treated HMDMs during engulfment of anoikic ARPE-19 cells. Altogheter, significant changes in the expression of 56 out of the investigated 95 genes were found in multiple comparisons between the different groups. The expression level of MFGE8, THSB1, ADORA3 and MERTK of the analyzed 95 genes- increased significantly after TC treatment in HMDMs. There was also a heterogenous group of genes with high level of expression in the macrophages that did not change during TC treatment ($0.5<FC<2$): ANXA5, CALR, CD68, ITGB2. The relative expressions of apopto-phagocytic genes in HMDMs of different

donors were also compared. It was observed that non-variable genes, whose relative expression level varied only 20% amongst donors (ADORA2B, ANXA1, ATG5, CD14, CIAS1, ELMO1, ICAM3, IL1B, IRF1, IRF5, ITGAM, ITGAV, ITGB3, PYCARD, TRIO) was present, and there were highly variable ones, whose expression level varied in 2-3 orders of magnitude (ADORA1, ASGR1, CARD15, CD68, IL23A, IL6, MAP1LC3A, MFGE8, OLR1, TGM2, TYRO3). The expression level of the non-variable genes was typically higher and some of them were down-regulated upon TC treatment (ANXA1, ICAM3, IL1B, ITGAM, ITGB3). Based on these data, it was assumed that these genes encode proteins that are essential to macrophage function and most of them get induced during TC treatment. Engulfment of anoikic ARPE-19 cells resulted in significant expression changes in 43 out of the investigated 95 genes in the macrophages, but in the case of ANXA5 the expression change did not reach the two-fold limit (FC>2, p<0.05). TC treatment can highly increase the phagocytic capacity of macrophages toward anoikic RPE cells. According to the data obtained and based on studies in macrophages from a large pool of donors in the presence of 1 μM TC, there was a 2-fold enhancement of the phagocytosis. As a result of the TC treatment, ADORA3 and THBS1 were significantly upregulated as compared to macrophages grown in the absence of TC during engulfment of anoikic ARPE-19 cells, while the expression of 55 genes altered significantly as compared to TC-treated macrophages. The TC-induced genes encode either receptors (ADORA1, ADORA3, ALOX5, AXL, CALR, FCGR2B, ITGAM, ITGAV, ITGAX, ITGB2, ITGB5, LRP1, MERTK, PTGER2, PTK2, PTX3, TYRO3) or bridge-building proteins (ANXA1, C1QA, C2, C4A, ICAM3, MFGE8, PROS1) between phagocytes and apoptotic cells. It should be noted that 2 of them (ADORA1, MFGE8) were from the group of genes whose expression was quite variable among donors, and the other 17 of them (ADORA3, ALOX5, AXL, C1QA, C2, C4A, CALR, FCGR2B, ITGAX, ITGB2, ITGB5, LRP1, MERTK, PROS1, PTGER2, PTK2, PTX3) were in the intermediate group regarding their variance. To investigate whether TC-induced upregulation had a direct effect on the enhanced phagocytic capacity of macrophages, a selection of the upregulated genes were silenced using siRNA. The transfection procedure did not cause significant loss of cells and did not have an adverse effect on the phagocytosis capacity. The silencing efficiency of the target genes varied among different macrophage donors and replicates of living ARPE-19 cells as described previously. Although the weakest silencing effect in macrophages was achieved for the members of TAM family (about 20-50%), the phagocytosis of only the TC-treated MERTK and AXL knock-down macrophages decreased significantly. The combination of MERTK silencing with siRNAs for AXL or any of the other TC upregulated genes as well as bridging molecules with each other did not show a significant synergistic effect. Furtermore, silencing of MERTK and AXL in living TC treated ARPE-19 cells resulted in a significant decrease in non-professional clearance of anoikic ARPE-19 cells. An investigation was then carried out to check whether blocking of MerTk with an antibody can verify its key role in TC-induced increase of phagocytosis: it was found that while the MerTk blocking antibody did not inhibit phagocytosis by macrophages differentiated in the absence of TC, the TC-induced increase of phagocytic capacity of macrophages could be prevented by treatment with this antibody. The distribution of the MerTk receptor on the macrophages was in punctated structures at the cell borders of human macrophages and anoikic ARPE-19 cells, and it was accumulated at the connecting points between TC-treated macrophages and dying ARPE-19 cells in a larger extent that could be detected in 30-50% of the engulfment gates of three different macrophage donors. Besides that, the expression of MerTk protein was confirmed by immunoblotting showing lower expression in both untreated cell types and much more elevated expression as a result of TC treatment in macrophages even during engulfment of anoikic ARPE-19 cells. Furthermore, it was checked whether blocking of MerTk with an antibody had an impact in the TC-induced increase of non-

professional phagocytosis performed by living ARPE-19 cells and it was observed that while the MerTk blocking antibody did not inhibit phagocytosis by non-professional phagocytes in the absence of TC, their TC-enhanced phagocytic capacity could be significantly decreased by treatment with this antibody administered either on the side of the phagocytes or to the anoikic ARPE-19 cells. MerTk was also found in punctated structures at the cell borders of phagocytosing ARPE-19 cells, and it was accumulated at the engulfment portals between TC treated phagocytes and dying ARPE-19 cells in a larger extent that could be detected in 20-40% of the engulfment gates of three different replicates. The expression of MerTk protein was visualized by immunoblotting showing lower expression in TC untreated cells and much more elevated expression as a result of the engulfment process even during TC treatment of the phagocytosing ARPE-19 cells. In parallel, the expression of MERTK and AXL genes in living ARPE-19 cells slightly increased upon TC treatment while they show a significantly elevated expression during the engulfment of anoikic ARPE-19 cells in TC-treated non-professional phagocytes. In addition, an *in vitro* model for studying the effect of TC treatment on the clearance of human primary RPE cells by professional phagocytes was established. In the study, TC treatment of macrophages 48 hrs prior the phagocytic assay enhanced the macrophage-mediated clearance of dying retinal cells: an augmentation of about 1.5-2 times in the engulfing capacity of anoikic hRPE cells occurred at 4 hrs of co-incubation. The administration of blocking antibody acting on MerTk significantly reduced the engulfment of dying hRPE cells by TC treated macrophages similarly to the in vitro clearance model of anoikic dying ARPE-19 cells described previously. Finally, it was checked whether the augmentation of MerTk by its ligand - the bridging molecule Gas6, could affect the clearance of anioikic ARPE-19 and hRPE cells: untreated macrophages moderately increased their phagocytosis capacity upon Gas6 treatment, while a remarkable phagocytosis 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. Altogether, the findings suggest that specific agonists of the tyrosine kinase receptors may have a potential role as phagocytosis enhancers in the retina and serve as future targets for AMD therapy.

During the third year of the project, the induction of autophagic cell death and clearance of such dying RPE cells was studied in detail *in vitro*. Time- and concentration-dependent induction of autophagy was determined by Western blot analysis of light-chain-3 (LC3) expression. In both, ARPE-19 and hRPE cells, the ratio of 17 kDa LC3-II (the autophagosomal membrane-bound form of LC3) and 19 kDa LC3-I (the free cytosolic form) peaked at 2h of serum deprivation and H_2O_2 treatment, contrary to p62 expression (additional autophagosomal membrane associated marker for detecting autophagy), which decreased. Autophagic vesicles that were likely autophagosomes were observed by TEM upon 2 h of 1mM H_2O_2 treatment. The presence of double-membraned autophagic vacuoles containing cytosolic components (black arrow) from which some were beeing fused with the lysosomes (white arrow), could be also confirmed.

The lysosomal inhibitor CQ has been used to measure the endogenous LC3-II turnover. Autophagic flux can be determined by immunoblotting showing the induction in the increase of LC3-II expression after CQ treatment. Inhibition of the autophago-lysosomal fusion by CQ significantly increased the LC3-II/LC3-I ratio alluding to the fact that autophagic flux was present in H_2O_2 treated ARPE-19 and hRPE cells.

Induction of autophagy in ARPE-19 cells led to the accumulation of perinuclear GFP-LC3 positive aggregates or autophagic vacuoles (AVs) which could be detected by fluorescence microscopy. Ring-shaped autophagic vacuoles were visualized by fluorescent

microscopy. The number and size of the GFP-LC3-positive AVs peaked at 2 h of 1mM H_2O_2 treatment. Furthermore, more and larger GFP-LC3-positive AVs were found as a result of CQ treatment. 40.9±8.4% of the cells contained GFP-LC3-positive vacuoles counted manually and 23.9±1.2 % of the cells were GFP-LC3-positive quantified by FACS analysis. In line with this, it was found that ARPE-19 and hRPE cells died as a result of serum deprivation and H_2O_2 co-treatment in a time and concentration dependent manner using a high-throughput flow cytometry based method. Cells that are viable are both Annexin-V^+ and PI^-. While cells that are Annexin-V^+ and PI^-, Annexin-V^- and PI^+ indicate early apoptosis and necrosis, respectively. In addition, Annexin-V^+/PI^+ positivity is a sign of late apoptosis. After 2h of 1mM H_2O_2-treatment the percentage of living ARPE-19 cells, compared to the untreated control ones, significantly decreased from 91.4±1.7% to 28.6±14.2%. In the case of hRPE cells the ratio of living cells changed from 87.1±4.9% to 51.6±3.6%. In parallel, the percentage of annexin-V^+ ARPE-19 cells increased from 2.1±2% to 41±10.8%. It was observed that 17.7±12.7% of hRPE cells became annexin-V^+ as a result of induction of autophagic cell death while the untreated control contained only 3.2±2.8% of early apoptotic cells. In addition, the rate of H_2O_2-induced cell death of ARPE-19 cells which contain LC3-positive autophagic vacuoles and show PS externalization was detected. ARPE-19 cells were transiently transfected with mCherry-LC3 plasmid, treated with H_2O_2 (1mM, 2h) and then annexin V-FITC labelling was used. The percentage of $LC3^+$ untreated ARPE-19 cells was 15.02% and it was increased to 28.76% upon H_2O_2-treatment, meanwhile, the percentage of Annexin-V^+ cells increased from 3.38% to 17.51%. Moreover, it was found that 52.31% of $LC3^+$ cells were Annexin-V^+ as well and 83.88% of Annexin-V^+ cells were $LC3^+$ likewise. These data suggest that autophagy has been induced in most of the dying ARPE-19 cells as a results of H_2O_2 treatment.

Although phagocytosis of apoptotic, autophagic dying- and necrotic cells has been extensively studied in other organ systems, and we have recently shown the dynamics of the clearance of apoptotic/anoikic RPE cells *in vitro,* no data are available on how autophagic dying cells get removed from the retina. In the latter experiments, non-dying ARPE-19 cells, macrophages and DCs could engulf autophagic dying RPE cells with increasing number of phagocytes containing cell corpses over a 24 h period demonstrated by time-lapse microscopy and quantified by flow cytometry.

Non-dying ARPE-19 cells removed autophagic dying ARPE-19 cells efficiently, reaching an average phagocytosis frequency of 3.8±1.1% at the 8h time point. Similarly, the rate of phagocytosis of autophagic ARPE-19 cells by macrophages was 6.9±1.7%. The engulfment of autophagic dying primary hRPE cells by macrophages was more efficient, the phagocytic capacity was 21.2±3.3 % after a 8h co-incubation period. TC treatment further enhanced the engulfment process under most of the conditions. On the other hand, DCs engulfed autophagic ARPE-19 cells more effectively: the phagocytic rate was 26.7±10.8% in the case of iDCs, and 21.4±6.4% when DCs were activated by IL-1β (5 ng/ml), IL-6 (100 ng/ml), TNFα (10 ng/ml), PGE2 (1 μg/ml), GMCSF (80 ng/ml) after 8 h of co-incubation. Next, the cell death and subsequent phagocytosis as a result of inhibition of autophagy in ARPE-19 cells was investigated. 10 mM 3-MA pre-treatment for 24 h partially blocked the conversion of LC3-I to LC3-II, which proved that 3-MA could inhibit the autophagic process in H_2O_2-treated ARPE-19 cells. In addition, this inhibitor significantly increased the number of living cells, and significantly decreased the number of annexin-V positive and PI positive, and double positive H_2O_2-treated ARPE-19 cells. Thus, 3-MA pre-treatment inhibited the autophagic cell death of ARPE-19 cells. As a next step, inhibition of autophagy by 3-MA

significantly decreased the engulfment of autophagic dying ARPE-19 cells by untreated and TC-treated macrophages.
To determine whether the engulfed cells were actually the autophagic dying cells and not any other type of dying cells, the engulfment of GFP-LC3 transfected, H_2O_2-treated ARPE-19 cells was quantified. The actual phagocytic rate was 9.3±3.7%, comparable to the engulfment when CFDA-SE stained, H_2O_2-treated ARPE-19 cells were co-incubated with the macrophages. In addition, the GFP-LC3 positive and negative RPE cells were sorted out and co-incubated with macrophages for 8 h. The rate of phagocytosis of non-autophagic, GFP-LC3 negative RPEs was negligible (0.6±0.2%). In contrast to that, macrophages engulfed the GFP-LC3 positive RPEs at the same rate (13.7±3.3%) as they did the non-sorted autophagic dying cells, thus confirming their fate in the engulfment process. In addition, TC treatment significantly enhanced the engulfment of GFP-LC3 transfected, H_2O_2-treated ARPE-19 cells. The engulfment of autophagic dying RPE cells induced secretion of IL-6 and IL-8 pro-inflammatory and pro-angiogenic cytokines by the macrophages.
In summary, autophagy was revealed by elevated light-chain-3 (LC3) expression and electron microscopy, while autophagic flux was confirmed by blocking the autophago-lysosomal fusion using chloroquine. The autophagic dying RPE cells were engulfed by human macrophages and DCs as well as live RPE cells. Inhibition of autophagy by 3-methyladenine decreased the engulfment of these dying cells by macrophages, while sorting out the GFP-LC3 positive cell population or treatment by the glucocorticoid TC enhanced it. Autophagic dying RPE cells induce a pro-inflammatory and pro-angiogenic cytokine release in macrophages which may explain the source of the background inflammation seen in AMD. These data suggest that cells dying through autophagy may engage in distinct sets of clearance mechanisms in professional and non-professional phagocytes and contribute to the pathogenesis and inflammatory nature of AMD.