RPE Phagocytosis

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Introduction

The retinal pigment epithelium (RPE) forms a polarized monolayer in the retina. Its basolateral aspect adheres to a specialized basement membrane, Bruch's membrane, and neighbors the choroidal vasculature, while its apical surface extends microvilli into the subretinal space and faces photoreceptor rods and cones of the neural retina. RPE cells perform numerous roles in support of photoreceptors specifically and the neural retina generally that are essential for vision including directed transport of ions, water and metabolites, absorption of light, secretion of growth factors and other signaling proteins, participation in the visual cycle and phagocytosis of spent photoreceptor outer segment fragments (POS) [1]. As both photoreceptors and RPE cells are post-mitotic and non-migratory their interactions persist in the mammalian eye and individual RPE cells must continue to perform their support activities for life.

Clearance phagocytosis of POS by the RPE is a daily task that is critical to maintain photoreceptors. Lack of or abnormal RPE phagocytosis caused by mutations in genes encoding proteins of the RPE phagocytic machinery impairs retinal function and integrity in experimental animal models [2–5] and causes retinitis pigmentosa (RP) in human patients (recently reviewed by Parinot and Nandrot (2016)) [6]. Moreover, failure to efficiently degrade engulfed POS by RPE cells compromised by oxidative stress and likely other age-related changes contributes to accumulation in the RPE of modified proteins and lipids in cytoplasmic lysosome-derived storage organelles known as lipofuscin [5–8]. Excess lipofuscin accumulation is harmful to the aging RPE and retina in human and experimental animals and is thought to contribute to age-related macular degeneration [9, 10].

In the vertebrate retina, the process of photoreceptor outer segment renewal continuously turns over the light sensitive outer segment portions of photoreceptors thought to bear damaged proteins and lipids. Permanent photoreceptor cells rejuvenate but maintain constant length of their outer segments by coordinating shedding of distal, most aged outer segment tips with precisely balanced formation of new membrane disks at the proximal end of outer segments [11]. The RPE participates in this process by removing shed photoreceptor debris by receptor-mediated phagocytosis [12].

Molecular mechanisms that promote synchronized POS tip shedding are only poorly understood. At the time of rod shedding the plasma membrane of POS tips externalizes

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phosphatidylserine (PS), an "eat me" signal also displayed by cells undergoing apoptosis [13]. RPE cells recognize exposed PS opsonized by extracellular bridge ligands with their phagocytic receptors. Moreover, RPE cells also enhance synchronized PS externalization in a diurnal rhythmic pattern to match the peak of their phagocytic activity. Thus, synchronized outer segment tip shedding is not photoreceptor cell autonomous but requires activities of the RPE.

In mammals, RPE clearance of POS occurs in a diurnal rhythm entrained by light and circadian mechanisms [14]. Both rods and cones shed POS [12, 15–17]. Different mammalian species shed cone POS either at the onset of light or of the dark period [18–25]. In contrast, in all species studied, rods shed POS at light onset. As rods make up the vast majority of photoreceptors in both rodent and human retina, rod POS shedding prompts a burst of phagocytic activity and uptake by the RPE at light onset followed by a period of relative phagocytic inactivity during which RPE cells process engulfed POS in phagolysosomes [5, 14]. RPE cells enzymatically break down POS proteins and POS-derived lipids, some of them for metabolism and others for recycling to photoreceptors [26–30].

Altogether, photoreceptors and RPE collaborate to achieve the necessary coordination of their activities that ensures maintenance of functional outer segments for life. The recurring challenge with spent POS requires RPE cells to engulf and degrade POS daily for life. This unique and enormous phagocytic load renders RPE cells the most highly phagocytic cell type in the body.

Experimental Approaches to Quantify the Phagocytic Function of the RPE

RPE phagocytosis can be investigated either by examining the RPE *in situ* or by feeding experimental phagocytic particles to RPE cells grown in culture. Both approaches have unique advantages that make them complementary. In the following we will discuss advantages of each strategy and the methods used.

Phagocytic challenge of RPE cells in culture has three distinct advantages over examining RPE phagocytosis *in situ*. (1) RPE cells may be manipulated before or during phagocytosis assays using genetic or pharmacological approaches. (2) Feeding wild-type POS to mutant RPE and vice versa will identify if a given genetic change affects activities of RPE or photoreceptors or both during outer segment renewal. (3) Cell culture assays can experimentally separate the distinct phases of the phagocytic process, particle recognition/binding, internalization, and digestion yielding insight into the dependence of each step on particular sets of genes and molecules. RPE cells grown in culture can maintain avid phagocytic activity specifically towards isolated POS [31]. However, phagocytic activity of RPE in culture may vary with cell culture condition. It is thus imperative to maintain strict culture protocols and experimental planning. POS for feeding to RPE in cell culture phagocytic assays can be obtained from fresh retinas e.g. from pig, cow, or rat following established protocols [32, 33]. Larger batches of POS may be prepared and stored as deep-frozen aliquots ensuring that particle quality and quantity show little variation from experiment to experiment. POS may be covalently labeled with a fluorescence dye or radiolabeled before use. Alternatively, POS may be detected labeling fixed samples with antibodies specific to POS, such as transducin or rhodopsin. Most commonly antibodies to rhodopsin are used, as opsin is by far the most abundant protein of POS and reliable monoclonal antibodies against well-defined epitopes of rhodopsin are commercially available [34, 35]. RNA silencing reducing candidate protein levels before or pharmacological modulators before or during phagocytic challenge will help identifying roles for specific molecules in the phagocytic process. Of note, RPE cells require extracellular molecules as stoichiometric bridge ligands between RPE surface receptors and POS (see sections "Recognition and Binding of POS by RPE Cells" and "Cell Surface Receptors and RPE Signaling Pathways Mediating POS Internalization" for more details). While present in the retina at physiological levels, such factors must be supplemented in

experimental POS phagocytosis assays to promote POS binding and engulfment, either in the form of purified ligand proteins or by addition of fetal bovine serum, which provides a mix of potent but poorly defined molecules supporting RPE phagocytosis [31, 36].

Examining specifically binding, internalization, and degradation phases of RPE phagocytosis can be accomplished by choosing appropriate duration of POS challenge, POS post-challenge incubation (in a discontinuous, pulse-chase type POS phagocytosis assay) selective addition of binding receptor versus internalization receptor bridge proteins, and taking advantage of the fact that rodent RPE cells in culture bind POS at temperatures above ~17 °C (but now below) while internalization does not proceed at temperatures below ~25 °C [37, 38]. For example, POS incubation in the presence of binding receptor ligand at 20 °C will allow only POS binding allowing studying the binding process in itself or yielding RPE cells with pre-bound POS, which following removal of excess POS may be supplemented with internalization receptor ligand and shifted to 37 °C to monitor specifically the internalization step.

Flow cytometry may be used to quantify the amount of phagocytosed POS by RPE cells in culture. Including manipulations removing or quenching the fluorescence of POS that are bound but not internalized will allow discriminating bound and internalized POS material [39, 40]. Flow cytometry-based analysis may quantify POS uptake by large numbers of cells to yield insight into levels of POS per cell and the fraction of cells in a population that takes up POS.

We prefer to study POS phagocytosis using immunofluorescence microscopy of fixed intact RPE monolayers since it allows us to distinguish bound and internalized POS fragments while monitoring cell morphology in the same sample. Selective immunofluorescence labeling of surface-bound POS after non-permeabilizing fixation can discriminate bound and internalized POS [41]. In RPE cells that are grown to differentiate into polarized monolayers with a cuboidal shape, confocal microscopy can discriminate surface-bound from engulfed fluorescent POS based on their position relative to the tight junction marker ZO-1 or F-actin. To accomplish this analysis, x-y image stacks comprising the entire cell are separated into two non-overlapping apical and central z-stacks. The apical z-stack is chosen such that it shows only POS signal located above tight junction/apical microvilli F-actin marker staining, which are bound POS. The central stack is chosen such that it includes only cell aspects below the tight junction or apical F-actin marker. Maximal projections of these separated stacks allow quantification and counting of bound and engulfed POS in the same sample (Fig. 3.1) [42].

Besides the method outlined above and illustrated in Fig. 3.1, experimental approaches to POS binding and engulfment by RPE cells in culture based on fluorescence microscopy or immunoblotting quantification may provide similar insight or additionally focus on POS phagosome acidification [43, 44].

Unlike POS phagocytosis by RPE cells in culture, the phagocytic activity of the RPE in the retina is directly linked to re-growth and shedding of the photoreceptor outer segments. In the healthy retina, rod POS shedding occurs at light onset prompting immediate POS clearance by the RPE. Any defect in POS shedding will affect POS phagocytosis secondarily. Moreover, animal model studies indicate that, conversely, abnormal RPE phagocytosis secondarily affects POS shedding [13]. Measures of phagocytic uptake by the RPE *in situ* in animal models may thus reflect abnormalities in either or both cell types involved. As intact outer segments and shed POS are located directly adjacent to the apical surface of the RPE a bona fide POS binding process does not take place during RPE phagocytosis *in situ*. However, recognition of extracellular bridge proteins by RPE surface receptors activates the same signaling pathways leading to internalization as in RPE in culture [5]. Studies of RPE phagocytosis *in situ* take advantage of the fact that outer segment renewal is highly synchronized and subjected to a strict diurnal rhythm. In cross sections of the retina, electron microscopy identifies engulfed POS based on morphology and location [7, 45], while

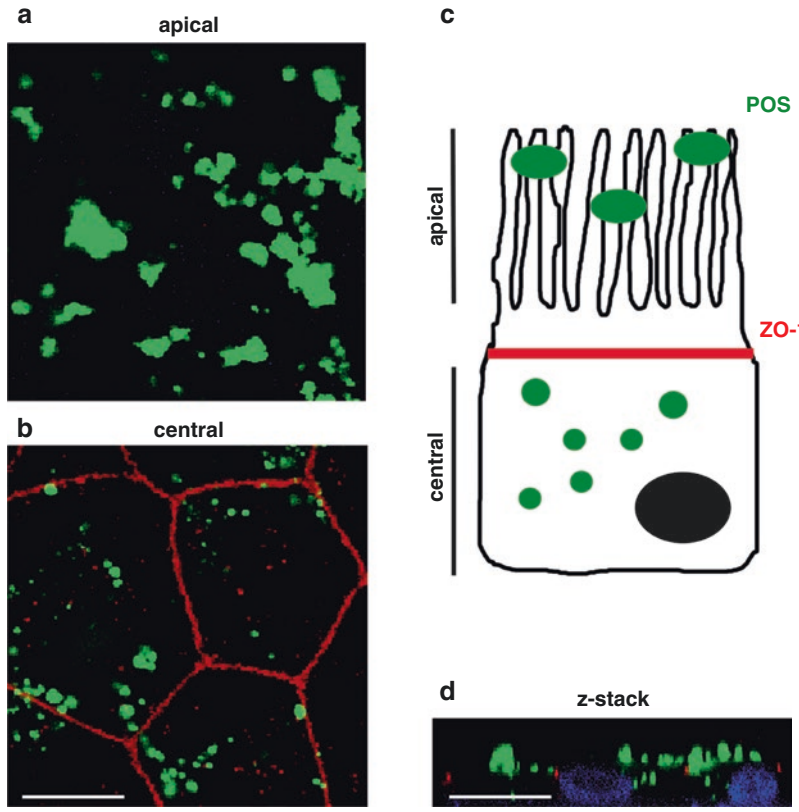

Fig. 3.1 Confocal microscopy analysis of phagocytosed photoreceptor outer segment fragments (POS) distinguishes bound and engulfed particles. (**a**) Apical x-y confocal projection shows POS (green) located above tight junctions indicated by lack of ZO-1 marker labeling in this area. These are quantified as bound POS. (**b**) Central x-y projections show tight junctions (ZO-1 labeling in red) and engulfed POS (green). (**c**) Scheme illustrating separate quantification of bound and engulfed POS based on location relative to tight junction marker labeling by confocal microscopy. The RPE cell nucleus is indicated in black. (**d**) The x-z confocal projection shows relative distribution of nuclei (shown in blue), ZO-1 stained tight junctions (shown in red) and POS (shown in green). POS below the tight junction are engulfed. Reproduced from Davis et al. 2017

light microscopy identifies POS based on POS marker immunoreactivity [46]. Counting POS phagosomes in the RPE of experimental animals at specific time points in relation to light onset allows quantifying the phagocytic load obtained by the RPE at light onset, a measure of phagocytic capacity, and following the decrease in POS phagosomes in the RPE at later time points reflecting progression of POS digestion [47]. Side-by-side comparison of POS phagosome counts with age- and strain-matched control animals provides insight into effects of altering genotypes or experimental treatments. Besides cross sections RPE phagocytosis can be examined in RPE flat mount preparations as well. Following removal of the retina, eyes may be observed live for detection of acidified POS phagosomes [48] or fixed and processed for POS marker immunofluorescence microscopy [47, 49].

Taken together, analysis of RPE phagocytosis in cell culture allows identification of molecular players in this process by separately probing and

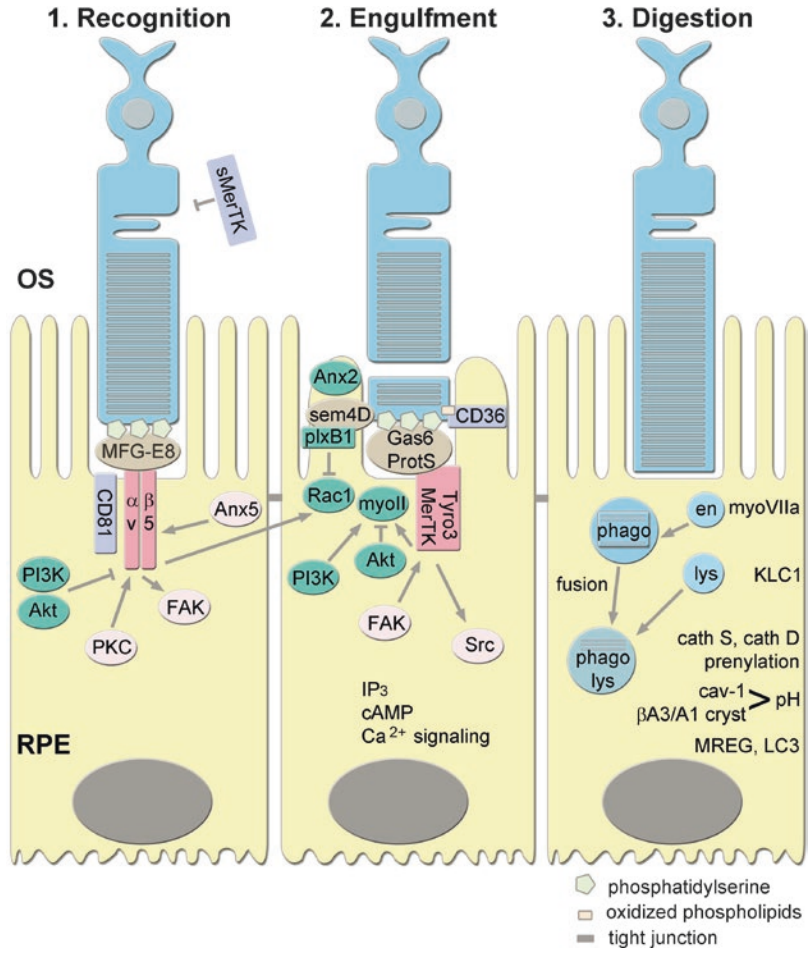

Fig. 3.2 Molecules and mechanisms known to date to contribute to the three different steps of RPE phagocytosis. In each step receptors at the apical RPE surface are highlighted in pink and purple, proteins related with cytoskeletal rearrangement in green, intracellular signaling components in rose, digestion in blue and extracellular ligands in grey. For brief description of protein roles and abbreviations please see Table 3.1. Details of protein roles are provided in the main text. Not all proteins contributing to RPE phagocytosis may be included

interfering with binding/recognition, internalization, and digestion step. In contrast, observation of RPE phagocytosis *in situ* illuminates a molecule's relevance for retinal structure and function under physiological conditions providing important complementary insight.

Molecular Mechanisms of RPE Phagocytosis

RPE phagocytosis belongs to a group of non-inflammatory clearance phagocytosis mechanisms that other phagocytes in the body use to remove apoptotic cells and cell debris and that are conserved from worm to man [38, 50, 51]. Recognition/binding and engulfment steps of phagocytosis require engagement and downstream signaling of specific phagocyte surface receptor proteins. While professional phagocytes of the immune system like macrophages or dendritic cells possess numerous surface receptors that can trigger or participate in clearance phagocytosis, RPE cells possess and use only a limited repertoire of molecules for POS phagocytosis. The scheme in Fig. 3.2 summarizes our current knowledge of the molecular mechanisms of RPE phagocytosis of POS.

Table 3.1 Proteins contributing to RPE phagocytosis as summarized in Fig. 3.2

Protein (abbreviation)	Role	References
AKT kinase (Akt)	Cytosolic signal transducer with functions in F-actin recruitment and POS engulfment	[80]
Annexin A5 (Anx5)	Cytosolic regulator of αvβ5 integrin surface levels	[55]
Annexin A2 (Anx2)	Cytosolic signal transducer with role in POS uptake synchronization	[49]
αvβ5 integrin (αvβ5)	RPE cell surface recognition receptor recognizing PS-bearing POS	[5, 52–54]
βA3/A1 crystallin	Cytosolic signal transducer with role in phagosome maturation (acidification)	[108, 109]
Cathepsin D, cathepsin S	Lysosomal enzymes with roles in POS protein (opsin) degradation	[8, 30, 102, 103]
Caveolin-1 (cav-1)	Cytosolic signal transducer with role in phagosome maturation (acidification)	[47]
CD36	RPE cell surface receptor that recognizes oxidized phospholipids	[73–77]
CD81	Tetraspanin co-receptor of αvβ5 integrin	[57]
Focal adhesion kinase (FAK)	Cytosolic signal transducer mediating activation of MerTK	[5, 36, 78]
Gas6, proteinS	Secreted ligands for MertK/Tyro3 in the subretinal space	[67–70]
Kinesin-1 light chain-1 (KLC1)	Cytosolic motor with role in phagosome transport	[101]
Melanoregulin (MREG)	Cytosolic signal transducer with role in phagosome maturation (LC3 association)	[110, 111]
Mer tyrosine kinase (MerTK)	RPE cell surface engulfment receptor triggering engulfment and limiting POS recognition	[2, 3, 56, 61–64]
Milk fat globule-EGF8 (MFG-E8)	Secreted ligand for αvβ5 integrin in the subretinal space	[36, 58]
Myosin7a (myoVIIA)	Cytosolic motor with role in post-engulfment phagosome transport	[97]
Non-muscle myosin II (myoII)	Cytosolic motor with role in POS engulfment	[79]
Phosphoinositide 3-kinase (PI3K)	Cytosolic signal transducer with role in POS engulfment	[80]
Plexin B1 (plxB1)	RPE cell surface receptor coordinating diurnal termination of RPE phagocytic activity	[96]
Protein kinase C (PKC)	Cytosolic signal transducer regulating αvβ5 integrin anchorage to F-actin	[38]
Rab escort-protein-1 (REP-1)	Cytosolic signal transducer with role in phagosome-lysosome fusion	[104]
Rac1 GTPase	Cytosolic signal transducer regulating F-actin assembly in phagocytic cup	[43]
Semaphorin 4D	Activating ligand of plexin B1 in the subretinal space	[96]
Soluble MerTK extracellular fragment (sMerTK)	Decoy MerTK receptor with inhibitory role in vitro	[70]
Src kinase (Src)	Cytosolic signal transducer with role in POS engulfment	[90]
Tyro3 receptor tyrosine kinase (Tyro3)	MerTK paralog; if expressed by RPE may substitute for MerTK	[65, 66]

Recognition and Binding of POS by RPE Cells

In the recognition/binding step of POS clearance, RPE cells respond to phosphatidylserine (PS) exposed by distal ends of outer segments, which serves as an "eat me" signal [13]. POS recognition requires an active phagocytic machinery at the apical side of RPE cells. The integrin receptor αvβ5 is the only integrin family receptor localized to the apical aspect of RPE cells. *In vivo* and cell culture experiments have provided complementary results indicating that αvβ5 serves as primary POS recognition receptor in human and rodent RPE [5, 52–54]. Ligand binding activity of αvβ5 integrin is highly regulated. RPE cells use a cytosolic protein kinase C (PKC) dependent pathway to promote anchorage of αvβ5 receptors to the F-actin cytoskeleton, which is required for its function in POS recognition [38]. Cell surface levels of αvβ5 integrin are regulated by its interaction with cytosolic annexin A5 (Anx5 in Fig. 3.2) [55]. Moreover, feedback mechanisms between αvβ5 integrin and the internalization machinery impact activity of αvβ5 such that increasing surface levels of αvβ5 integrin does not promote a proportional increase in POS binding [56]. Finally, αvβ5 activity is dependent on its plasma membrane co-receptor, the tetraspanin CD81. CD81 does not function as a binding receptor for POS itself, but inhibition or overexpression of CD81 reduces or increases particle binding by αvβ5 integrin, respectively [57].

Activated αvβ5 receptors do not bind their substrates, PS-bearing POS, directly, but via extracellular bridge proteins that opsonize POS. The subretinal space contains secreted PS-binding glycoproteins, including Protein S, Gas6, and milk fat globule-EGF8 (MFG-E8). Of these, MFG-E8 specifically acts to bridge POS and αvβ5 integrin receptors of the RPE [36]. MFG-E8 may be secreted into the subretinal space by both photoreceptors and RPE [58]. The blockade of αvβ5 integrin as well as lack of αvβ5 or MFG-E8 greatly reduces POS binding by RPE cells. However, a bona fide binding process does not take place in the intact retina, where outer segments, POS and apical surface of the RPE with its αvβ5 receptors are in close contact at all times. However, mice lacking β5 integrin or MFG-E8 do not show the characteristic morning peak of POS phagosomes in the RPE after light onset. Instead, phagocytosis occurs at a reduced but constant level at all times of day [5, 36]. Thus, the binding of MFG-E8-opsonized POS to αvβ5 integrin receptors and their ligation promotes the synchronized peak of POS engulfment. That aged β5 null mice show reduced vision and accumulation of autofluorescent lipofuscin-like material in the RPE illustrates that the rhythmicity of POS clearance is vital to long term health and function of the retina [5].

Cell Surface Receptors and RPE Signaling Pathways Mediating POS Internalization

Antibody blockade of αvβ5 integrin greatly reduces POS binding, but internalization of surface-bound particles is unaffected [53]. Signaling downstream of αvβ5 is necessary but not sufficient for engulfment of αvβ5–bound POS. Thus, the internalization step of POS phagocytosis requires signaling stimulated by POS binding to αvβ5 integrin in addition to a distinct set of surface receptors and their downstream signaling.

Studies on POS phagocytosis and specifically its internalization step have been greatly facilitated by the availability of an animal model that lacks this activity. The Royal College of Surgeons (RCS) rat was first identified in 1938 and has since been widely studied as model of hereditary blindness since [3, 59]. Mullen and LaVail in 1976 showed that RCS retinal degeneration is caused by a defect of clearance phagocytosis by the RPE rather than a photoreceptor defect [3, 41, 60]. In 2000, the causative mutation in the RCS rat was identified to disable the gene for the receptor tyrosine kinase Mer (MerTK). A deletion mutation in the *MERTK* gene in the RCS rat yields a shortened transcript and absence of MerTK protein [61, 62]. Adenoviral delivery of MerTK to RCS RPE rescues the phagocytic

defect and improves RCS retinal integrity *in vivo* [63, 64]. Targeted mutation has generated a MerTK knockout mouse model that phenocopies the RCS rat [2]. In both RCS rat and MerTK knockout models photoreceptors continue to grow from the inner segment side, but RPE cells are unable to engulf POS. As a result, outer segments briefly elongate and distort before outer segment debris and possibly shed POS accumulate in the subretinal space further causing distress of photoreceptors, which eventually die. Notably, lack of MerTK does not cause retinal degeneration in mice with increased expression of Tyro3 in the RPE [65], a receptor tyrosine kinase that is very similar in structure and ligand binding activity to MerTK [66]. Altogether these data illustrate the critical importance of MerTK or equivalent RTK activity for POS phagocytosis by RPE cells.

Like αvβ5 integrin, MerTK or Tyro3 do not interact directly with spent or shedding POS but are ligated by soluble bridge proteins. Protein S and Gas6 are members of a protein family with binding domains shared by both RTKs as well as a binding domain for the PS exposed by POS. Gas6 or ProteinS knockout mouse retinas are phenotypically normal but deletion of both ligands leads to photoreceptor cell death as seen in MerTK rats and mice with rapid early onset retinal degeneration [67, 68]. These *in vivo* data suggest overlapping functions for the RTK ligands. Indeed, both proteins may lead to MerTK receptor ligation, activation and POS internalization by RPE in culture [69]. In contrast, other cell culture experiments have suggested that ProteinS and Gas6 may exert distinct MerTK downstream signals [70]. Further unrelated bridge ligands, e.g. tubby and tubby-like protein 1, and galectin-3 have been proposed but their physiological significance remains untested [71, 72]. With ProteinS/Gas6 together evidently necessary and sufficient for POS internalization via MerTK, additional experiments will be needed to clarify role or contributions of additional molecules and mechanisms.

In addition to RTK activity, RPE may employ CD36 receptor ligation to activate POS engulfment. CD36 is expressed at the apical, phagocytic surface [73]. It recognizes oxidized lipids or lipoproteins. In the phagocytic process CD36 acts in a post binding step and independent of the POS binding receptor αvβ5 integrin [74]. Dependence of CD36 on specific oxidized phospholipids that are generated in retina subjected to high intensity light and the possibility of pro-inflammatory signaling downstream of CD36 suggests that CD36 may contribute to RPE phagocytic activity in damaged or distress retina rather than in routine diurnal outer segment renewal in the healthy retina [75–77].

Ligation of phagocytic receptors of the RPE by bridge proteins opsonizing shed POS elicits cytosolic signaling pathways that ultimately reorganize the cell for particle engulfment. Signaling activities downstream of both, αvβ5 integrin and MerTK/Tyro3 are required.

The cytosolic tyrosine kinase focal adhesion kinase (FAK) is a crucial signaling component between αvβ5 integrin and MerTK linking binding and engulfment mechanisms. FAK mediates the activation of MerTK directly or indirectly by increasing phosphorylation and therefore activity of this receptor [78]. FAK resides in a complex with αvβ5 at the apical plasma membrane domain of the RPE, the site of POS phagocytosis. αvβ5 integrin ligation during POS binding first increases FAK recruitment to the apical complex, in which it is phosphorylated at multiple tyrosine residues. Phosphorylated FAK then dissociates from the complex and redistributes from the apical membrane to the RPE cytoplasm. In rodent retina, *in vivo* FAK phosphorylation and activity peak right after light onset. Synchronized tyrosine phosphorylation of MerTK can be detected subsequently, 2 hours after light onset. These activity peaks are absent in β5 integrin and MFG-E8 knockout mice demonstrating the requirement for MFG-E8-αvβ5 signaling for elevating MerTK activity during the diurnal burst of POS clearance [5, 36].

As much as αvβ5 signaling regulates MerTK activity, reverse receptor cross talk also takes place in RPE cells: RPE cells use a MerTK dependent feedback mechanism to limit phagocytic particle binding by αvβ5 integrin [56]. RPE cells that transiently or permanently lack the

expression of MerTK bind excessive numbers of POS via surface αvβ5 receptors. A small fraction of MerTK is cleaved and released as soluble extracellular fragment (sMerTK) during POS phagocytosis *in vivo* and *in vitro* and may further contribute to the regulation of the RPE's phagocytic capacity. sMerTK may act as decoy receptor blocking effects of MerTK ligands on RPE cells, but cell culture assays found that sMerTK affects mainly POS binding [70].

Like for all forms of phagocytosis, F-actin cytoskeletal re-arrangement is the cardinal process required for particle engulfment by RPE cells [51]. The RPE's long apical microvilli are based on F-actin and reach into the subretinal space interdigitating with intact photoreceptor outer segments. These structures are likely distinct from the *de novo* F-actin recruitment beneath surface-bound phagocytic particles for formation of structures called "phagocytic cups" that are needed during phagocytic processes. POS internalization requires closure of phagocytic cups, which depends on further F-actin reorganization together with plasma membrane fusion.

Both αvβ5 integrin and MerTK signaling contribute to F-actin dynamics during POS clearance by RPE cells. Activated Rac1, a member of the Rho GTPase family of primary cellular regulators of the F-actin cytoskeleton dynamics, promotes the recruitment of F-actin cytoskeletal elements. MFG-E8-ligated αvβ5 integrin is required for both MerTK activation via FAK and F-actin recruitment via Rac1. Both pathways are required for phagocytic clearance, but Rac1 activation does not require MerTK [43]. Annexin A2 (Anx2 in Fig. 3.2), a cytosolic Ca^{2+}- and phospholipid binding protein and regulator of F-actin dynamics serves in early stages of phagocytosis during phagocytic cup closure [49]. Annexin A2 is recruited to nascent phagocytic cups in RPE cells in culture and dissociates once phagosomes have been internalized. Tyrosine phosphorylation of Annexin A2 is increased at the peak of phagocytic activity in wild-type mice. In annexin A2 knockout mice POS phagocytosis is slightly attenuated and the peak of FAK activation is delayed but not eliminated. These observations suggest that annexin A2 recruitment to the forming phagosome leads to the activation of Src kinase, which is required for the activation of FAK and downstream MerTK activation.

MerTK also directly contributes to F-actin cytoskeletal rearrangement in a way that mobilizes non-muscle myosin II (myoII in Fig. 3.2) from the RPE cell periphery to sites of POS engulfment [79]. Akt signaling contributes to regulation of this process as Akt inhibition increases both the number of phagocytic cups and the recruitment of F-actin and myosin II to individual phagocytic cups in RPE cells in culture [80]. This role of Akt is independent and contrasts with the contribution of PI3 kinase in POS engulfment: PI3 kinase inhibition during POS binding weakens F-actin association with bound POS, has no effect on myosin-II recruitment, and inhibits engulfment. Note that unrelated to their opposing roles in internalization, inhibition of either Akt or PI3 kinase increases POS binding by cultured RPE cells. Taken together, Akt functions downstream of PI3 kinase in POS binding, while its inhibitory role in early events of F-actin assembly and rearrangement beneath bound particles that promotes OS engulfment is distinct from PI3-kinase.

Signaling mediators of the IP_3/Ca^{2+} intracellular signaling system and the cAMP second messenger system have both been reported to regulate RPE phagocytosis *in vitro*. Pharmacological increases in intracellular cAMP levels in cultured RPE reduce phagocytosis of ROS, while similar increases in cGMP had no effect [81–83]. Stimulation of RPE adenosine A2 receptors, which induces generation of intracellular cAMP, also reduce POS internalization by RPE cells in culture [84]. MerTK ligation during POS phagocytosis by RPE cells in culture may increase IP_3, which in turn activates ingestion of bound POS [78, 85, 86]. Elevating IP_3 levels pharmacologically is sufficient to increase phagocytic activity of RCS RPE cells in culture [87]. As expected from RTK/IP_3 signaling, RPE phagocytosis also involves a rise in intracellular free Ca^{2+} [88]. Ca^{2+} oscillations in RPE monolayers in regions with bound POS have been reported [89]. However, the specific and essential targets of IP_3 signaling remain to be uncovered.

Src kinase interacts with MerTK and its phosphorylation and activation is increased after phagocytic challenge of cells and downstream of MerTK [90]. Downstream of Src signaling, MerTK dependent tyrosine phosphorylation of GDP dissociation inhibitor alpha (GDI1) may regulate Rab GTPase dependent membrane dynamics such as vesicle fusion [91].

Another downstream effect of Src kinase signaling during RPE phagocytosis is activation of L-type Ca^{2+} channels [92]. Blocking L-type channels in cultured RPE cells reduces phagocytosis, and ligation of POS binding receptor integrins activates L-type Ca^{2+} channel activity [40]. Further, MaxiK Ca^{2+} dependent K^+ (BK) ion channels, L-type Ca^{2+} channels and bestrophin-1 contribute to intracellular Ca^{2+} homeostasis and affect POS phagocytosis by RPE cells in culture [93]. Lack of BK or $Ca_v1.3$ L-type Ca^{2+} channels in mice leads to a shift in phagocytosis rhythm and shortened outer segments suggesting an imbalance of POS shedding and outer segment growth [94]. Moreover, a recent report showed cAMP dependent circadian rhythms in Ca^{2+} spiking frequencies and bead uptake by human RPE cells in culture [95]. If and how these signaling processes are linked to the known phagocytic machinery for POS remains to be shown.

Altogether, the ligation of αvβ5 integrin and MerTK via the complex signaling mechanisms discussed above result in post-translational protein activity changes through altered protein phosphorylation and changes in GTP load that allow re-arrangement of the cytoskeleton and plasma membrane required for POS intake.

In the eye, these processes occur after light onset in a synchronized and highly coordinated fashion that promote the characteristic phagocytic burst. Importantly, elevated signaling is swiftly terminated by RPE cells at the end of its morning phagocytic activity [5, 43]. The molecular mechanisms used in the retina to inactivate phagocytic signaling after the daily phagocytic burst remain only partly understood. They include the synchronized activation of the RPE surface receptor plexin B1 (plxnB1; plxB1 in Fig. 3.2) by its ligand semaphorin 4D (sema4D; Sem4D in Fig. 3.2), which is found in the rat retina especially in or on cone photoreceptors [96]. In wild-type but not in RCS phagocytosis-defective rat retina, sema4D levels and, likely as a result, plxnB1 phosphorylation are reduced 1 hour after light onset as compared to either 1 hour before or 3 hours after. Mice lacking either plxnB1 or sema4D show increased numbers of POS in the RPE 1 hour after light onset suggesting a physiological function of sema4D/plxnB1 signaling in attenuating the phagocytic burst. Mechanistically, plxnB1 signaling acts on inhibiting F-actin dynamics required for phagocytosis: adding purified Sema4D to RPE cells in culture during POS challenge prevents Rac1 GTPase activation abolishing POS internalization without affecting POS binding.

Processing and Degradation of Phagocytosed POS by RPE Cells

RPE cells need to digest internalized POS material promptly and completely to prevent gradually buildup of undigested debris that is toxic for the RPE and may contribute to age-related retinal dysfunction. Phagosomes (phago in Fig. 3.2) move from apical to basal RPE regions to mature and fuse with lysosomes (lys in Fig. 3.2), forming phagolysosomes (phago lys in Fig. 3.2), and degrade their content. The molecular control of these phagolysosomal digestion processes remains only poorly understood.

Post-engulfment phagosome transport inside RPE cells depends on both F-actin and microtubule dependent processes. In the shaker-1 mouse model, RPE cells lacking functional F-actin motor myosin VIIA (myoVIIa in Fig. 3.2) show delay of phagosomes exit from the F-actin-rich apical region [97, 98]. Human myosin VIIA mutations cause Usher 1B, a deaf-blindness disorder [99, 100].

Having exited the apical F-actin cytoskeleton on their way towards central and basal areas of the RPE cell, POS phagosomes move bidirectionally along microtubules while associated with kinesin-1 light chain 1 (KLC1) [101]. Lack of KLC1 results in defects of phagosome trafficking, possibly decreasing the probability of

phagosome fusion with other vesicles. Impaired POS phagosome degradation eventually increases accumulation of RPE and sub-RPE deposits resulting in a pro-oxidative, pro-inflammatory environment.

The degradation of engulfed POS protein and lipid components requires enzymatic hydrolyses. Earlier studies focused on degradation of POS opsin, the by far most abundant protein in POS. Early during maturation und movement towards the cell body phagosomes transiently interact with endosomes (en in Fig. 3.2) such that opsin undergoes limited proteolysis even before bona fide phagosome-lysosome fusion [35]. Activities of cathepsin D and cathepsin S proteases (cath S, cath D in Fig. 3.2) and phagosomal acidification have been shown to be essential for efficient lysosomal opsin degradation [30, 45]. In transgenic mice expressing a mutant form of cathepsin D resulting in impaired processing of internalized POS RPE cells accumulate autofluorescent opsin-positive inclusion [8]. Ultimately, fusion with cathepsin D positive lysosomes promotes step-wise opsin degradation [35]. Synchronized appearance of cathepsin D in phagolysosomes correlates with decreasing levels of detectable opsin and progressive acidification [102]. Cathepsin D activity fluctuates in the RPE with its diurnal maximum at the time of peak phagocytic activity [103].

Recent studies have shed light on the enormous importance of POS lipid digestion. For disposal of POS lipids and recycling of metabolic intermediates back to the outer retina RPE cells use fatty acid β-oxidation and ketogenesis pathways, which support the cells energy demand and prevent buildup of lipid accumulation, which causes oxidative stress and mitochondrial dysfunction. POS phagosome maturation and processing are linked to ketogenesis and release of β-hydroxibutyrate (β-HB) [27]. Cultured RPE cells release increased levels of the ketone body β-HB apically after challenged with POS. Mouse RPE/choroid explants mainly release β-HB levels after light onset at the time of the daily burst of phagocytic activity. Animal models of delayed phagosome processing or abnormal phagosome lipid content show a time shift in the β-HB release illustrating the importance of tight temporal regulation for long term retinal health.

Our understanding of the control of POS phagosomal processing remains limited but there is evidence that it is precisely regulated at the level of organelle fusion. Rab escort-protein-1 (REP-1) supports posttranslational isoprenyl modification of Rab GTPases that control vesicle formation, movement, docking and fusion. RPE cells in culture lacking REP-1 internalize POS like control cells but show delayed POS protein clearance [104]. This suggests that absence of REP-1 inhibits POS specific phagosomal-lysosomal fusion events through aberrant Rab GTPase activities. In general, fusion with lysosomes is a required step in ensuring that the pH of POS phagosomes decreases sufficiently to allow enzymatic hydrolyses. Inhibition of the vacuolar-type ATPase (v-ATPase) proton pump by Bafilomycin A1 prevents POS degradation even if cathepsin D is present in phagosomes [102]. Acidification is affected by signaling through receptors such as adenosine A2 receptors, P2X7 receptors, and CFTR although it remains to be tested if such signaling is dynamic or specifically controlling diurnal POS clearance [105–107]. The scaffolding protein caveolin-1 (cav-1 in Fig. 3.2) contributes to POS phagolysosomal acidification. Caveolin-1 resides on maturing phagolysosomes in RPE cells *in vivo* and in cell culture and is essential for phagolysosomal POS degradation [47]. RPE-specific deletion of caveolin-1 *in vivo* reverses rhythmic profiles of levels and activity of lysosomal enzymes and impairs photoreceptor function. Lowering caveolin-1 protein levels in RPE cells in culture is sufficient to impair lysosomal acidification decreasing lysosomal and phagolysosomal enzyme activities.

Like caveolin-1, βA3/A1 crystallin (βA3/A1 cryst in Fig. 3.2) is localized to lysosomes and required for degradation of POS [108]. In RPE-specific βA3/A1 crystallin knockout mice V-ATPase activity is decreased and lysosomal pH elevated, resulting in undigested POS accumulation. Mechanistically, βA3/A1 crystallin affects lysosomal acidification by interacting with and presumably regulating activity of v-ATPase proton pump components [109].

In addition to completing POS renewal every 24 hours for life, RPE cells must coordinate processes of POS renewal with other cellular maintenance activities. Specifically, regulated routine organelle maintenance and degradation of protein aggregates via autophagic processes also employ and occupy lysosomes. Indeed, molecular mechanisms of POS degradation partly overlap with macroautophagy and the two degradative pathways via shared use of lysosomes influence each other. The shared presence of microtubule-associated protein 1 light chain 3 (LC3) on POS phagosomes and autophagosomes in RPE cells has recently led to a new classification of RPE phagocytosis as belonging to "LC3-associated phagocytosis" (LAP) pathways [110]. A key protein coordinating POS turnover and macroautophagy in RPE cells is melanoregulin (MREG), an intracellular sorting protein that is hypothesized to play a role in organelle biogenesis including lysosome maturation and intracellular trafficking. Lack of MREG results in reduced cathepsin D activity and delayed degradation of engulfed POS by RPE cells *in vivo* and in culture [7]. MREG links and may balance macroautophagic and phagocytic processes by interacting with LC3 and coordinating its association with phagosomes in the RPE [111].

Taken together, RPE cells tightly regulate degradation of engulfed POS to ensure both coordination with autophagy and completion within 24 hours and in time for the next phagocytic burst. How intracellular processing is triggered by phagocytic surface receptors and their signaling pathways remains to be elucidated.

Defects in POS Clearance Phagocytosis by RPE Cells and Human Retinal Disease

Animal models with specific molecular or engineered defects in POS renewal show retinal abnormalities ranging from rapid early onset and complete retinal degeneration of the RCS rat to abnormal but gradual accumulation of undigested POS debris followed by photoreceptor dysfunction [5] or of little impact on visual function within the short life span of a rodent [7]. In human patients, inherited defects in engulfment of POS and subsequent degradation are also associated with inherited retinal degenerative diseases.

Disease causing mutations in the engulfment receptor MerTK were identified in patients with early onset retinitis pigmentosa who suffer from severe retinal degenerations [112]. MerTK mutations have also been linked to rare retinal dystrophies and severe rod cone dystrophy [113, 114]. The very rapid and complete retinal degeneration found in the RCS rat is not seen in human RP due to MerTK mutation found so far, which suggests that POS phagocytosis takes place to some extent in affected patients, either through partly active MerTK or through alternate pathways.

Another progressive degeneration of RPE, photoreceptors and choroid, choroideremia (CHM) is caused by mutations in the CHM gene encoding REP-1 [115]. CHM patients have less or no REP-1 protein [116, 117] and show accumulation of unprocessed POS material in the RPE and excess inflammatory cells in the choroid [104].

RPE cells are post-mitotic with high phagocytic activities and high metabolic demand. Partly degraded and oxidized debris material gradually accumulates in autofluorescent lipofuscin granules in human RPE with age. Lipofuscin is rich in oxidized lipids including retinoid derivatives some of them directly and specifically toxic to RPE and harmful to its phagocytic activity [118, 119]. Lipofuscin arises from gradual and long-term accumulation of incompletely degraded, oxidized remnants of POS phagolysosome and/or autophagosome content. Although still poorly understood, all evidence suggests that excessive lipofuscin accumulation in the aging human eye impairs RPE function and health and secondarily affects vision as support for the neural retina by compromised RPE cells fails.

Outlook

Exciting technical and conceptual progress in understanding POS phagocytosis has provided the foundation for the development of new treat-

ments for RPE pathologic conditions. Specifically, MerTK mutation associated retinal degenerations have been the focus of gene and cell replacement therapeutic approaches as well as drug studies. AAV hMerTK vector treatment introduced photoreceptor rescue in the RCS rat and MerTK null-mouse animal models [120, 121]. Clinical trials for different forms of MerTK-associated retinal dystrophies are on the way [122]. Complete replacement of RPE cells generated from various stem cell sources like pluripotent (hESC), induced pluripotent (iPSC) and adult RPE stem cells (RPESC) is also under development [123]. Further, patient specific iPSC derived RPE models have become available that allow screening approaches to potential new treatments like in a nonsense variant of MerTK-RPE readthrough inducing drugs to restore production of a full length protein [124].

On the basic research side, it has become clear that strict coordination of photoreceptor growth and shedding, phagocytosis of spent POS tips and degradation and waste removal must create a precise homeostatic balance that is essential for photoreceptor and RPE health. Many open questions remain on the signaling mechanisms used by both RPE and photoreceptors to communicate and fine tune these processes including how RPE cells contribute to shedding. Much remains to be learned about phagosome processing, degradation, recycling and transport processes towards the retina and the choroid. Recent methodological advances will surely allow addressing these important issues in the near future.

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