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Rab GTPases in the differential processing of phagocytosed pathogens versus efferocytosed apoptotic cells

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Summary. Phagocytosis is an important feature of innate immunity in which invading microorganisms are engulfed, killed and degraded - and in some immune cells, their antigens presented to the adaptive immune system. A closely related process, efferocytosis, removes apoptotic cells, and is essential for the maintenance of homeostasis. Both phagocytosis and efferocytosis are tightly regulated processes that involve target recognition and uptake through specific receptors, followed by endolysosomal trafficking and processing of the internalized target. Central to the uptake and trafficking of these targets are the Rab family of small GTPases, which coordinate the engulfment and trafficking of both phagocytosed and efferocytosed materials through the endolysosomal system. Because of this regulatory function, Rab GTPases are often targeted by pathogens to escape phagocytosis. In this review, we will discuss the shared and differential roles of Rab GTPases in phagocytosis and efferocytosis.

Key words: Phagocytosis, Efferocytosis, Rab GTPase, Phagosome, Efferosome

Overview of the phagocytic and efferocytic process

Phagocytosis is an evolutionarily conserved mechanism which is thought to have arisen as a feeding mechanism for single-celled organisms such as amoeba (Yutin et al., 2009). In metazoans, phagocytosis plays a central role in both host defense and maintenance of tissue homeostasis. Phagocytosis is defined as the cellular uptake of microorganisms and other foreign particulates (>0.5 μm) into a plasma membrane-derived vacuole known as a phagosome (Esteban et al., 2015). In metazoans, phagocytosis not only targets foreign objects,

but also functions in mediating the uptake and clearance of apoptotic cells via a separate but similar process known as efferocytosis (Gordon, 2016). Phagocytosis and efferocytosis consist of four main steps: recognition of the target particle, receptor signaling and internalization, phagosome formation, and target particle degradation through phagosome maturation (Nordenfelt and Tapper, 2011).

Phagocytosis and efferocytosis are performed predominantly by dedicated professional phagocytes such as monocytes, macrophages, neutrophils, dendritic cells, eosinophils and osteoclasts. However, in certain tissues and under specific circumstances, phagocytosis and efferocytosis can be conducted by non-professional phagocytes such as endothelial cells, epithelial cells and fibroblasts (Sarode et al., 2017). Generally, these non-professional phagocytes engage efferocytosis and not phagocytosis (Rosales and Uribe-Querol, 2017). Phagocytes recognize target particles through a variety of cell surface receptors, which upon binding to a cognate ligand initiate signaling cascades that result in engulfment of the target into an intracellular vacuole termed the phagosome or efferosome. These receptors can be divided into two general classes: opsonic and non-opsonic receptors. Opsonic receptors do not recognize their targets directly, instead relying on host-derived opsonins (e.g. antibodies, mannose-binding lectin, Gas6) that bind to the target (Kuhlman et al., 1989; Scott et al., 2001). Non-opsonic phagocytosis or efferocytosis occurs via direct ligation of a receptor on the phagocyte with a ligand on the target particle - e.g. binding of dectin-1 to β-glucans on fungal cell walls, or Tim-4 to phosphatidylserine (PtdSer) on apoptotic cells (Miyanishi et al., 2007; Taylor et al., 2007). These receptors often engage in cooperative signaling with non-phagocytic pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), to enhance target detection and uptake (Yadav and Schorey, 2006; Shin et al., 2008).

Following ligation of the target particle, a signaling cascade is initiated by the phagocytic receptors, resulting

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in an actin remodeling process that extends the phagocyte plasma membrane to engulf the target particle (Kaplan, 1977; May et al., 2000). This engulfment process starts with the initial formation of a phagocytic cup; an invagination of the plasma membrane beneath the target particle (Lee et al., 2007). The distal margin of the cup is driven around the target by a combination of sequential engagement of phagocytic receptors, organization of these receptors into a phagocytic synapse, and the coordinated extension of the plasma membrane via actin reorganization (Freeman and Grinstein, 2014). For larger particles, focal exocytosis delivers additional membrane to the phagocytic cup to ensure that sufficient membrane is available for the complete envelopment of the target (Di et al., 2003). The resulting phagosome/efferosome then proceeds through a tightly-regulated endolysosomal maturation pathway which delivers the catabolic enzymes and other proteins that degrade the engulfed target. This degradative pathway is mediated by the sequential fusion of the phagosome or efferosome with early endosomes, late endosomes, and lastly lysosomes (Gordon et al., 1992; Berg et al., 1998).

Degradation of the engulfed target occurs predominantly during the phagolysosomal stage - i.e. after the phagosome or efferosome has undergone fusion with lysosomes. Within the phagolysosome, a combination of lysosomal hydrolases, acidification of the phagolysosomal compartment by vacuolar ATPases, and the production of reactive oxygen species (ROS) via the NADPH oxidase complex, act together to degrade the engulfed target (Jutras and Desjardins, 2005). In some cell types this is the final stage of phagocytosis, with the resulting material thought to be either absorbed by the phagocyte, or expelled from the cell (Yin et al., 2019). However, professional antigen presenting cells, notably dendritic cells and macrophages, add an additional step to the phagocytic process - formation of the MHC II loading compartment (MIIC) (Uribe-Querol and Rosales, 2017). The MIIC is derived from the phagolysosome, wherein short antigenic peptides derived from the phagocytosed target are loaded onto MHC II molecules. These are then exported to the cell surface, where alongside costimulatory molecules and cytokines also produced by the professional antigen presenting cell, they mediate activation and polarization of CD4^{+} helper T cells, thereby initiating an antigen-targeted adaptive immune response (Underhill and Goodridge, 2012). In contrast, phagolysosomes containing innocuous materials such as degraded apoptotic cells do not evolve into a MIIC. Rather, these apoptotic cell contents are absorbed or otherwise disposed of by the phagocyte without initiation of MIIC formation or the production of an adaptive immune response (Yin et al., 2016, 2019; Penteado et al., 2017). Inappropriate processing of apoptotic cell-derived material has been associated with a range of pathological processes including autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus, as well as chronic inflammatory disorders such as atherosclerosis (reviewed in Doran et al., 2019).

All stages of phagocytosis and efferocytosis - from the engulfment of the target, through to the final disposition of its remains - are tightly-regulated by members of the Rab (Ras related in brain) family of small GTPases. Rab GTPases are the largest family of small GTPases, and serve as master regulators of intracellular vesicle formation, trafficking and fusion (Stenmark, 2009). There are ~70 Rab subfamilies in humans, many of which are associated with phagocytic and/or efferocytic processes (Murray et al., 2005; Husebye et al., 2010; Flannagan et al., 2012). In this review, we discuss critical functions of Rab GTPases in phagocytosis, efferocytosis, and in the differential processing of phagocytic versus efferocytic cargos.

Rab GTPases

Rab proteins are master regulators of vesicle formation, trafficking and fusion. To mediate these events, Rabs interact with a broad range of effector proteins, with Rab activity tightly regulated through upstream signaling (Mizuno-Yamasaki et al., 2012). Rab's function as nucleotide-dependent switches, possessing both GDP-bound 'off' and GTP-bound 'on' forms, with conversion from the GTP-bound to GDP-bound state induced by the Rab's intrinsic GTPase activity (Toma-Fukai and Shimizu, 2019). Inactive GDP-bound Rabs are recognized by a Rab escort protein (REP), which serves both as a chaperone for the Rab protein, and recruits a geranylgeranyl transferase (GGT), which catalyzes the geranylgeranylation of the Rab protein (Alexandrov et al., 1994; Müller and Goody, 2018). This geranylgeranylated, GDP-bound Rab protein is then bound by Rab GDP dissociation inhibitor (GDI) and GDI displacement factor (GDF), which function together to target the Rab to the appropriate intracellular membrane. This targeting is generally via a guanine nucleotide-exchange factor (GEF) which is recruited to the target membrane, often via phosphoinositide-specific PH domains or other targeting motifs. Once recruited to the target membrane, the GEF catalyzes the displacement of bound GDP on the Rab for GTP, thereby activating the Rab (Oesterlin et al., 2012).

Upon Rab activation by its GEF, the Rab protein proceeds to recruit its effectors, with effector binding resulting in increased GEF activity. This Rab-GEF-effector positive feedback loop stabilizes the activated Rab protein on its target membrane, where the Rab can then mediate its activity via its effectors. There is a large plethora of Rab effectors, many of which are beyond the scope of this review. For a detailed review of Rabs and their effectors, see these excellent reviews (Hutagalung and Novick, 2011; Pylypenko et al., 2018). Depending on the Rab, the recruited effectors can mediate a number of cellular events, including the recruitment of dyneins and other proteins involved in the budding of vesicles off of target membranes, interactions with myosins and

kinesins to mediate the intracellular movement of vesicles, and interactions with soluble NSF attachment protein receptors (SNAREs) to mediate vesicle-vesicle fusion (Söllner et al., 1993; Park and Loh, 2008). Rab proteins are inactivated by the activity of GTPase-activating proteins (GAP's), which stimulate the hydrolysis of GTP, converting the Rab protein back to its inactive GDP-bound state (Bollag and McCormick, 1991; al-Alawi et al., 1993; Li and Marlin, 2015).

Particle uptake

Phagocytic uptake is a receptor-mediated and actin-driven process, requiring significant reorganization of the actin cytoskeleton beneath the forming phagocytic cup (Lee et al., 2007). The importance of membrane remodeling during phagocytosis in terms of both lipid composition changes and delivery of new membrane to the site of particle binding is well characterized (reviewed in Rougerie et al., 2013). The extension of the plasma membrane around the target particle requires that new membrane be added to the growing phagocytic cup. This addition of membrane occurs through the focal exocytosis of intracellular vesicles derived from the Golgi and endocytic system (Vashi et al., 2017). This exocytic process is regulated by Arf6, a GTPase which regulates trafficking to and from recycling endosome compartments (Aikawa and Martin, 2003; Vashi et al., 2017). While Arf6 is not itself a Rab GTPase, it is a member of the larger small GTPase family, and functions in a similar manner to Rab GTPases (Chardin et al., 1996; Donaldson and Jackson, 2011). While Arf6 is the primary mediator of focal exocytosis at the phagocytic cup, its activity is regulated by Rab GTPases that also localize to the phagocytic cup. These Rabs are recruited by the presence of specific phosphatidylinositol species generated by phagocytic signaling (reviewed in Gutierrez, 2013). Specifically, Rab8a, 8b 10, 13, 27a and 35 are recruited by phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which is generated in the phagocytic cup from plasma membrane phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) (Tamura et al., 2009). This lipid phosphorylation is performed by receptor-activated type I phosphatidylinositol-3-kinases (PI3Ks) whose activity is stimulated by their binding to phosphorylated (activated) phagocytic receptors (Byekova et al., 2010; Yeo et al., 2016; Demirdjian et al., 2018). While multiple Rabs are recruited to the forming cup, knockdown of only Rab13 or Rab35 inhibits phagocytosis, suggesting that the other Rabs recruited to the phagocytic cup are either functionally redundant, or that their recruitment is dispensable for phagocytosis (Yeo et al., 2016). Rab35 directly controls focal exocytosis at the phagocytic cup through mediating recruitment of the Arf6 GAP ACAP2, thus controlling the activity of Arf6 (Egami et al., 2011). The role of Rab13 is less well defined, but studies have suggested that Rab13 is recruited to the phagocytic cup by the Rab35 effector MICAL-L1, and that Rab13 may be

required for the focal exocytosis of phagocytic receptor-bearing vesicles during phagocytic cup formation (Kobayashi et al., 2014). In addition, Rab13, via its effector MICAL-L2, recruits the actin-regulatory GTPase RhoA, and therefore may also contributes to RhoA-mediated actin reorganization at the phagocytic cup (Ioannou and McPherson, 2016). While the roles of these Rab GTPases at the forming phagocytic cup remain only partially elucidated, it is clear that these proteins play a critical regulatory role necessary for the extension of the plasma membrane around the phagocytic target.

Phagosome maturation

Once phagocytic targets are internalized, they are confined within a plasma membrane-derived vesicle known as a phagosome which undergoes maturation through fusion with an increasingly acidic series of endolysosomal vesicles, resulting in the degradation of the phagocytosed cargo (Lukacs et al., 1990; Canton et al., 2014). Some pathogens modulate Rab activity to promote their intracellular growth, typically by blocking or altering the phagosome maturation pathway. Bacteria including *Mycobacterium tuberculosis, Helicobacter pylori, Salmonella,* and *Chlamydia* all disrupt phagosome maturation through the selective retention or exclusion of Rabs on vacuolar membranes (Brumell and Scidmore, 2007), highlighting the importance of Rab-mediated regulation of phagosome maturation in pathogen killing. Phagosome maturation is a step-wise process, in which the phagosome progresses through early, late and phagolysosomal maturation stages (Fig. 1), with each stage and the transition between stages regulated by Rab GTPases. More than 20 Rab proteins have been detected on phagosomes (Table 1), with the known roles of these GTPases reviewed below.
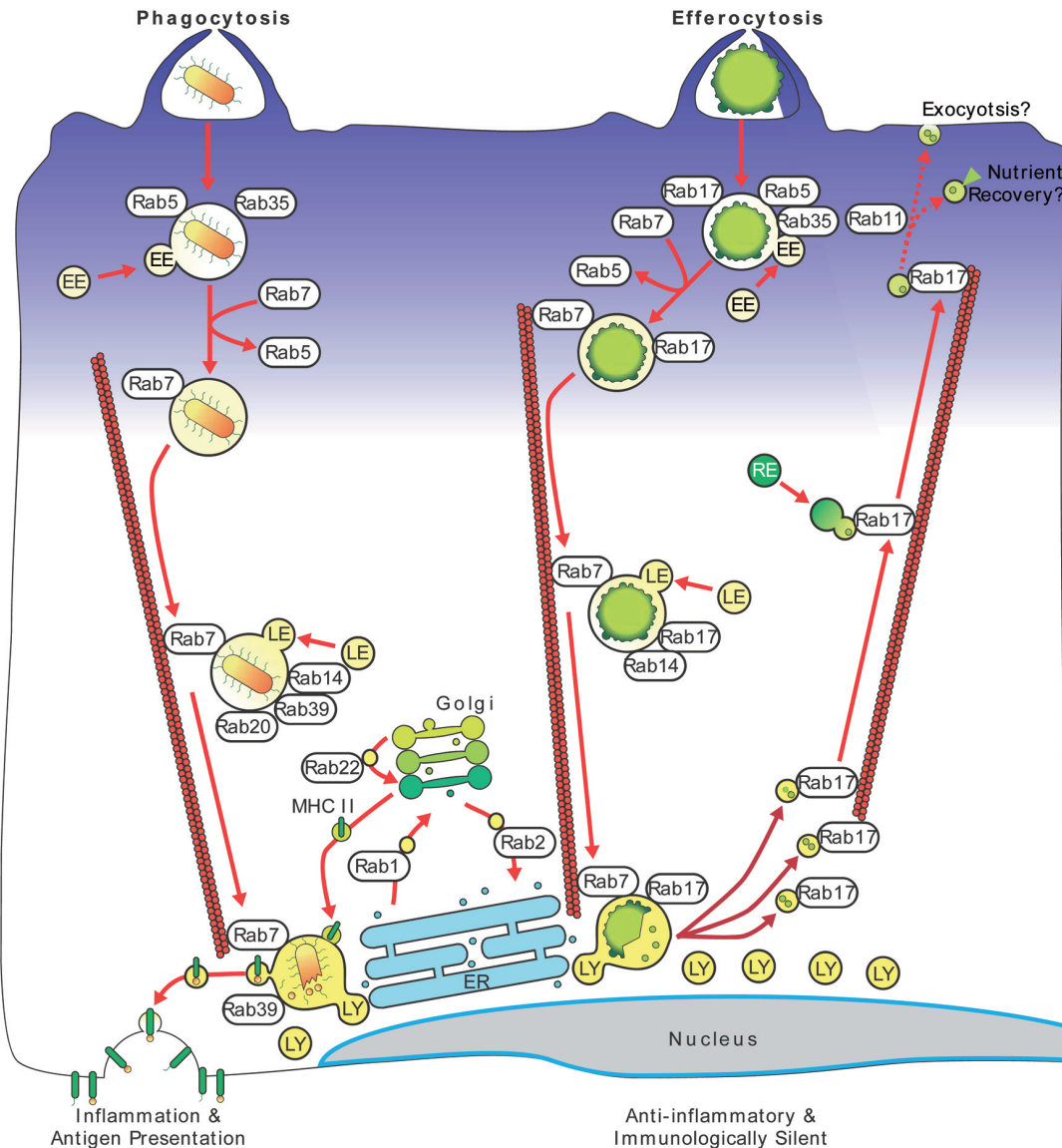
Early phagosome

Early phagosomes are characterized by the presence of Rab5 on the phagosomal membrane. Initially, the GEF Rabex-5 is recruited to early phagosomes by Rab22; an early endosome Rab GTPase which is targeted to the phagosome via a currently unknown mechanism (Roberts et al., 2006). Active Rab5 recruits additional Rabex-5 as well as another Rab5 effector, Rabaptin-5, which enhances the activity of Rabex-5, generating a positive feedback loop for Rab5 activation (Zhu et al., 2007). During phagosome closure, $PI(4,5)P_2$ and PIP_3 are dephosphorylated by lipid phosphatases such as PTEN and INPP5, producing an early phagosome bearing phosphatidylinositol as its predominant inositide species (Kamen et al., 2007; Serezani et al., 2012; Segawa et al., 2014b). One of the first activities conducted by Rab5 on the early phagosome is activation of the type III PI3K Vps34 that generates phosphatidylinositol 3-phosphate (PI(3)P) on the cytosolic leaflet of early phagosomes (Kinchen et al.,

2008). PI(3)P serves to dock and activate Rab5 effectors including endosomal early antigen 1 (EEA1) and rabenosyn-5 (Simonsen et al., 1998; Nielsen et al., 2000). Rab5 and its effectors then mediate the formation of the large class C core vacuole/endosomes tethering (CORVET) complex, comprised of a core of Vps11, 16, 18, 33 plus the CORVET-specific Vps3 and 8 subunits (Balderhaar and Ungermann, 2013). This complex mediates the tethering of early endosomes to the maturing phagosome, through bridging active Rab5 on the phagosome to active Rab5 on the early endosome. This bridging of an early endosome to the phagosome sets the stage for fusion, which is initiated by the SNARE syntaxin 13, with fusion requiring SNARE activation by Rab5 and NSF (Collins et al., 2002; Becken et al., 2010). Fusion with early endosomes begins the delivery of vacuolar ATPases to the phagosome, which reduces the pH of the phagosome from that of the extracellular milieu (pH 7.4) to a pH of 6.5 (Davies et al., 1994; Flannagan et al., 2009).

Other Rab proteins that are involved in the regulation of early phagosome maturation include Rab22a and Rab14. As discussed above, Rab22a, which is highly homologous to Rab5, induces recruitment of Rabex-5 and Rab5 activation, promoting fusion of early endosomes with the nascent phagosome (Zhu et al., 2009). Rab14 also has been shown to play a role in the regulation of fusion between the phagosome and early endosomes (Gutierrez, 2013). While the role of these Rab GTPases are not as defined as the roles of Rab5, their function is critical for phagosome maturation, and indeed, mycobacterial pathogens manipulate Rab14 and

Fig. 1. Model illustrating the role of Rab GTPases in phagocytosis and efferocytosis. Both processes involve maturation of the phagosome or efferosome through sequential fusion with early endosomes (EE), late endosomes (LE) and lysosomes (LY). In phagocytosis, following lysosomal fusion, the phagolysosome matures into a MHC II loading compartment through delivery of newly synthsized MHC II to the phagolysosome. In contrast, in efferocytosis, the late efferosome breaks into smaller vesicles which traffick to the cell periphery where they undergo fusion with the recycling endosome system (RE).

Rab22 to block phagosome maturation at the early (e.g. minimally microbicidal) phagosome stage (Kyei et al., 2006; Roberts et al., 2006).

Late phagosome

Following fusion with early endosomes, Rab5 triggers the next stage of phagosome maturation by facilitating its exchange with Rab7 (Vieira et al., 2003). The transition of Rab5 to Rab7 is driven by Mon1 and Ccz1, which together serve as the Rab7 GEF (Nordmann et al., 2010). Recruitment of the Mon1/Ccz1 complex terminates Rab5 activation by displacing Rabex-5 from the early phagosome, while also recruiting and activating Rab7 (Kinchen and Ravichandran, 2010; Poteryaev et al., 2010). Rab7, through its effectors Rab-Interacting Lysosomal Protein (RILP) and oxysterol-binding protein related-protein 1 (ORP1L), recruits dynein/dynactin to the phagosome, causing the phagosome to migrate along microtubules to the perinuclear region (Toyohara and Inaba, 1989; Blocker et al., 1998; Johnson et al., 2016). Here, Rab7 seeds the formation of the HOPS complex which shares the same core as the CORVET complex (Vps11, 16, 18 and 33), plus bears the HOPS-specific effectors Vps39 and 41 (Harrison et al., 2003; Johansson et al., 2007). Like the CORVET complex, the HOPS complex serves as a bridge between the phagosome and intracellular vesicles, but differs in that the HOPS complex tethers vesicles bearing Rab7, allowing the phagosome to fuse with late endosomes and lysosomes (Balderhaar and Ungermann, 2013). These fusion events deliver the hydrolytic enzymes required for killing and degradation of the phagocytosed pathogen, as well as additional vacuolar ATPases, reducing the luminal pH to 5.0 or lower, thereby activating these hydrolases (Johnson et al., 2016). Following fusion with late endosomes and lysosomes the phagosome matures into a fully microbicidal phagolysosome (Pauwels et al., 2017).

Phagolysosome

In the last phagosome maturation stage, late phagosomes fuse with lysosomes to become phagolysosomes, which are regarded as the ultimate microbicidal organelle (Harrison et al., 2003). Phagolysosomes, through a number of mechanisms, drive the killing and degradation of phagocytosed microorganisms. Defects in the process of phagolysosome formation and maturation cause a number of negative consequences. For instance, mutations in lysosome-associated membrane protein-2 (LAMP2) recruitment to the late phagosome is associated with impaired fusion of phagosomes with lysosomes, and contributes to the development of severe periodontitis early in life due to reduced bacterial killing by phagocytes such as neutrophils (Beertsen et al., 2008).

The recruitment of large numbers of V-ATPase molecules on the phagolysosomal membrane is responsible for establishing the highly acidic environment of the phagolysosomal lumen that drives pathogen killing and degradation (Marshansky and Futai, 2008). Fusion of lysosomes results in the delivery of hydrolytic enzymes including glycosidases, lipases, DNases and proteases such as cathepsins to the phagolysosomes. Cathepsins and other lysosomal proteases require low pH for optimal activity, and therefore work synergistically with vATPase-mediated acidification of the phagolysosome (Strasser et al., 1999; Pauwels et al., 2017). Phagolysosomes also contribute to microorganism destruction through concentrating ROS and reactive nitrogen species (RNS) which have microbiocidal properties (Castrillo et al., 2001; Winterbourn et al., 2006). Finally, previous studies have shown that antimicrobial peptides including defensins and cathelicidins are delivered to the phagolysosomes to disrupt the integrity of phagocytized pathogens (Selsted et al., 1993; Larrick et al., 1994; Bensch et al., 1995).

Rab7, 20, 22b, 32, 34, 38 and 43 are all known to be involved in the delivery of hydrolases such as cathepsin D to the phagosome (Seto et al., 2011). Rab27 has been reported to function on later phagolysosomes by regulation of phagosomal pH and NADPH production, thus promoting antigen cross-presentation (Jancic et al.,

Table 1. Rab GTPases involved in the regulation of phagosome and efferosome maturation.

Rab GTPases	Function	Pathway	Reference
Rab1	ER to Golgi transport	Phagocytosis	Saraste, 2016
Rab2	ER to Golgi transport	Phagocytosis/ Efferocytosis	Saraste, 2016
Rab5	Required from nascent phagosome to Rab7(+) stage	Phagocytosis/ Efferocytosis	Mottola, 2014
Rab7	Required from Rab5(+) to LAMP	Phagocytosis/ Efferocytosis	Bröcker et al., 2012
Rab11	Transport from Golgi to polarized membrane, v-ATPase transport	Phagocytosis	Jing and Prekeris, 2009
Rab14	Phagosome and early endosome fusion, trafficking between early endosomes and Golgi	Phagocytosis/ Efferocytosis	Yeo et al., 2016
Rab17	Efferosome to recycling endosome trafficking	Efferocytosis	Yin et al., 2016
Rab20	Required for late phagosome	Phagocytosis	Egami and Araki, 2012
Rab22	Trans-Golgi-endosome transport	Phagocytosis	Weber and Faris, 2018
Rab35	Required for early phagosome	Phagocytosis/Efferocytosis	Yeo et al., 2016
Rab39	Required for late phagosome	Phagocytosis	Yeo et al., 2016

2007). Additionally, Rab34 promotes phagolysosome biogenesis through interaction with RILP (Wang and Hong, 2002).

MHC II loading compartment formation

Professional antigen presenting cells add an additional step to phagosome maturation - conversion of the phagolysosome into an MIIC. The trafficking events driving this process are not well understood, in part because the pathway allowing for newly synthesized MHC II to be delivered to the phagolysosome remains unclear (Saric et al., 2015). MIIC formation begins with MHC II transcription and assembly of MHC II along with the invariant chain in the endoplasmic reticulum. The invariant chain binds to the antigen binding grove of the MHC II heterodimer, preventing the inadvertent loading of peptide prior to the complex reaching the phagolysosome (ten Broeke et al., 2013). Several studies have suggested that this complex is exported to the cell surface, and is then endocytosed and delivered to the MIIC via the canonical endolysosomal trafficking route (Schmid et al., 2007; Blander, 2018). This model is predicated on the observation that MHC II and invariant chain can be found on the cell surface; however, this complex is not observed colocalizing with endosomal markers such as Rab5, leading to the alternative hypothesis that the MHC II/invariant chain complex is instead delivered via direct Golgi-to-phagolysosome trafficking (Dörfel et al., 2005; Schmid et al., 2007). It is unclear at this time as to the extent these two trafficking routes contribute to the transport of MHC II to the phagolysosome, and consequentially, the Rab GTPases regulating this process remain to be described (Yin et al., 2016; Penteado et al., 2017). Regardless, once delivered to the phagolysosome, the invariant chain is cleaved by lysosomal proteases, leaving a small fragment in the MHC II antigen binding grove. Chaperones then mediate the exchange of this fragment for a phagolysosome-derived peptide, thereby loading the MHC II molecule (Stumptner and Benaroch, 1997; Landsverk et al., 2009). The antigen/MHC II complex is then exported to the cell surface, allowing for antigen presentation to CD4^{+} T helper cells (Rock et al., 2016).

Recycling endosome

While not directly involved in engulfment and killing of bacterial pathogens, the recycling endosome plays a critical role in phagocytosis. The recycling endosome is a highly dynamic system of endosomal structures and associated regulatory molecules that is responsible for recycling of internalized phagocytic receptors back to the cell surface to permit rapid, sequential clearance of multiple phagocytic targets (reviewed in Goldenring, 2015). Unsurprisingly, Rab GTPases are involved in multiple components of the recycling endosome system. Rab11 and Rab14 are known to regulate membrane receptor transport from the recycling endosome to the cell surface in phagocytic cells (Agola et al., 2011). Of these, Rab11 is the best studied and mediates the transport of vesicles from the trans-Golgi network to the recycling endosome, and from there to the cell surface (Horgan and McCaffrey, 2012). In particular, Rab11 activity is required for FcR-mediated phagocytosis, as macrophages expressing an inactive form of Rab11 have impaired FcR-mediated phagocytosis and reduced cell surface FcR expression (Cox et al., 2000). Rab10 is another critical regulator of phagosome recycling, and is recruited to the phagosomal membrane prior to the acquisition of Rab5. Here, Rab10 plays an indispensable role in facilitating the recycling of phagosomal GPI-linked proteins back to the plasma membrane (Cardoso et al., 2010). Rab10 overexpression partially rescues phagosomal maturation arrest induced by Mycobacteria, suggesting Rab10 is a potential target that can restore normal phagosome maturation during infection (Cardoso et al., 2010).

Efferosome maturation

The recognition, uptake and degradation of apoptotic cells through efferocytosis is a crucial mechanism in the maintenance of tissue homeostasis (Henson, 2017). In most cases, efferocytosis is performed by professional phagocytic cells — macrophages in particular are responsible for apoptotic cell clearance in many tissues of the body (Gordon and Plüddemann, 2018). Efferocytosis removes apoptotic cells from the body, thus preventing apoptotic cells from undergoing secondary necrosis - a pro-inflammatory process due to the release of proinflammatory cytosolic contents (Wickman et al., 2013; Henson, 2017; Morioka et al., 2019). Efferocytosis shares many parallels with phagocytosis, and utilizes many of the same Rab GTPases and vesicular trafficking events to engulf and degrade apoptotic cells (Fig. 1). However, even in professional antigen presenting cells, efferosomes do not mature into a MIIC, thus limiting the presentation of apoptotic cell derived antigens (e.g. self-antigens) to the adaptive immune system (Yin et al., 2016, 2019).

Although the process of apoptotic cell recognition, binding of the phagocyte to the apoptotic cell and subsequent apoptotic cell internalization and degradation is similar to the processes utilized during the phagocytosis of pathogens, efferocytosis utilizes a distinct set of receptors that recognize and bind to unique chemical motifs present on the surface of apoptotic cells. Although we provide a brief overview of apoptotic cell recognition and internalization below, these topics have recently been reviewed in detail by Flannagan et al. (2012) and Penberthy and Ravichandran (2016).

Recognition and uptake of efferocytic targets

Efferocytosis is initiated upon recognition of apoptotic cells by phagocytes. Efferocytic cells are

capable of recognizing specific ligands on the surface of the apoptotic cells, called "eat-me signals" (Elliott and Ravichandran, 2016). A number of eat-me signals have been identified, including exposure of PtdSer on the outer leaflet of the plasma membrane of apoptotic cells, alterations in the glycosylation patterns on the apoptotic cell surface, changes in ICAM-1 epitopes on the cell surface, and exposure of the endoplasmic protein calreticulin on the cell surface (reviewed in Green et al., 2016; Nagata, 2018). Of these eat-me signals, PtdSer exposure is the best understood. In healthy cells, PtdSer is sequestered on the cytoplasmic face of the plasma membrane through the activity of flippase enzymes (Kimani et al., 2014). During apoptosis, the amount of PtdSer exposed on the cell surface increased by more than 280-fold, comprising up to 10% of the exofacial lipid content (Borisenko et al., 2003). This large change in PtdSer distribution is driven by two caspase-mediated processes: the cleavage-induced inactivation of the flippases ATP11A and ATP11C, and the parallel cleavage-induced activation of the scramblase XRK8 (Suzuki et al., 2013; Segawa et al., 2014a; Suzuki et al., 2016).

Exposed eat-me signals on the surface of apoptotic cells are recognized by specialized efferocytic receptors (Freeman and Grinstein, 2014; Gordon and Plüddemann, 2018). Most known efferocytic receptors function by binding to PtdSer, either directly or through one or more opsonins. Among the efferocytic receptors that bind directly to PtdSer are members of the TIM family of receptors (including TIM-1, TIM-3 and TIM-4), brain angiogenesis inhibitor 1 (BAI1), Stabilin-2, members of the CD300 family of receptors, and the receptor for advanced glycation end products (RAGE) (Park et al., 2007; Miyanishi et al., 2007; Park et al., 2008; He et al., 2011; Nakahashi-Oda et al., 2012). Opsonin-dependent efferocytosis receptors include multiple integrins - recognizing apoptotic cells via MFG-E8 and sCD93, and the TAM family of receptors - recognizing apoptotic cells via the opsonins Gas6 and Protein S (Savill et al., 1990; Van der Meer et al., 2014; Blackburn et al., 2019).

Following recognition and binding, the phagocyte engulfs the apoptotic cell. The exact nature of this uptake mechanism is unclear, with *in vitro* experiments often observing the uptake of intact cells (Yin et al., 2016, 2019), while *in vivo* studies often observe a piecemeal disassembly of the apoptotic cell - often by multiple phagocytes (Wang et al., 2017). Regardless, the signaling and engulfment mechanisms appear to closely parallel pathogen phagocytosis: initial binding and receptor activation leads to the protrusions of the phagocyte's plasma membrane around the target cell, culminating in engulfment of the apoptotic cell (or cell fragment) into an intracellular efferosome (Karaji and Sattentau, 2017).

Early efferosome

Following closure of the phagocytic cup, the apoptotic cell becomes fully internalized within the efferosome, a plasma membrane-derived vacuole analogous to the phagosome (Flannagan et al., 2012). The efferosome undergoes a series of highly-regulated biochemical modifications to efficiently degrade the internalized apoptotic cell (Kinchen and Ravichandran, 2008). Similar to phagosome maturation, efferosome maturation is characterized by both sequential fusion with early and then late endosomes, along with progressive acidification of the efferosome lumen (Kinchen and Ravichandran, 2008). The regulation of the early stages of efferosome maturation is similar to that involved in early phagosome maturation and is characterized by the recruitment of the small GTPase Rab5 to the efferosome surface (Kinchen and Ravichandran, 2008). To date no studies have investigated the role of focal exocytosis by Rab13 and Rab35 during apoptotic cell engulfment, meaning that the earliest known efferosome maturation event following apoptotic cell internalization is the recruitment of Rab5 to the nascent efferosome (Kitano et al., 2008). However, in *C. elegans* Rab35 mutants do not effectively clear apoptotic cells and have delayed Rab5 accumulation on the efferosome surface, suggesting a similar role for Rab35 in efferocytosis and phagocytosis (Haley et al., 2018).

The stages and timing of the early efferosome maturation are very similar to phagocytosis. Indeed, our group has directly compared these processes, and aside from a slight delay in the Rab5-Rab7 transition on efferosomes, the early maturation steps appear identical (Yin et al., 2016). Many of the other events in phagosome maturation have been observed on efferosomes, including the Mon1/Ccz1 mediated exchange of Rab5 for Rab7, lysosome fusion, and efferosome acidification (Kinchen and Ravichandran, 2008). However, while phagocytosis results in the induction of an inflammatory immune response and presentation of antigens from the internalized pathogen, efferocytosis is immunologically silent and does not result in antigen presentation (McColl et al., 2009; Birge et al., 2016). Therefore, key differences must exist in the regulation of the late maturation of efferosomes compared to phagosomes to achieve these disparate outcomes.

Late efferosome

As in phagocytosis, replacement of Rab5 by Rab7 marks the transition from the early efferosome to the late efferosome (Kinchen and Ravichandran, 2008). Rab7 then recruits effectors that have crucial roles in driving apoptotic cell degradation. These mediators include RILP and ORP1L, which as in the late phagosome, interact with the dynein/dynactin motor complex to traffic late efferosomes to the perinuclear region (Harrison et al., 2003; Yin et al., 2019). Perinuclear localization is necessary for efficient fusion with lysosomes and the delivery of lysosomal hydrolases to the efferosome (Cantalupo et al., 2001; Johnson et al.,

2016). RILP further mediates v-ATPase assembly on the efferosome surface and drives efferosome acidification and activation of lysosomal hydrolases to complete degradation of the internalized apoptotic cell (De Luca et al., 2015). Thus far, late efferosome maturation closely resembles phagosome maturation. However, it is at this point where the two processes diverge (Fig. 1).

While pathogen-bearing phagolysosomes mature into a MIIC, efferosomes do not, and instead late efferosomes interact with the recycling endosome system (Yin et al., 2019). The small GTPase Rab17 is an essential regulator of this process (Yin et al., 2016, 2019; Ono et al., 2020). Rab17 has previously been characterized in the literature as a regulator of apical-to-basolateral transport during transcytosis in hepatic cells, and in the exocytosis melanosomes from melanocytes (Hunziker and Peters, 1998; Zacchi et al., 1998; Beaumont et al., 2011). Following apoptotic cell internalization, Rab17 is recruited to the maturing efferosome in parallel with Rab5, but unlike Rab5, Rab17 persists on the efferosome throughout the entire efferosome maturation process (Yin et al., 2016). Following lysosome fusion in the perinuclear region, Rab17 mediates the outwards migration of the efferosome towards the cell periphery. Here, the efferosome intermixes with recycling endosomes, presumably to complete the absorption of the apoptotic cell or to exocytose the degraded apoptotic cell debris (Yin et al., 2019). Recent work by Ono and colleagues has presented evidence that Rabex-5 and another Rab17-interacting protein, ALS, recruit Rab17 to the efferosome surface, with Rabex-5 acting as a Rab17 GEF (Mori et al., 2012; Ono et al., 2020). When a dominant negative Rab17 mutant is expressed, trafficking of degraded apoptotic cell contents to the recycling endosome does not occur, and instead, the apoptotic cell containing phagolysosome remains in the perinuclear area where it accumulates markers of an MIIC (Yin et al., 2016, 2019; Ono et al., 2020). Clearly, late efferosome maturation diverges from late phagosome maturation, with the selective retention of Rab17 to efferosomes playing a central role in the trafficking of efferocytosed apoptotic cell contents away from the MIIC and into the recycling endosome system, and in doing so, limits the loading of self-antigens onto MHC II and their subsequent presentation to CD4$^+$ T cells.

Recycling endosome

In addition to aiding in the final dispossession of apoptotic cells, the recycling endosome system plays other important roles during efferocytosis. For example, Chen et al. demonstrated that in *C. elegans* mutants lacking sorting nexins involved in vesicle trafficking within the recycling endosome system had impaired recycling of the efferocytic opsonin MFG-E8, resulting in lower levels of extracellular MFG-E8, poorer apoptotic cell opsonization, and a resulting increase in the numbers of uncleared apoptotic cells during germline development (Chen et al., 2010). Recruitment

of Rab17 to the recycling endosome occurs in a Rab11-dependent manner, suggesting that Rab11 activity may be critical to transport of degraded apoptotic cell contents from the late efferosome to the recycling endosome compartment (Ono et al., 2020). However, Rab11 activity may oppose efferocytosis under certain circumstances. Jiang et al. showed that reduction of Rab11 expression enhances the clearance of apoptotic neutrophils by macrophages in a murine model of airway inflammation (Jiang et al., 2017). They suggest that this was due to Rab11-mediate recycling of the metalloproteinase ADAM17, which when localized to the cell surface cleaved the efferocytic receptor CD36 (Jiang et al., 2017). Another Rab protein involved in the regulation of apoptotic cell clearance and endocytic recycling is Rab2 (Mangahas et al., 2008). Rab2 was initially identified through a loss-of-function screen as playing a role in apoptotic cell clearance in *C. elegans*, and was proposed to be a direct regulator of efferosome maturation. However, subsequent evidence has suggested that Rab2 is instead involved in targeting proteins to the recycling endosome system. In phagocytic cells, defective Rab2 activity results in the aberrant trafficking of endosome-associated proteins such as RME-1, resulting in their mis-localization to the cell surface (Lu et al., 2008; Mangahas et al., 2008).

Conclusion

Phagocytosis and efferocytosis are highly conserved processes that involve the binding of a target particle, internalization of the target into a phagosome or efferosome, and the maturation of the phagosome/efferosome through sequential fusion with endosomes and lysosomes. However, while phagocytosis is involved in pathogen elimination and results in inflammation and antigen presentation, efferocytosis removes apoptotic cells and is immunologically silent. As central regulators of membrane and vesicular trafficking, the Rab family of small GTPases play crucial roles in the regulation of the maturation processes of phagosomes and efferosomes, and help determine the fate of phagocytic and efferocytic cargo (Fig. 1). Unsurprisingly, pathogens often target Rab GTPases and their effectors to evade phagocytic killing, and Rab GTPases are putative targets for treating autoimmunity through manipulating the presentation of self-antigens (Lam et al., 2016). Future research is crucial for improving our understanding of how pathogens evade phagocytic killing, and how the aberrant presentation of apoptotic cell-derived antigens contribute to autoimmune disease.

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