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How macrophages deal with death

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Abstract

Tissue macrophages rapidly recognize and engulf apoptotic cells. These events require the display of 'eat-me' signals on the apoptotic cell surface, the most fundamental of which is phosphatidylserine (PtdSer). Externalization of this phospholipid is catalyzed by scramblase enzymes, several of which are activated by caspase cleavage. PtdSer is detected both by macrophage receptors that bind this phospholipid directly, and by receptors that bind a soluble 'bridging protein' that is independently bound to PtdSer. Prominent among the latter receptors are the Mer and Axl receptor tyrosine kinases. Eat-me signals also trigger macrophages to engulf virus-infected or metabolically traumatized, but still living cells; and this 'murder by phagocytosis' may be a common phenomenon. Finally, the localized presentation of PtdSer and other eat-me signals on delimited cell surface domains may enable the phagocytic pruning of these 'locally dead' domains by macrophages, most notably by microglia of the central nervous system.

Introduction

In long-lived organisms, abundant cell types are often short lived. In the human body for example, the lifespan of many white blood cells — including neutrophils, eosinophils, and platelets — is less than two weeks. For normal healthy humans, a direct consequence of this turnover is the routine generation of more than 100 billion dead cells each and every day of life[1,2]. This macroscopic mass of cell corpses, which is largely the product of apoptosis, must be recognized and cleared. These quotidian functions are carried out continuously, in a 'silent' non-inflammatory fashion, by tissue-resident macrophages, the dedicated undertakers of the immune system[3]. These often highly specialized cells mediate tissue homeostasis in all organs, and include marginal zone macrophages of the spleen, Kupffer cells of the liver, alveolar macrophages of the lungs, Langerhans cells of the skin, and microglia of the central nervous system[4]. In settings of fulminant infection or severe tissue

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trauma where cells may also die by immediate necrosis, the dead cell burden reaches even higher levels, but tissue-resident macrophages are again mobilized to eat these cells. Although apoptosis and necrosis are morphologically and physiologically distinct death processes — apoptotic cells shrink and their plasma membranes bleb but remain intact, whereas necrotic cells swell and their plasma membranes rupture[5] — the principal phagocytes that deal with both dead cell types are macrophages.

As apoptosis accounts for the bulk of the everyday dead cell burden, this Review focuses on recent findings with respect to the phagocytosis of apoptotic cells by tissue-resident macrophages — a process termed 'efferocytosis', from the Latin *efferre*, to carry to the grave[6] — and on the molecules that mediate this process. Efferocytosis is a remarkably efficient business: macrophages can engulf apoptotic cells in less than 10 minutes, and it is therefore difficult experimentally to detect free apoptotic cells *in vivo*, even in tissues where large numbers are generated[7]. Many of the molecules that macrophages and other phagocytes use to recognize dead cells are themselves the products of apoptosis, and are often generated via the action of the cysteine-aspartic acid proteases, most notably caspase 3 and caspase 7, that are the ultimate executioners of apoptotic signalling cascades[8]. Since defects in efferocytosis have significant consequences for tissue integrity, homeostasis and function, and can lead to the development of autoimmunity[9,10], identifying these molecules and elucidating their mechanisms of action is of genuine importance to understanding and treating human disease.

Finding dead cells

Find-me signals.

Many macrophages are embedded within tissues in which apoptosis occurs continuously (for example, the neurogenic regions of the adult brain)[11,12] or in which dead cells are delivered directly to phagocytes by the circulation (for example, the marginal zones of the spleen)[13]. In other settings, however, tissue-resident and blood-borne macrophages, or the processes of these cells, appear to be recruited and to migrate to apoptotic cell loci in response to cues. This may also be the case for tissues in which the number of resident macrophages is low relative to that of the cells surrounding them. In these settings, macrophages *in vitro*, and to a much more limited extent *in vivo*, have been shown to respond to a set of so-called 'find-me' signals[14,15], released by injured and dying cells, and to use these signals to migrate toward sites of cell death. The most-studied find-me signals have been, firstly, the lysophospholipids lysophosphatidylcholine (LPC)[16,17] and sphingosine-1-phosphate (S1P)[18], and secondly, the nucleotides ATP, AMP, and UTP[19]. LPC production is triggered by caspase 3 activation of phospholipase A2 (PLA2)[16]. PLA2 generates LPC via partial hydrolysis (removal of one of the fatty acid chains) of phosphatidylcholine, and so its activation by caspase-mediated cleavage may be an intrinsic component of the apoptotic cascade. LPC release from cells is dependent on the ATP-binding cassette transporter 1 (ABCA1)[20]. This is consistent with the fact that ABCA1 is a homologue of ced-7, which was among the first gene products identified in genetic screens for mutations that perturb the engulfment of dead cells in *Caenorhabditis elegans*[21].

ATP, AMP, and UTP are released from apoptotic but still intact cells via a mechanism that may also be caspase-dependent. This release involves export of the nucleotides through connexin-like Pannexin-1 channels in the plasma membrane, the gating of which is controlled by cleavage of the C-termini of oligomeric Pannexin-1 subunits by caspase 3 and caspase 7[22]. These auto-inhibitory C-termini normally occlude the channel, and their proteolytic removal in mid-to-late apoptosis therefore opens Pannexin-1 for nucleotide transport[23–25].

In addition to lysophospholipids and nucleotides, several other find-me signals have been investigated, although most analyses have been carried out in cells in culture, and the case for the *in vivo* biological relevance of many of these additional signals is therefore less clear. Among the strongest candidates is the chemokine CX_3CL1 (also known as fractalkine), which has been implicated as a find-me signal that mediates macrophage chemotaxis towards apoptotic B cells in germinal centres[26].

Find-me receptors.

Macrophages are thought to detect and respond to find-me signals using an array of receptor systems that have been reviewed previously[15]. For lysophospholipids, the G protein coupled receptor (GPCR) G2A appears to play a role in the chemotactic response of macrophages to LPC[27], although the extent to which LPC binds to G2A is unclear, and the precise pathway that transduces LPC signalling in macrophages remains to be elucidated. There are five S1P receptors in the mouse, but only limited data as to which of these might control macrophage chemotaxis *in vivo*[28]. For nucleotides, tissue macrophage populations express several different P2Y purinoceptors, which are GPCRs that bind ATP, ADP, UTP, and UDP[29,30]. These include the P2Y2, P2Y6, P2Y12, P2Y13, and P2Y14 receptors, some of which show expression that is highly restricted to specific macrophage subsets[31,32]. The G_i-coupled P2Y12 receptor, for example, is very abundantly expressed in CNS microglia, where it plays important roles in the earliest stages of microglial chemotaxis and process extension[30,33], but is not expressed by most other tissue macrophages. As such, P2Y12 is now used as a specific marker of ‘homeostatic’ as opposed to activated microglia[33,34]. How various receptors might mediate a response to gradients of find-me signals such that macrophages move in the right direction; that is, toward increasingly higher levels of a find-me signal, remains controversial and unresolved[35]; and *in vivo* gradients of these signals (in tissues where apoptotic cells are cleared) have not been quantified.

Distinguishing live cells from dead

Phosphatidylserine, flippases, and scramblases.

Once macrophages are close enough to actually touch apoptotic cells, they rely on the expression of a set of cell surface molecules that tag these cells as dead. These tags are the so-called ‘eat-me’ signals for phagocytosis. Multiple eat-me candidates have been advanced (discussed below), but the most ubiquitous, efficacious, pleiotropic, and important of these is, without a doubt, phosphatidylserine (PtdSer)[1,36,37]. This humble glycerophospholipid is a component (at varying levels) of many different membranes – including those of the endoplasmic reticulum, the mitochondria, the Golgi apparatus, and the plasma membrane –

within each and every cell of the body. Given this, it is difficult, *a priori*, to imagine how PtdSer might function as a signal for efferocytosis – or anything else. It can do so only because it is normally highly polarized with respect to its membrane bilayer localization (Box 1). In the plasma membrane of healthy cells, for example, nearly 100% of PtdSer is confined to the inner, cytoplasm-facing leaflet of the bilayer. (Phosphatidylinositol and phosphatidylethanolamine are also highly enriched in the inner leaflet.) Except in the special circumstances discussed below, PtdSer is never seen by the extracellular world[38,39]. When it is externalized on the plasma membrane surface, PtdSer serves as a signal — most generally to indicate that the cell has died by apoptosis or is en route to this end. As discussed below, externalized PtdSer is recognized by several different ligand/receptor systems on macrophages, but all of these systems function based on their detection of this everyday phospholipid.

The remarkable PtdSer membrane asymmetry of healthy cells is established by the action of a family of ion-channel-like, 10-transmembrane-domain phospholipid translocases commonly referred to as ‘flippases’[40,41] (Box 1). These flippases are P4-type ATPases. There are 14 such intramembrane ATPases in human cells, distributed among different tissues and membrane compartments[40,42]; and several of these proteins — including ATP8A1, ATP8A2, ATP10A, ATP11A, and ATP11C — have been found to catalyze the translocation of phospholipids[41,43,44]. In general, flippases display substrate specificity either for aminophospholipids, such as PtdSer and phosphatidylethanolamine, or alternatively, for phosphatidylcholine[41]. At the plasma membrane, ATP11A and ATP11C flip essentially all of the PtdSer to the inner leaflet of the plasma membrane bilayer[45,46]. In some settings, flippase expression has been shown to be essential for the normal development of cell lineages. Mice that lack ATP11C, for example, present with a severe B cell deficiency that results from the fact that ATP11C-deficient B cell precursors display high levels of surface PtdSer, and are therefore aberrantly recognized and eaten by macrophages[47]. Flippases require a chaperone, generally CDC50A, in order to take up residence at their appropriate membrane locations[41], and cells that lack CDC50A have been shown to lose all plasma membrane flippase activity and to constitutively expose PtdSer on the cell surface[46,48].

If flippases set up PtdSer bilayer asymmetry, how is this asymmetry ever disrupted? Although a subset of flippases, notably ATP11A and ATP11C, are inactivated by caspase cleavage during the course of apoptosis[45,46], this inactivation alone is insufficient for the exposure of PtdSer on the cell surface, as the inner-to-outer transmembrane exchange of any phospholipid has a high energy barrier (15–50 kcal/mol) and thus does not occur spontaneously[49,50]. Instead, the movement of PtdSer from the inner to the outer leaflet of the plasma membrane requires yet another set of phospholipid translocases – so-called ‘scramblases’ – which catalyze this reverse translocation (Box 1). It is these ATP-independent enzymes that allow PtdSer to act as a signal. There are now thought to be two major classes of scramblases — those of the TMEM16 and XKR families[51–53]. The latter has at least three members, XKR4, XKR8, and XKR9, that are cleaved and *activated* by caspase-3 and/or caspase-7 during apoptosis[54,55], and correspondingly, caspase inhibitors antagonize PtdSer externalization by these XKR scramblases. XKR8 is thought to be especially important for the externalization of PtdSer on the plasma membrane of apoptotic

cells[52]. A large second set of scramblases, those of the transmembrane protein 16 (TMEM16) family, are Ca^{2+}-activated[56–58]. Also referred to as Anoctamins (they were originally thought to be anion channels with 8 transmembrane helices), these 10-transmembrane domain proteins are particularly interesting. Although two family members — anoctamin 1 (ANO1) and (ANO2) — appear to function exclusively as Ca^{2+}-activated ion (chloride) channels, there is now good evidence that ANO3, ANO4, ANO5, ANO7, ANO9, and especially ANO6 (also known as TMEM16F) function as Ca^{2+}-dependent phospholipid scramblases[50,51,53,58]. TMEM16F plays a key role in PtdSer externalization on activated platelets during blood coagulation[59]. As discussed below, the localized activation of XKR and/or TMEM16 family scramblases may allow for the localized externalization of PtdSer on only a small segment of the plasma membrane, as opposed to across the entire surface of the cell.

Receptors for apoptotic cell uptake

TIM4.

As PtdSer is the only universal apoptotic cell-intrinsic eat-me signal, the most intensively studied of the macrophage cell surface proteins that mediate apoptotic cell recognition are those that bind, either directly or indirectly, to this phospholipid. Prominent among these are the transmembrane receptors of the T cell immunoglobulin- and mucin-domain-containing molecule (TIM)[60] and Tyro3, Axl and Mer (TAM)[61] families (Fig. 1). The TIM family has three members in the human genome and eight in the mouse, but the best-studied of these with respect to macrophage efferocytosis is TIM4 (Fig. 1). TIM4 is a heavily glycosylated single transmembrane protein with a short (42-residue) cytoplasmic tail[62]. It binds PtdSer — but not phosphatidylcholine, phosphatidylinositol, or phosphatidylethanolamine — tightly and directly, with low nanomolar affinity, in a Ca^{2+}-dependent reaction. When transduced into heterologous cells, such as NIH3T3 cells and mouse embryonic fibroblasts, TIM4 strongly supports efferocytosis of apoptotic cells[62]. This activity requires a secondary intracellular signalling transducer, as the short cytoplasmic domain of TIM4 is dispensable for signalling in response to PtdSer binding. TIM4 is presumed to use the TAM tyrosine kinase activities for signal transduction, since when it is introduced into fibroblasts that lack a TAM receptor (see below) it is unable to support efferocytosis[63]. Similarly, phagocytosis studies using PtdSer-coated beads and AD293 cells that heterologously expressed TIM4 suggested that TIM4 may also use β1, β3, and β5 integrins as co-receptors, and in turn, the cytoplasmic signalling proteins that are associated with these integrins (for example, Src-family kinases), as downstream intracellular effectors[64]. As discussed below, the β3 and β5 subunits also play important roles in other PtdSer-dependent phagocytosis pathways.

AlthoughTIM4 is expressed in large peritoneal macrophages, which are frequently studied due to their ease of isolation, it is not detectably expressed by several other highly phagocytic macrophage populations, including alveolar macrophages and microglia[32], and so its stimulation of efferocytosis is not common to all macrophages. Mice deficient in TIM4 do not display detectable accumulation of apoptotic cells in the spleen or other tissues, and do not present with splenomegaly or lymphadenopathy, although modestly elevated circulating levels of anti-dsDNA autoantibodies have been reported[65,66]. The related TIM1

and TIM3 proteins, which are prominently expressed in T helper (Th2) cells, plasmacytoid DCs, and Th1 cells, have also been reported to bind PtdSer[62,67–69], but these proteins are not generally expressed by macrophages.

TAM receptors and their ligands.

The TAM proteins Tyro3, Axl, and Mer (gene name *Mertk*) are cell surface receptor tyrosine kinases (RTKs)[61,70]. These receptors do not bind PtdSer directly, but instead rely on their activating ligands — growth arrest specific Gas6 and Protein S (Pros1) – for this activity (Fig. 1)[61,71–74]. As such, Gas6 and Pros1 may be viewed as co-receptors that act in concert with the TAM RTKs to mediate PtdSer recognition and signalling. The C-terminal sex hormone-binding globulin (SHBG) domains of the ligands bind to the extracellular domains of the TAMs, while their N-terminal γ-carboxyglutamic acid (GLA) domains bind to PtdSer[61,70,71,75,76]. Gas6 binds and activates all three TAMs, whereas Pros1 binds and activates only Tyro3 and Mer[71]. In a very unusual arrangement, Gas6 is constitutively bound to Axl in tissues *in vivo*, and moreover, is entirely dependent on Axl for its stable expression in these tissues[74,77]. Both Gas6 and Pros1 only function as effective activators of TAM tyrosine kinase activity and drivers of phagocytosis when, first, they are simultaneously bound to PtdSer via their GLA domains and to a TAM receptor via their SHBG domains; second, the γ carbons of glutamic acid residues within their GLA domains are carboxylated in a vitamin K-dependent reaction; and third, 6–7 divalent cations (probably Ca^{2+}) are also bound to the GLA domains[61,71,73,78]. In this way, the TAM ligands ‘bridge’ a TAM receptor-expressing phagocyte (for example, a macrophage) to a PtdSer-expressing phagocytic target (for example, an apoptotic cell).

The TAM receptors and their ligands are the most broadly expressed PtdSer recognition system in macrophages. Unlike TIM4, Mer appears to be universally expressed on all phagocytic macrophages at steady state[31,32], and antibodies to Mer and the high-affinity $F_C\gamma$ receptor are now routinely used as a marker pair to define and sort these cells. Multiple studies have shown that Mer is a critical mediator of efferocytosis by tissue macrophages throughout the body[11,79–84]. Axl is more restricted in its macrophage expression under basal conditions – to red pulp macrophages, Kuppfer cells, and alveolar macrophages, among others[74]. However, Axl expression is markedly up-regulated in all macrophages by most inflammatory stimuli, including type I interferons and interferon-γ (IFNγ), lipopolysaccharide (LPS), and poly I:C[74,80]. Dramatic Axl up-regulation is also seen when tissue macrophages *in vivo* are activated by trauma, disease, or viral infection[11,80]. Similarly to other RTKs, including MET, ERBB4 and EPHB2, Axl exhibits ‘shedding’ of a soluble version of its extracellular domain, which is generated via the action of ADAM family metalloproteases, subsequent to tyrosine kinase activation[86,87]. As such, soluble Axl in the blood, generated by proteolytic cleavage of the Axl ectodomain subsequent to receptor activation, is now commonly used as an inflammation indicator[85].

Thus, macrophage Mer is thought to handle the bulk of steady state apoptotic cell phagocytosis that is associated with continuous tissue turnover and homeostasis, whereas Axl participates in apoptotic cell phagocytosis during the resolution of inflammation subsequent to infection and tissue trauma[74,80]. Correspondingly, the two receptors

frequently — though not always — display a ‘yin and yang’ relationship with respect to their regulation in macrophages: when one goes up, the other tends to go down[74]. This is also true with respect to their expression in most DCs, where Axl is more prominent than Mer[74,88]. Tyro3 expression in macrophages is very limited, but this RTK is expressed in select DC populations, where it plays a role in the inhibition of type 2 immunity[74,89]. Importantly, mice with mutations in TAM receptor genes, most notably loss-of-function mutants in the *Mertk* gene, show prominent accumulation of apoptotic cells in multiple tissues — and develop splenomegaly, lymphadenopathy, autoantibodies, glomerular nephritis, rheumatoid arthritis and broad-spectrum autoimmune disease — in the absence of any other overt perturbation or challenge[11,70,71,76,90–92].

Also unlike TIM4, the TAM RTKs do not need an accessory intracellular signal transducer to promote phagocytosis, as they carry very strong tyrosine kinases[74,88]. Indeed, the kinase activities of Mer and Axl are absolutely required for their stimulation of apoptotic cell phagocytosis by macrophages[74]. The TAMs are not mere signal transducers, however, since their kinase activity alone is not sufficient to stimulate efferocytosis. The TAM kinases can be strongly activated artificially using extracellular domain antibodies that cross-link the receptors in the absence of Gas6 or Pros1, but this artificial activation has no stimulatory effect on the phagocytosis of apoptotic cells by cultured bone marrow-derived macrophages in the absence of a TAM ligand[74]. It appears that in order for the TAM system to function in efferocytosis, the entire assembly of PtdSer exposure on apoptotic cells, TAM ligand bridging between apoptotic cells and macrophages, and TAM receptor kinase activation within macrophages must operate (Fig. 1). As noted at the outset, in most settings, routine efferocytosis is immunologically silent, in that it is not associated with the secretion of inflammatory cytokines. The TAM receptors also appear to play an essential role in this effect, as the activation of Mer and Axl in macrophages and DCs has repeatedly been found to be potently immunosuppressive[83,88,91,93–95].

MFG-E8 and integrins.

In a manner that is very much analogous to the TAM ligands Gas6 and Pros1, the soluble extracellular matrix glycoprotein MFG-E8 (milk fat globule-EGF factor 8, also named lactadherin)[96] functions during efferocytosis to bridge PtdSer on the surface of apoptotic cells to receptors that are expressed by phagocytic macrophages[97,98] (Fig.1). In this case, these receptors are the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, which bind to MFG-E8 via an Arg-Gly-Asp (RGD) motif located within its second EGF-like repeat. MFG-E8 ‘bridging’ of these macrophage-expressed integrins to PtdSer on the surface of apoptotic cells is mediated by a C-terminal discoidin-like domain rather than a Gla domain (which is not present in the protein)[99,100] (Fig.1). Both MFG-E8 and the integrin proteins to which it binds are widely expressed in many cell types in addition to macrophages and their phagocytic targets, but introduction of these proteins into heterologous cells often strongly potentiates efferocytosis[97]. Consistent with its activity and expression, mice that lack MFG-E8 can display apoptotic cell accumulation in the spleen and lymph nodes, and often go on to develop splenomegaly, nephritis, autoantibodies and a systemic lupus erythematosus (SLE)-like autoimmune disease[98,101]. Recent studies suggest that glucocorticoid-mediated

amelioration of autoimmunity in mice may be dependent on the ability of glucocorticoids to up-regulate MFG-E8 expression[102].

Additional ‘eat-me’ signals and receptors.

There are additional receptor systems that have been found to play a role in direct PtdSer binding and recognition, and consequently, in the efferocytosis of apoptotic cells by select subsets of tissue macrophages (Fig. 1). These include the single Ig-domain type I transmembrane protein CD300b[103], a member of the large CD300 receptor family[104], which is expressed by peritoneal macrophages and neutrophils, but not microglia. This protein also binds PtdSer directly, is localized to the phagocytic cup of engulfing macrophages, and uses the adaptor protein DAP12 to nucleate intracellular signal transduction events that mobilize F-actin for phagocytosis[103]. Similarly, brain-specific angiogenesis inhibitor 1 (BAI1), an adhesion-related GPCR with a relatively large extracellular domain that carries an RGD motif and multiple thrombospondin type I repeats, has been reported to bind PtdSer, cardiolipin, phosphatidic acid, sulfatide and LPS, and to promote the phagocytosis of apoptotic cells (and Gram-negative bacteria) by coupling to downstream effectors[105,106]. While BAI1 is highly expressed in the brain, its expression in many macrophage populations is very low; and although mice deficient in BAI1 exhibit deficits in synaptic plasticity[107], they do not, except upon challenge, display significant apoptotic cell accumulation in most tissues where clearance is mediated by macrophages[108]. An additional direct PtdSer binder implicated in apoptotic cell clearance, notably that of spent erythrocytes, is the large, 19-EGF-domain-containing, transmembrane protein Stabilin-2[109,110]. While highly expressed in red pulp macrophages and present in Kuppfer cells, it is not generally expressed by tissue macrophages.

Finally, there are several sets of plasma proteins that have been shown to decorate the surface of apoptotic cells and have been tied to efferocytosis. Among the most intriguing of these are proteins of the complement cascade, notably C1q and C3b[111–113]. C1q is a very large, 18-chain hexameric plasma glycoprotein assembly, structurally similar to mannose binding lectin and ficolins, which is an essential upstream activator of the classical complement cascade[114]. As might be expected given its size and abundance in serum, C1q has been reported to associate with many molecules (for example, β amyloid, calreticulin, fibronectin, DNA) in addition to its well-described binding to IgM and IgG immune complexes[114]. There are conflicting reports as to its ability to bind to PtdSer, and the macrophage/phagocyte receptors for C1q remain the subject of investigation[114,115]. C1q has nonetheless received considerable attention, as the majority of human patients with C1q deficiency eventually develop lupus[116], and C1q binding to macrophages in culture is immunosuppressive[117]. C3b has also been found to decorate the surface of apoptotic cells, and to account for much of the ability of serum to potentiate apoptotic cell phagocytosis[118,119]. The phagocyte receptors for C3b engagement are thought to be CR3 (CD11b/CD18; $\alpha_M\beta_2$ integrin) and CR4 (CD11c/CD18; $\alpha_x\beta_2$ integrin)[114]. Interestingly, reduced plasma levels of the TAM RTK ligand Pros1 are observed in patients with SLE that have serositis, haematological, neurological and immune disorders, and these reduced Pros1 levels are in turn correlated with reductions in plasma levels of C3, which is consistent with complement consumption, in these patients[120].

Don’t eat me.

Although most attention has been focused on signals that *promote* efferocytosis, a countervailing, phagocytosis-inhibiting activity, or ‘don’t eat-me’ signal, has been proposed to function as a negative regulator of engulfment. This activity is exhibited by the transmembrane, immunoglobulin-related cell surface protein CD47[121] (Fig. 1). In addition to interacting with integrins and thrombospondin-1 to regulate neutrophil migration, neuronal axon growth and T cell co-stimulation, CD47 has been found to bind to signal regulatory protein alpha (SIRPα) on macrophages to inhibit phagocytosis[122,123]. This inhibition appears to require phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the SIRPα cytoplasmic domain, leading to the activation of the SHP1 and SHP2 phosphatases[124], although the precise mechanism of inhibition remains the subject of investigation[125]. Tumour cells have frequently been found to up-regulate CD47 expression as a potential mechanism of immune evasion[126], and antibodies that target the CD47–SIRPα interaction have shown efficacy as therapeutics in some mouse pre-clinical cancer models[127,128]. Correspondingly, four of these antibodies are now in clinical trials as therapeutics for haematological malgnancies[129]. The relative power of CD47 as a don’t eat-me signal versus PtdSer as an eat-me signal has not been measured, but it should be noted that B cell precursors deficient in the ATP11C flippase (mentioned above), which strongly express both signals, are readily engulfed[47]. Apart from the efferocytosis of apoptotic cells, the CD47–SIRPα axis has also recently been implicated in the negative regulation of more local membrane excision events carried out by microglia during ‘synaptic pruning’ in the postnatal brain[130], as discussed below.

Phagocytic attack and engulfment

Efferocytosis requires: one, the engagement of signal transduction pathways that activate RAC1–CDC42 GTPases and dramatically re-organize the macrophage actin cytoskeleton; two, the extension of a phagocytic cup around the target cell; three, the internalization of this doomed cell into the macrophage; four, the routing of the internalized cargo to endosomes and lysosomes; and five, its eventual enzymatic degradation and turnover[131–134]. The genetic and biochemical pathways underlying these intracellular engulfment events are beyond the scope of this Review, but have recently been discussed[135–137]. The remainder of this Review will focus on our growing understanding of how it is not just dead and dying cells that are targeted by macrophages, but also, in some settings, the living.

Eaten alive.

Do eat-me signals only trigger macrophage phagocytosis of dead cells? Indeed, they do not. Recent findings indicate that macrophages do not always wait for cells to die before initiating phagocytic attack, and may kill living cells by eating them, if these cells express sufficient levels of externalized PtdSer. These findings harken back to much earlier observations in *C. elegans*, in which mutation of engulfment genes, including *ced-6* and *ced-7*, whose products are required for the phagocytosis of dead and dying cells, led to the long-term survival of ‘half-dead’ embryonic cell populations (expressing a hypomorphic *ced-3* allele) that would normally have been eliminated[138,139]. The recent findings support the hypothesis that scramblase activation and the externalization of PtdSer may result in a

'murder by phagocytosis' phenomenon that is both PtdSer- and TAM-dependent (Fig. 2). For example, the precursor B cells that are aberrantly cleared, by macrophages, in mice deficient for the ATP11C flippase, are alive at the time of their engulfment; and genetic and tyrosine kinase inhibitor analyses indicate that this fatal engulfment is dependent on the action of Axl and Mer[47]. In the adult brain, when dividing progenitor cells in the subventricular zone (SVZ) are pulse-labelled with BrdU during cell division, many more healthy, functional, BrdU-labelled neurons are observed in the olfactory bulb, to which newborn SVZ cells migrate, one month after labeling in $Axl^{-/-}Mertk^{-/-}$ mice than in wild-type[11]. Conditional inactivation of the *Mertk* gene only in microglia again indicates the cells responsible for this effect are the brain's macrophages[11]. Similarly, adenovirus-transduced astrocytes have been shown to transiently externalize PtdSer after infection. Over the course of the next several days, many of these PtdSer-displaying transduced cells are phagocytically cleared by activated microglia[140]. This phagocytosis is not observed in $Axl^{-/-}Mertk^{-/-}$ mutants or when PtdSer externalization is inhibited[140]. Importantly, these cleared cells are unambiguously alive at the time of phagocytosis, since the surviving transduced cells seen in the $Axl^{-/-}Mertk^{-/-}$ mutants persist as healthy functioning astrocytes for many months after infection – if they are not eaten[140] (Fig. 2).

These findings of microglial murder by phagocytosis are also consistent with analyses of tissue recovery following focal brain ischemia, in which areas of neuronal atrophy were found to be markedly reduced in size, and functional recovery to be enhanced and accelerated, in $Mertk^{-/-}$ mice relative to wild-type animals[141] (Fig. 2). They may also be relevant to neurodegenerative disease, since neurons in the P301S-tau mouse model of frontotemporal dementia were recently shown to externalize PtdSer and, as a consequence, to be phagocytosed by microglia while still alive[142]. In this instance, MFG-E8 appears to play an important role in microglial recognition of the externalized PtdSer[142] (Fig. 2). This may also be the case for microglial phagocytosis of viable neurons during brain inflammation, since neuronal loss subsequent to *in vivo* LPS injections is also reduced in $Mfge8^{-/-}$ mice relative to wild-type[143]. Finally, recent work suggests that macrophage engulfment of HIV-1-infected but still living CD4+ T cells can occur, and that this engulfment of live cells is TAM-dependent, since it is triggered by; one, PtdSer externalization after infection; two, Pros1 or Gas6 binding to this externalized PtdSer; and three, engagement of macrophage Mer by these bound TAM ligands[144]. It is possible that the phagocytic killing of PtdSer-expressing but nonetheless living cells by microglia and other tissue macrophages, a process that is sometimes referred to as 'phagoptosis'[145,146], may be a common and numerically substantial phenomenon whose general significance has not heretofore been widely appreciated.

Phagocytosis of membrane segments.

There are select settings in which only small membrane segments of cells, as opposed to entire cells, are phagocytically engulfed. The best-understood of these selective 'pruning' phenomena occurs in the retina. For a few hours each morning around subjective dawn, the retinal pigment epithelial (RPE) cells of the eye, which are not macrophages but rather neuroectoderm-derived epithelia, nibble off the distal ends of photoreceptor outer segments[147,148] (Fig. 3). This localized phagocytosis disposes of toxic oxidized proteins and

lipids that are produced by reactive oxygen species during phototransduction; these oxidized products normally accumulate in the outer segments and would kill the photoreceptors if not removed. Students used to be taught that photoreceptors 'shed' the distal tips of their outer segments on a daily basis, but this is not entirely correct: these tips are instead actively eaten by RPE cells, the most vigorous phagocytes in the body[148].

This RPE phagocytosis displays an absolute requirement for Mer: RPE cells express Mer (and Tyro3), and mice, rats and humans with complete loss-of-function mutations in *MERTK* are blind due to a failure in RPE phagocytosis and the consequent death of most photoreceptors[149–152]. Many different human *MERTK* mutations account for inherited forms of retinitis pigmentosa[151], and clinical trials involving subretinal injection of Mertk-expressing adeno-associated virus vectors have been initiated in patients carrying these mutations[153]. Gas6 and Pros1 function in concert as Mer ligands for this RPE-mediated phagocytosis[154]. The MFG-E8–$\alpha_v\beta_5$ integrin system highlighted above also plays a role, albeit a secondary one, in RPE phagocytosis of these photoreceptor outer segments[155]. Very importantly, the membrane segment that is phagocytosed by an RPE cell appears to be delimited by the precisely localized exposure of PtdSer at only the tips of photoreceptor outer segments around subjective dawn[156]. This externalized PtdSer provides a binding site for both the GLA domains of Gas6 and Pros1 (which bridge to RPE-expressed Mer) and the discoidin-like domain of MGF-E8 (which bridges to RPE-expressed $\alpha_v\beta_5$ integrin). That is, externalized PtdSer tells RPE phagocytes exactly how much of the outer segment membrane to eat, and is thus the essential signal for this localized phagocytosis.

Macrophages also prune. Microglia selectively phagocytose pre-synaptic elements (boutons) of axonal membrane during postnatal brain development, in a process termed 'synaptic pruning'[157–159] (Fig. 3). This very selective phagocytosis is known to be driven by neuronal electrical activity and is thought to be mediated at least in part by the deposition of complement proteins C1q, C3, and C4 on pre-synaptic elements that are to be engulfed by microglia[159,160]. Indeed, defects in synaptic pruning associated with complement deposition may contribute to neurodegenerative and neuropsychiatric disease[161].

Complement proteins are not intrinsic to the neuron, however, and so how they might specifically decorate pre-synaptic boutons of the axon that are to be pruned, while not decorating others, has remained unclear. One recently advanced hypothesis is that PtdSer might again be an important signal[75] (Fig. 3). Neuronal activity is associated with Ca^{2+} influx into boutons, which might in principle locally activate Ca^{2+}-dependent scramblases that would in turn result in the local externalization of PtdSer — to which C1q has been reported to bind[75,115]. Interesting experimental support for this hypothesis has very recently been provided by the demonstration of preferential association of C1q with pre-synaptic membrane vesicles that are both positive for Annexin V binding; that is, have externalized PtdSer, and cleaved (that is, activated) caspase 3[162]. As highlighted above, PtdSer scramblases of the XKR family are activated by caspase 3 proteolysis. Together, these observations suggest that highly localized externalization of PtdSer on pre-synaptic axonal boutons that are not paired with a post-synaptic element (to form a complete synapse) may serve as a platform for the binding of Gas6 and/or Pros1, MFG-E8 or complement proteins to trigger microglial engulfment of these boutons (Fig. 3). How PtdSer externalization might

be confined to small membrane domains of cells — unpaired pre-synaptic boutons or the tips of photoreceptor outer segments — remains the subject of speculation. Possibilities include the localized clustering of either plasma membrane or intracellular Ca^{2+} channels, which would locally activate Ca^{2+}-dependent scramblases, and/or the localized expression of the scramblases themselves, and/or the localized cleavage (activation) of caspase 3 and caspase 7. Locally externalized PtdSer would in theory be prevented from diffusing within the plane of the plasma membrane by its immediate binding to the panoply of extracellular agents — for example, Gas6/Pros1-TAM, MFG-E8-integrin, TIM4 – described above, all of which are physically connected to another cell.

PtdSer-dependent phagocytosis of membrane segments highlights the vast scale over which PtdSer and TAM receptor signalling operates: most of the apoptotic and live cells illustrated in Fig. 1 and Fig. 2 have volumes ranging from 1,000 to 100,000 μm^3, whereas the tips of photoreceptor outer segments that are engulfed by RPE cells have volumes of roughly 10 μm^3, and the engulfed pre-synaptic boutons of neuronal axons measure in the range of 0.2 to 1μm^3. PtdSer- and TAM-dependent phagocytosis of enveloped virus particles also occurs — in a process termed 'apoptotic mimicry'[163,164] — and these viruses have volumes on the order of 0.005 μm^3. Together, these diverse phagocytic events, all of which are initiated by PtdSer externalization and most of which are known to be TAM-dependent, can capture engulfment targets whose sizes span over seven orders of magnitude.

Conclusion: what could go wrong?

The consequences of defective efferocytosis by macrophages, which leads to secondary necrosis and the presentation of autoantigens, are commonly assumed to be dire. However, the evidence for this with respect to disease in humans, as opposed to mouse models, is largely circumstantial. Deficient efferocytosis has been observed in advanced human atherosclerotic plaques, leading to the formation of highly inflammatory necrotic lesions[165,166]. In human inflammatory lung diseases, including chronic obstructive pulmonary disease, inadequate efferocytosis has also been strongly implicated in the exacerbation of disease pathology[167]. And defects in the clearance of apoptotic cells from the germinal centers of the lymph nodes of patients with SLE have been a consistently reported feature of the disease[9,168]. Nonetheless, these observations of apoptotic cell accumulation can only be said to correlate with disease, as human beings are not, except in the context of clinical trials, experimental animals.

As noted above, mice in which the TAM system, the MFG-E8/integrin system, or the enzymes that externalize PtdSer are either partially or fully disabled do indeed display very substantial accumulations of apoptotic cells and often develop severe autoimmune disease. *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mertk*$^{-/-}$ triple mutant mice, for example, are a mess. They present with prominent apoptotic cell accumulation and activated lymphocytes in many tissues (both lymphoid and non-lymphoid), are plagued by massive splenomegaly, rheumatoid arthritis, psoriasis, and nephritis, are infertile as males due to severely impaired phagocytosis of apoptotic germ cells by Sertoli cells in the testes, and are blind due a failure in RPE phagocytosis of photoreceptor outer segments in the retina[90,91,154]. In humans, there are now descriptions of a plethora of *MERTK* gene mutations that result in inherited retinal

diseases[152], but patients carrying these mutations have not been evaluated for immune dysfunction. Particularly for those patients with complete loss-of-function mutations in *MERTK*, such evaluations — for example, tests for splenomegaly and/or the presence of anti-dsDNA, anti-nuclear antigen, or anti-phospholipid autoantibodies in the circulation — are clearly warranted. Finally, the TAM RTKs play very important roles in the initiation, growth, metastasis and resistance of many different cancers, and several small molecule kinase inhibitors of these receptors are now in development or in clinical trials as cancer therapeutics[169]. Given the macrophage biology highlighted above, evaluation of the effects of these inhibitors on the development of autoimmune disease should also be a priority.

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Glossary terms:

NIH3T3 cells	A cell line derived from mouse embryonic fibroblasts
AD293 cells	A cell line derived from the HEK293 human embryonic kidney cell line

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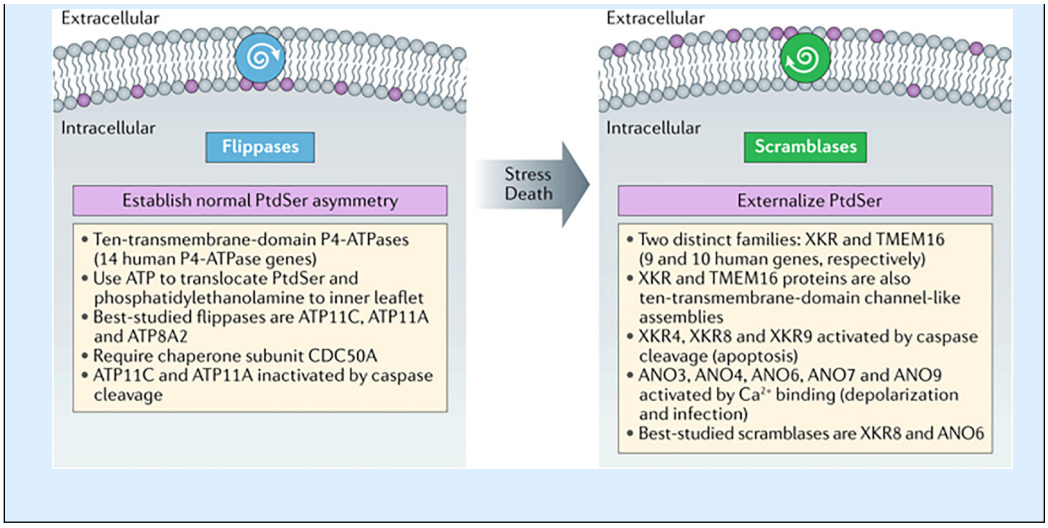
Box 1.

Stringent regulation of phosphatidylserine localization in the plasma membrane.

Mammalian genomes encode many 10-transmembrane-domain, channel-like enzymes that are devoted to the intra-bilayer translocation and localization of phosphatidylserine (PtdSer) and other phospholipids[1,38–40,42,50,56]. Although some members of the indicated families display other activities — the TMEM16A and TMEM16B proteins, for example, are Ca^{2+}-activated chloride channels — many are catalytically active phospholipid translocases. A subset of ATP8 and ATP11 proteins, notably ATP11C, use ATP hydrolysis to establish the pronounced asymmetric distribution of PtdSer and other phospholipids seen at the plasma membrane (and internal membranes) of all healthy cells. Importantly, nearly 100% of PtdSer is normally confined to the cytoplasmic leaflet of the plasma membrane bilayer. Scramblases are ATP-independent enzymes that move PtdSer to the extracellular leaflet, where it is displayed to the external world. Several XKR scramblases, notably XKR8, are activated by caspase cleavage and are thought to function during apoptosis. Several TMEM16 scramblases, notably TMEM16F, are activated by Ca^{2+} binding subsequent to membrane depolarization and other signaling events.



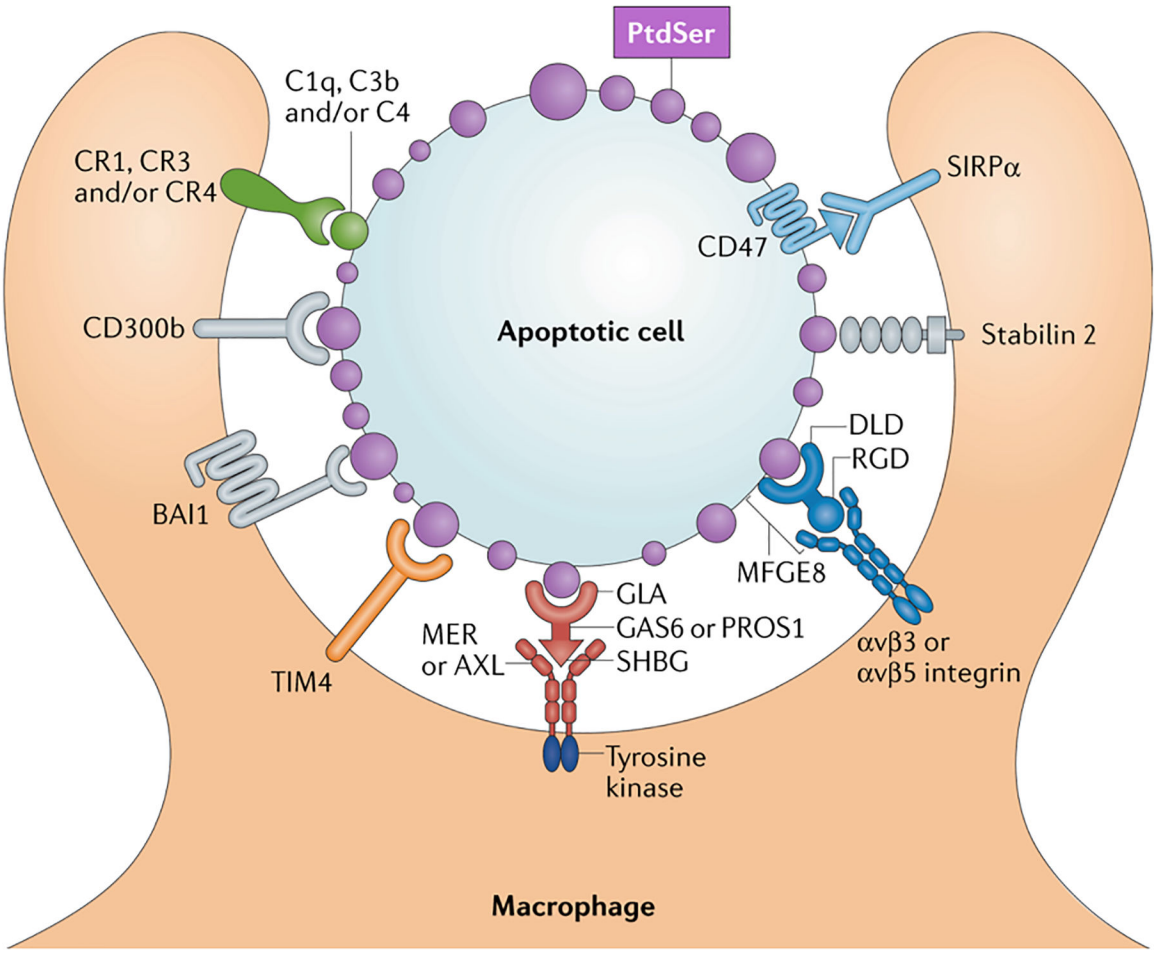


Figure 1. Receptors and ligands mediating apoptotic cell (AC) recognition and phagocytosis by macrophages.

Apoptotic cell surfaces are marked by a profusion of membrane blebs that universally display externalized phosphatidylserine (PtdSer, purple). PtdSer – the most potent 'eat-me' signal for apoptotic cell phagocytosis – is recognized by macrophage receptors that directly bind this phospholipid – including CD300b, BAI1, TIM4 (green), and Stabilin-2. PtdSer is also recognized by soluble, bi-functional 'bridging' proteins, including Gas6/Pros1 (red) and MFG-E8 (blue). These proteins carry one domain that binds PtdSer – the 'Gla' domains of Gas6/Pros1 and the Discoidin-like domain (Dld) of MFG-E8 – and a second domain that binds to phagocytic receptors expressed by macrophages – the SHBG domain of Gas6/Pros1 and an RGD motif within the second EGF-like domain of MFG-E8. The macrophage receptors for Gas6/Pros1 are the TAM receptor tyrosine kinases Mer and Axl (red), while those for MFG-E8 are $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin dimers (blue). Complement proteins, including C1q, C3b, and C4 (closed gray circle), also decorate the surface of apoptotic cells by mechanisms that remain under study, but may involve the recognition of PtdSer. These complement factors are recognized by the complement receptors CR1, CR3, and CR4. Some apoptotic cells also express CD47 (light blue), which negatively regulates phagocytosis by acting as a 'don't-eat-me' signal. Its receptor is SIRPα. All phagocytic receptors must engage signal transduction networks that ultimately activate Rho family GTPases, but the

only receptors that carry intrinsic signaling activity are the TAM receptors, which are robust, ligand/apoptotic cell-activated tyrosine kinases (TKs).

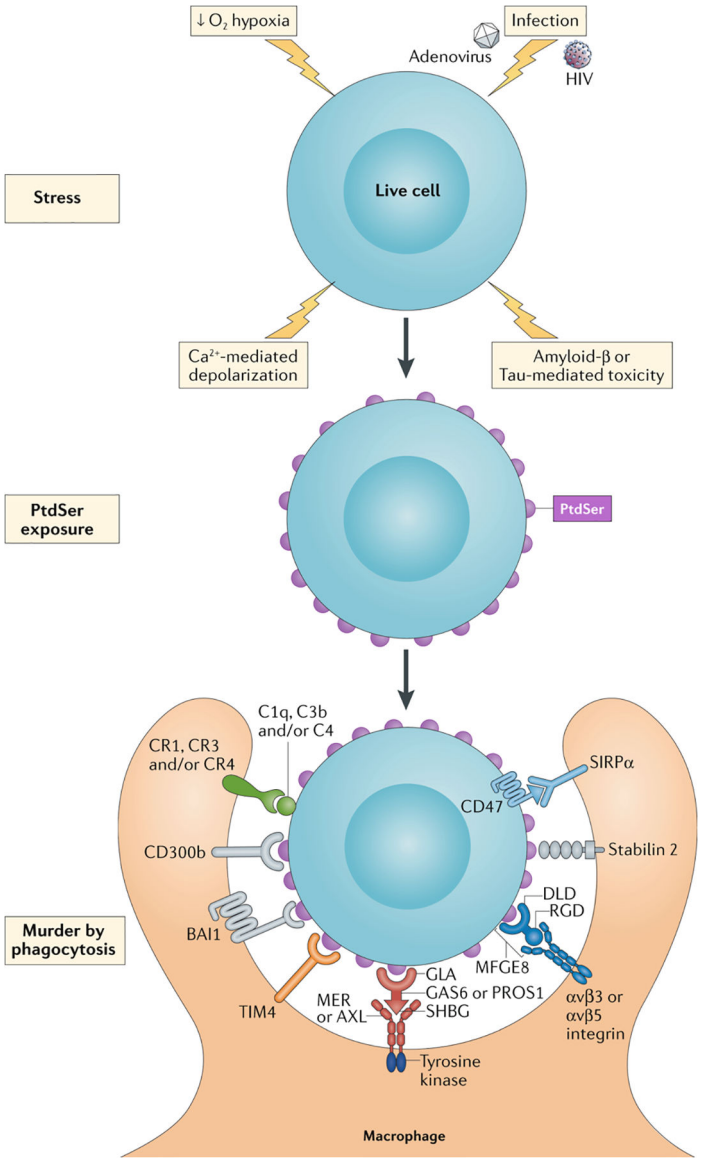


Figure 2. PtdSer-triggered phagocytic engulfment of living cells.

Recent findings indicate that a variety of stressors - including hypoxia precipitated by stroke, calcium influx triggered by excitatory and/or exitotoxic stimulation (depolarization), infection by HIV and adenoviruses (AdV), and the toxic effects of Amyloid β (Aβ) and phospho-Tau deposition (top) – can lead to the activation of XKR and TMEM16 scramblases and the exposure of PtdSer on the surface of living cells (middle). In some settings, this leads to the engulfment of stressed but still living cells – or murder by phagocytosis (bottom).

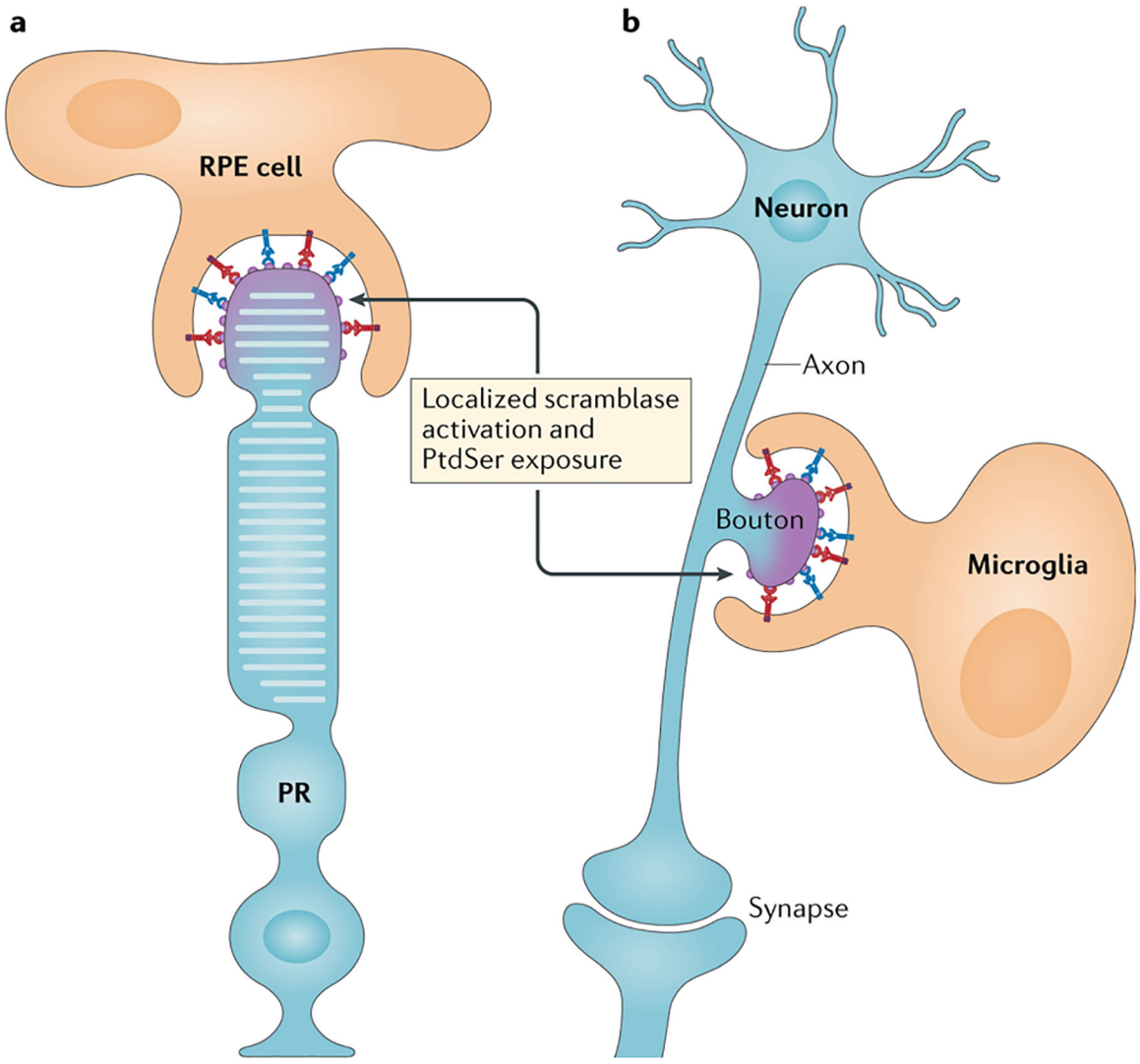


Figure 3. PtdSer-delimited phagocytosis of only small parts of cells.

a. The phagocytic retinal pigment epithelial (RPE) cells of the eye pinch of and engulf the distal ends of the rhodopsin-containing outer segments of photoreceptors (PR) each morning. This localized phagocytosis is entirely Mer-dependent (red) and is assisted by the MFG-E8 integrin system (blue). Patients with complete loss-of-function *MERTK* mutations have an inherited form of retinitis pigmentosa due to a failure in RPE phagocytosis. **b**. Microglia, the tissue macrophages of the brain, phagocytose unpaired pre-synaptic axonal boutons of the postnatal brain (those that are not part of an anatomically complete, functional synapse), in a process termed ‘synaptic pruning’. These boutons are decorated by several of the eat-me signals highlighted in Fig. 1, including complement proteins (gray). Importantly, the extent of the bouton to be pruned may again be delimited by the local externalization of PtdSer on its surface. The localized activation of scramblase activity for the events depicted in a and b is at this point a speculation.