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Review

Clearance of apoptotic cells by phagocytes

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Phagocytic clearance of apoptotic cells may be considered to consist of four distinct steps: accumulation of phagocytes at the site where apoptotic cells are located; recognition of dying cells through a number of bridge molecules and receptors; engulfment by a unique uptake process; and processing of engulfed cells within phagocytes. Here, we will discuss these individual steps that collectively are essential for the effective removal of apoptotic cells. This will illustrate our relative lack of knowledge about the initial attraction signals, the specific mechanisms of engulfment and processing in comparison to the extensive literature on recognition mechanisms. There is now mounting evidence that clearance defects are responsible for chronic inflammatory disease and contribute to autoimmunity. Therefore, a better understanding of all aspects of the clearance process is required before it can truly be manipulated for therapeutic gain.

Phagocyte Accumulation to Sites of Apoptotic Cell Generation

In the blood, apoptotic leucocytes and effete erythrocytes contact phagocytes in spleen and liver during normal flow and filtration enabling contact, recognition and subsequent clearance. Casual apoptosis of individual tissue cells likewise may result in recognition and uptake into neighbouring viable cells, as seen with the required apoptosis of defined cells during development, for example, in *Caenorhabditis elegans*.[1] In this context, it is important to note that many different mesenchymal and epithelial cell types have the capacity to engulf apoptotic cells[2] and may play major roles in their clearance under normal circumstances *in vivo*. The efficiency and rapidity of apoptotic cell removal and digestion[3] means that visual evidence of such clearance histologically represents but a tiny snapshot of what may be a large and often unappreciated, ongoing process (Figure 1). Only when clearance is defective, may the extent of these processes become apparent.

On the other hand, when large number of apoptotic cells are generated in tissue injury or inflammation, accumulation of mononuclear phagocytes is seen. The timely removal of the apoptotic cells before they undergo secondary necrosis necessitates phagocyte migration and this has led to the assumption that apoptotic cells release attraction signals that direct phagocytes to their location. In support of this attractive hypothesis, Lauber *et al.*[4] showed *in vitro* that apoptotic cells secrete a chemotactic signal that induces attraction of monocytic cells in a caspase-3-dependent fashion. They identified the phospholipid lysophosphatidylcholine (LPC) and showed that it was released from apoptotic cells due to caspase-3-mediated activation of the calcium-independent phospholipase A2. Whether LPC is a real attraction signal in inflamed solid organ tissue remains unclear considering that LPC is likely to be taken up rapidly by neighbouring cells. Another likely candidate are specific monocyte chemoattractant chemokines, such as MCP-1. For example, during apoptosis of the corpus luteum, local cells secrete MCP-1, which appears to be responsible for macrophage accumulation.[5] However, whether the chemokine is directly produced by the apoptosing cells or in response to them is not yet clear.

Another intriguing hypothesis is that the changes in membrane composition that are characteristic of cells undergoing apoptosis may initiate electric signals that attract phagocytes. Electrical fields play an important role in wound healing and drive migration of endothelial cells and neutrophils toward the wound centre.[6] The partial loss of membrane asymmetry in apoptosis generates a negative surface charge in apoptotic cells, which has been shown to induce endothelial sprouting toward the dying cell.[7] Furthermore, modified beads that mimic the surface charge of apoptotic cells have been used as simplified targets for phagocytosis assays and shown similar characteristics as apoptotic cells.

Still much work has to be performed to elucidate the specific mechanisms that direct professional and non-professional phagocytes toward the dying cells before they lose membrane integrity but the large number of cells that are

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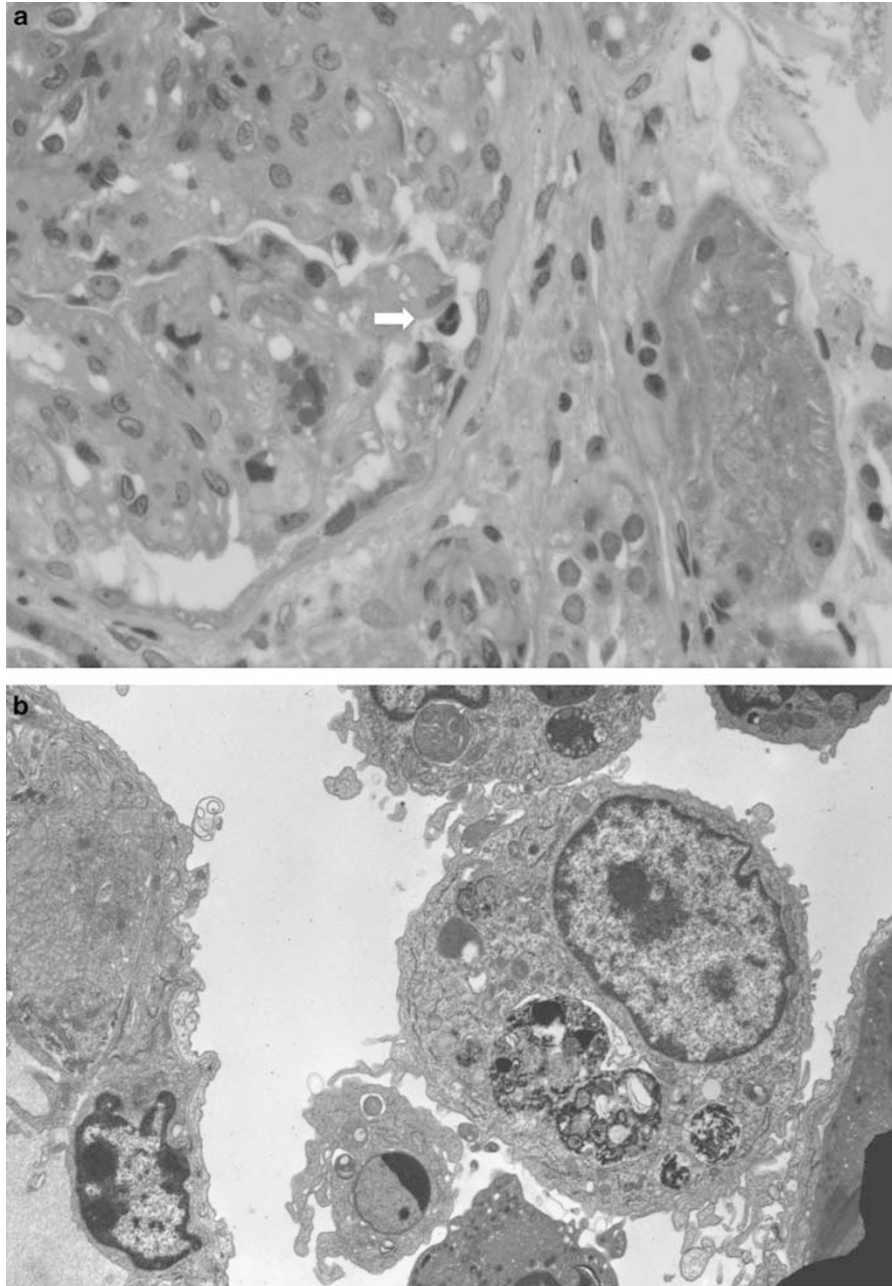
Figure 1 (**a**) Light microscopy showing an apoptotic body at the edge of the glomerulus in a patient with Lupus nephritis. Kindly provided by Paul Brown, University of Aberdeen, UK. (**b**) Electron micrograph showing apoptosis as seen in late inflammation in the mouse lung. Kindly provided by Jan Henson, National Jewish Medical and Research Center, Denver, CO, USA

effectively cleared make it highly likely that such mechanisms exist.

Recognition

The second part of the uptake process is the recognition of dying cells by professional and non-professional phagocytes. This process is facilitated by changes in the composition of the apoptotic cell membrane. Among the surface changes, the most universal and best characterised is the loss of phospholipid asymmetry and the translocation of phosphatidylserine (PS) to the outer leaflet of the lipid bilayer, which occurs very early in the apoptotic process.[8,9] This process involves activation of a nonspecific bidirectional phospholipid flip-flop along with inhibition of an aminophospholipid translocase that normally confines and returns PS to the inner leaflet.[10,11] Other molecules in addition to PS that are normally confined to the cytosolic side of the cell membrane appear on the cell surface. These include annexin I that colocalises with PS,[12] the endoplasmic reticulum protein

calreticulin (CRT) whose levels increase on the cell surface during programmed cell death by unknown mechanisms and exposure of DNA.[13] Recent observations suggest that autophagy, which is commonly observed during programmed cell deaths contributes to dead cell clearance by generating energy-dependent signals necessary for PS exposure and LPC production.[14]

There is also poorly described modifications of existing surface molecules by oxidation (e.g. phospholipids,[15,16]) and alterations in sugar chains and surface charge.[17,18] These modifications result in the generation of sites resembling oxidised lipoprotein particles, thrombospondin (TSP) binding sites, sites capable of binding lectins, the complement proteins C1q and C3b as well as various collectin-binding sites.[19–21]

These surface changes can then either interact directly with receptors on the macrophage surface or bind serum proteins that serve as links between the phagocyte and its apoptotic meal. These extracellular bridge molecules (opsonins) enhance the susceptibility of apoptotic cells to phagocytosis and provide additional recognition (binding site – receptor) arrangements. The bridge molecules milk-fat-globule-EGF-factor 8 (MFG-E8), growth arrest-specific 6 (Gas6), β2-glycoprotein I (β2-GPI) and serum protein S all bind to PS on the apoptotic cell surface. MFG-E8 can then be recognised by $\alpha v \beta$3 and $\alpha v \beta$5 integrins through its RGD motif,[22,23] Gas6 by receptor tyrosine kinases of the Axl, Sky and Mer family[3,24] and β2-GPI to the β2-GPI-receptor.[25] Other bridge molecules are linked to the recognition of altered sugars and/or lipids on the apoptotic cell surface and include the members of the collectin family surfactant proteins A and D, mannose-binding lectin and the collectin-like first component of the classical complement cascade C1q.[21,26] The collectin family of molecules are then recognised through their interactions of their collagenous tails with CRT, which in turn signals for uptake by the phagocyte through the low-density lipoprotein (LDL)-receptor-related protein (LRP-1/CD91).[27] As another example, the first bridge molecule identified was TSP-1,[18] an extracellular matrix glycoprotein and thought to bind to TSP-1 binding sites on apoptotic cells to then bind to a receptor complex on the phagocyte comprising the integrin $\alpha v \beta$3 and the scavenger receptor CD36.

Many other phagocyte receptors are thought to bind directly to structures exposed on apoptotic cells and these include a number of scavenger receptors in addition to CD36 such as the class A scavenger receptor,[28] the lectin-like oxidised LDL particle receptor (LOX-1)[29] and macrosialin CD68.[30] Integrin family members in addition to the aforementioned $\alpha v \beta$3 and $\alpha v \beta$5 are thought to bind directly to C3b/bi binding sites on the apoptotic cell and include $\alpha m \beta$2 and $\alpha x \beta$2 integrins also known as complement receptors 3 and 4.[31] Altered ICAM3 on the apoptotic cell surface has been suggested to facilitate apoptotic cell binding to the lipopolysaccharide receptor CD14 without inflammatory consequences.[32] ATP-binding cassette transporters have been implicated in transbilayer redistribution of PS in apoptotic cells and undoubtedly play a role in the recognition of apoptotic cells. ABCA1 was thought to be the mammalian orthologue of CED-7, which contributes to apoptotic cell clearance in *C. elegans* and earlier work suggests that ABCA1 promotes recognition and subsequent

engulfment.[33] A recent study by Jehle *et al.*[34] suggests that ABCA7 rather than ABCA1 may be the mammalian orthologue of CED-7 and provides evidence for its role in the clearance of apoptotic cells through interaction with LRP (Table 1).

Given the prominence of PS in the membrane changes occurring during apoptosis and the many studies showing blockade of apoptotic cell uptake by PS and/or by PS-binding proteins, it still remains likely that a specific PS receptor (or receptors) exists. This would presumably be able to directly bind to PS exposed on the apoptotic cell and from early work on PS inhibition, to be able to recognise the PS head group stereospecifically.[35] However, a previously implicated candidate receptor for PS (PSR) is now not thought to act as a surface receptor.[36,37] This molecule appears to be primarily localised in the cell nucleus and its possible role in apoptotic cell uptake remains to be elucidated.

The assumption that this large number of receptors and bridge molecules only exist to provide redundancy to ensure that clearance of apoptotic cells is accomplished without secondary necrosis is almost certainly too simplistic. It remains unclear which of these receptors act as signalling molecules, have tethering function or are directly involved in engulfment. Work by Hoffmann *et al.*[38] brings some structure into the large number of eat-me signals, bridge molecules and corresponding phagocyte receptors. They show that a number of the 'eat-me' signals mediate tethering rather than direct signalling for engulfment and that engagement of PS appears one of the key processes required for internalisation. This has led to a two-step model in which the apoptotic cell is first tethered to the phagocyte before other interactions transduce signals that initiate cell uptake, the so called 'tether and tickle' model.[38,39] It will be important to further elucidate the role of individual receptors and the expected interactions between them, in the actual processes of phagocytosis and intracellular processing.

Discrimination between living, dying and dead cells is an essential requirement for appropriate clearance of apoptotic cells. In this context, many of the ligands identified as candidates for recognition of apoptotic cells are also found on viable cells, particularly following activation (i.e. PS or CRT). One possibility to account for the discrimination is that apoptotic cell recognition and subsequent engulfment require not only the exposure of eat-me signals but also redistribution into patches. Many of the ligand interactions are likely of low affinity, requiring oligomerisation (increase in avidity) for optimal stimulation. This could lead to generation of a recognition 'synapse' between the apoptotic cell surface and the phagocyte.[40]

Similar recognition ligands also occur on necrotic, or post-apoptotic cells and of course it is important that the body has mechanisms for clearance of these structures as well. Increasingly, it is apparent that recognition and removal of necrotic cells and indeed of cells dying from various forms of programmed or non-programmed cell death are cleared by phagocytes by similar processes. What appears to be different is the subsequent response of the phagocyte to the state of the cell that is being ingested in that apoptotic cells normally induce an anti-inflammatory and anti-immunogenic response whereas 'necrotic cells' usually promote the gen-

Table 1 Summary of phagocyte receptors, bridge molecules and phagocyte binding sites on apoptotic cells

Recognition receptor on phagocyte	Bridge molecule	Binding site on apoptotic cell
Scavenger receptors	Thrombospondin	
SR-A		OxLDL-like sites
LOX-1		OxLDL-like sites
CD68		OxLDL-like sites
CD36		TSP-1 binding sites
CD 14		ICAM-3
		Unidentified glycoproteins
Unidentified lectins		
CD91-calreticulin		
	MBL	Collectin-binding sites
	SP-A	Collectin-binding sites
	SP-D	Collectin-binding sites
	C1q	C1q-binding sites
	SP-A, SP-D, MBL	Nucleic acid
Integrin receptors		
Vitronectin receptor $\alpha v \beta 3$	Thrombospondin	TSP-1 binding sites
$\alpha v \beta 5$	Thrombospondin	TSP-1 binding sites
Complement receptor 3 $\alpha m \beta 2$	C3b/bi	C3b/bi binding sites
Complement receptor 4 $\alpha x \beta 2$	C3b/bi	C3b/bi binding sites
PS-bridge molecule receptors		
Mer	Gas-6	Phosphatidylserine
$\beta 2$-GPI-receptor	$\beta 2$-GPI	Phosphatidylserine
Vitronectin receptor $\alpha v \beta 3$	MFG-E8	Phosphatidylserine
Not yet identified	Annexin1	Phosphatidylserine
Mer	Protein S	Phosphatidylserine
PS receptor		
Not yet identified		Phosphatidylserine
ATP-binding cassette transporter		ABCA7

eration of proinflammatory and proimmunogenic mediators.[41,42]

There is also evidence that inhibitors ('don't eat me' signals) play a role in this discrimination, that is the platelet endothelial cell adhesion molecule (PECAM/ CD31) interacts homotypically with CD31 on potential phagocytes to facilitate detachment of living cells and thereby potentially prevents engulfment.[43] Its inability to provide the same signals to apoptotic cells prevents detachment and enables ingestion. The integrin-associated protein/CD47 is expressed on the surface of many cells and can bind to the signal regulatory protein (SIRP-α), which is a critical immune inhibitory receptor. This interaction prevents uptake of target cells expressing CD47.[40] On many cell types, CD47 expression is reduced during apoptosis and redistributed into patches distant from those containing PS or CRT.[44] Consequently, apoptotic cells (or even viable CD47$^{-/-}$ cells) are unable to stimulate SIRP-α and the downstream SHP-1 and lose their ability to prevent uptake. This raises the intriguing possibility that cells may be constantly targeted for removal and have to prove their viability to prevent the initiation of the clearance process.

Engulfment

Phagocytosis is an extremely complex process and no single model can account for the diverse structures and outcomes associated with particle ingestion. Nevertheless, independent of the ingested cell (apoptotic or necrotic) and the receptors involved a number of common principles have been established: following receptor-mediated particle recognition actin polymerisation occurs at the site of ingestion and the particle is internalised via an actin-dependent mechanism. After internalisation, actin is shed from the phagosome and the phagosome matures by a series of fusion and fission events with components of the endocytic pathway culminating in the mature phagolysosome. Phagosome trafficking occurs primarily in association with microtubules and therefore requires the coordinated interaction of the actin- and tubulin-based cytoskeleton. The internalisation process however depends profoundly on the phagocytic receptor involved. Fcγ-receptor-mediated phagocytosis relies on the sequential interaction of IgG-coated particles with receptors, which in turn drives pseudopod extension from the phagocytes surface and results in a tight fitting phagosome with no solute uptake,[45] the so called 'zipper' mechanism.

There is now increasing evidence that apoptotic cell engulfment is different in that uptake of apoptotic cells initially involves formation of spacious phagosomes accompanied by membrane ruffling and associated uptake of a 'healthy gulp' of the surrounding fluid.[38,46] The process appears more akin to macropinocytosis than classical phagocytosis mediated by IgG or C3 opsonins (Figure 2). However, it should be noted that some investigators have observed what appear to be closer apposition of the phagosome membrane with the apoptotic cell surface during uptake[47] and it remains possible that different combinations of ligands may promote different physical modes of uptake. Furthermore, as noted below, the
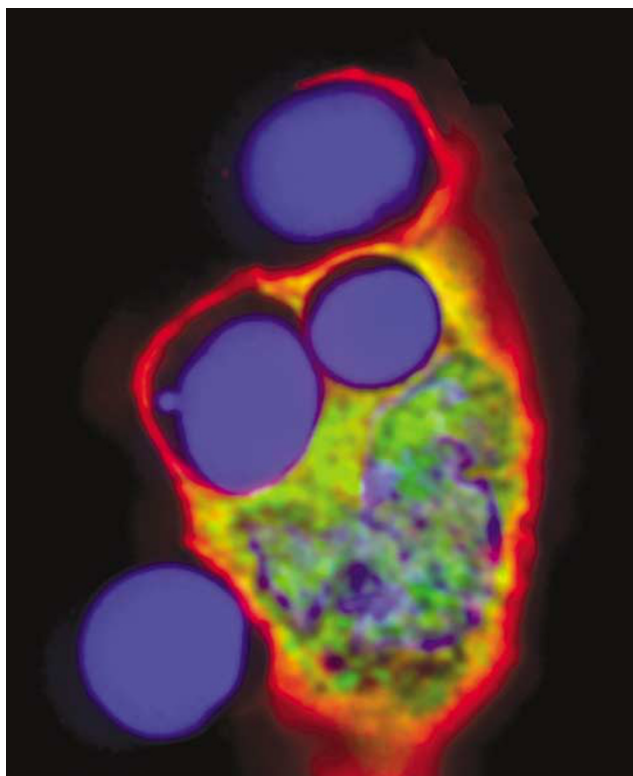
Figure 2 Fluorescent microscopy showing membrane extensions and ingested apoptotic cells (blue) in spacious phagosomes. Kindly provided by Yury Miller, University of California, SD, USA

signal pathways involved in uptake of apoptotic cells by either macrophages or non-professional phagocytes appear to be unique and very highly conserved evolutionarily. Consequently, we have coined the term efferocytosis (from effero – to carry to the grave, to bury) for apoptotic cell uptake.[44,46] Little, is known however about the underlying mechanisms that drive apoptotic cell phagosome formation and its consequences for subsequent apoptotic cell digestion.

Genetically tractable organisms such as *C. elegans* that allow the dissection of phagocytosis have shed some light on the signalling pathways underlying the engulfment of apoptotic cells. Seven independent genes have been identified in *C. elegans* that are required for efficient corpse removal. It is intriguing to note that most of these encode signalling molecules, not receptors. The observations support the possible extensiveness of ligand/receptor redundancy. Double mutant analyses have shown that the proteins encoded by these genes function in two partially redundant signalling pathways.[48] Interestingly, both of these pathways may play a role in promoting cytoskeletal reorganisation and activation of CED-10, which is homologous to the mammalian small GTPase Rac-1 (known to be essential in mammalian apoptotic cell removal). In the first pathway, the proteins CED-2, CED-5 and CED-12 (mammalian homologues CrkII, Dock180 and ELMO, respectively) function to activate CED-10 (Rac) and worms deficient in any of these protein can be rescued by overexpression of Ced-10.[49] In the second group, the candidate receptor CED-1 (CD91/LRP) probably recog-

nises an unknown ligand on the apoptotic cell and signals via its cytoplasmic tail to the adaptor protein CED-6 (hCED-6/GULP), whereas CED-7 (ABCA?) is thought to play a role in membrane dynamics. Hengartner *et al.* have shown that CED-1, CED-6 and CED-7 are required for actin reorganisation around the apoptotic cell corpse, and that the CED-10 (Rac) GTPase acts genetically downstream of these proteins to mediate corpse removal, functionally linking the two engulfment pathways as upstream regulators of Rac activation.[50] More recently, the importance of Rho-GTPases for the engulfment of apoptotic cells is further illustrated by a number of studies showing *in vitro* that Rac activation is required for phagocytes to ingest apoptotic cells whereas activation of RhoA inhibits uptake.[51] Furthermore, the initial Rac-dependent apoptotic cell uptake results fairly soon in activation of Rho, which explains previous observations that show that apoptotic cell uptake downregulates the ability of macrophages to ingest a second meal of apoptotic targets.[52] Ravichandran and co-workers[53] have identified another link between Ced-12 (ELMO) and cytoskeletal rearrangements possibly relevant for apoptotic cell engulfment and digestion by showing that ELMO is an ezrin–radixin–moesin (ERM)-binding protein. ERM proteins are enriched in membrane structures, where they link integral membrane proteins to the actin cytoskeleton and upon activation can also bind to the phagosomal membrane and facilitate actin assembly necessary for phagosome movement and maturation.[54]

Ultimately, we still know very little about the details of engulfment of apoptotic cells but it is apparent that signals leading to actin polymerisation and particle internalisation depend on the specific receptors that mediate the process and on additional modifying signals that can be generated by complex particles. Systems biology approaches (as recently used to characterise latex bead phagosomes[55]) may provide the entrée into this complex process before we can analyse specific signals that are critical for the subsequent processing of the ingested particle.

Digestion

Little is known about how the final step of apoptotic cell clearance is regulated and more importantly how it differs from the processing of classically opsonised or microbial cells, which employ a common underlying route of degradation from phagosomes to lysosomes. It has long been established that intracellular pathogens have developed multiple mechanisms to arrest phagosome development to aid their survival.[56] This raises the question whether dying cells employ similar mechanisms and whether phagosome maturation is dependent on the nature of the dying cell, the mechanism of engulfment and/or the receptors involved in recognition. This was first addressed by Shiratsuchi *et al.*[57] who showed that peritoneal macrophages from toll-like receptor 4 (TLR-4) null mice processed apoptotic thymocytes, Jurkat cells and opsonised thymocytes at a faster rate than wild-type macrophages. These observations are made in the context of no apparent changes in the macrophages ability to bind or engulf their apoptotic or necrotic targets and suggest that TLR-4 negatively regulates the degradation of engulfed cells in macrophages. On the other hand, Blander *et al.*[58] suggested

ER-Tracker DAPI   PKH -FITC   Lysotracker-CY3   3D-Composite
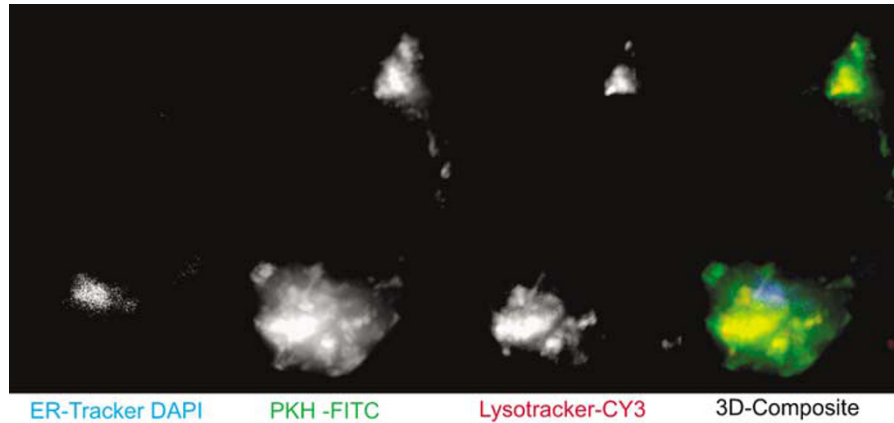
Figure 3 Fluorescent microscopy and 3D reconstruction of a green PKH-labelled apoptotic neutrophil ingested by a J774 macrophage. The macrophage has been coincubated with red lysotracker dye and blue ER-tracker before phagocytosis. The picture shows the marked colocalisation of the red lysotracker dye with the ingested apoptotic neutrophil 90 min after ingestion

that phagocytosis of bacteria but not apoptotic cells was impaired in the absence of TLR signalling. The authors deduced that two modes of phagosome maturation exist: first, a constitutive process as observed for apoptotic cells, which is slow and not influenced by signals received through TLRs and inducible as observed for bacteria, which is faster and can be further enhanced through TLR signalling. This interesting hypothesis would provide a mechanism for how the organism rapidly and efficiently clears potentially harmful bacteria. On the other hand, it is counterintuitive that apoptotic cells are cleared at a slow constitutive rate given the trillions of apoptotic cells cleared in tissue homeostasis every day (reviewed by Henson and Hume[59]) and the large number of apoptotic cells that are rapidly cleared in tissue injury without the appearance of a large numbers of apoptotic bodies. In contrast, a more recent study suggests no influence of TLR signalling (for all particles tested) on phagosome maturation in macrophages.[60] Therefore, it still remains unclear whether signals received through receptors not directly involved in uptake of apoptotic cells alter phagosome maturation.

Molecules and signalling pathways directly engaged in the clearance of apoptotic cells are more likely to be important for this process and we have recently provided evidence to support this hypothesis. We showed that phagosomes containing apoptotic cells mature more rapidly than those containing opsonised cells in many different phagocytic cell types, including primary macrophages, macrophage cell lines and fibroblasts and this effect was independent of the phagocyte species or the ingested target cell[61] (Figure 3). Furthermore, we have identified Rho as important for the maturation of phagosomes containing apoptotic cells and shown that its inhibition slowed the acidification rates of phagosomes containing apoptotic cells to those seen with opsonised cells. Finally, we identified ERM proteins as the downstream targets of Rho kinase and showed that dominant-negative inhibition of moesin had effects on acidification similar to inhibition of Rho signalling. Our data are consistent with a model in which the Rac and Rho pathways have mutual antagonistic effects, as previously shown for spatiotemporal activation and downstream signalling of Rho and Rac

regulating directional cell motility or engulfment of apoptotic cells.[62] Consequently, our data in the context of the existing literature suggest that the Rho/Rac balance in a given cell is not only important for apoptotic cell engulfment but also for phagosome maturation and may have direct consequences for antigen presentation.

Interestingly, dendritic cells (DC) acidify phagosomes at a slower rate than macrophages, independent of phagosome content.[61] This is in keeping with data showing that DC in comparison to macrophages generate low levels of lysosomal proteases and have a decreased ability to degrade internalised protein, which in turn favours their ability to present antigen.[63] It is therefore not surprising that apoptotic cells contained in DC phagosomes can be observed in the afferent lymphatics of the gut[64] en route to lymph nodes. The slow degradation of ingested material by DC may allow an extended period of time to sample the microenvironment for danger signals, which in turn instruct the DC to initiate an immune response or assist in the maintenance of self-tolerance. Autoimmunity can arise as a consequence of apoptotic cell recognition defects and clearance failure[65] and it is possible that the rate of apoptotic cell phagosome maturation and processing may influence this process.

Understanding the processes and regulation of apoptotic cell digestion in phagocytes provides an area for research with profound implications for the innate and adaptive immune response. Additional importance may be derived if one considers the redundancy of the recognition mechanisms, which make it conceivable that the highly conserved digestive process is the rate-limiting step for the clearance of apoptotic cells.

Concluding Remarks

Efficient clearance of apoptotic cells is a prerequisite for normal tissue homeostasis as well as immune response to injury. We have discussed the multistep process of apoptotic cell removal and how comparatively little we still know about the attraction signals, the engulfment process and subsequent digestion. It is suggested that the extensive knowledge

of the recognition receptors could be used to determine in detail whether and how signals derived from engagement of individual receptors modulate engulfment and subsequent processing of the internalised particle.

Apoptotic cell clearance can be efficiently manipulated to alter the immune response to injury as demonstrated by tumours and pathogens that employ this road to aid their survival. This in conjunction with the comprehensive literature detailing the anti-inflammatory consequences of apoptotic cell uptake (which was beyond the scope of this paper) raises an intriguing possibility. A better understanding of all the individual steps of the clearance process may enable us to harness the reparative properties of apoptotic cell clearance for therapeutic intervention.

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