Apoptotic cell clearance: basic biology and therapeutic potential

Ivan K. H. Poon[1,2], Christopher D. Lucas[3]*, Adriano G. Rossi[3] and Kodi S. Ravichandran[1]*

Abstract | The prompt removal of apoptotic cells by phagocytes is important for maintaining tissue homeostasis. The molecular and cellular events that underpin apoptotic cell recognition and uptake, and the subsequent biological responses, are increasingly better defined. The detection and disposal of apoptotic cells generally promote an anti-inflammatory response at the tissue level, as well as immunological tolerance. Consequently, defects in apoptotic cell clearance have been linked with various inflammatory diseases and autoimmunity. Conversely, under certain conditions, such as the killing of tumour cells by specific cell-death inducers, the recognition of apoptotic tumour cells can promote an immunogenic response and antitumour immunity. Here, we review the current understanding of the complex process of apoptotic cell clearance in physiology and pathology, and discuss how this knowledge could be harnessed for new therapeutic strategies.

Professional phagocytes
Professional phagocytes such as macrophages and immature dendritic cells can efficiently detect and engulf pathogens and dying cells.

[1]Center for Cell Clearance and the Department of Microbiology, Immunology, and Cancer Biology, University of Virginia, Charlottesville, Virginia 22908, USA.
[2]Department of Biochemistry, La Trobe Institute for Molecular Science, La Trobe University, Victoria 3086, Australia.
[3]MRC Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, UK.
**These authors contributed equally to this work.*
Correspondence to A.G.R. and K.S.R.
e-mails: a.g.rossi@ed.ac.uk; Ravi@virginia.edu
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In contrast to necrosis (BOX 1), apoptosis, also known as programmed cell death, occurs throughout life in essentially all tissues as part of normal development, homeostasis and pathogenic processes. Despite the constant turnover of cells through apoptosis, apoptotic cells are rarely seen under physiological conditions, even in tissues with high rates of apoptosis. For example, approximately 80% of developing thymocytes eventually undergo apoptosis, but free apoptotic cells are rarely observed in the thymus. This suggests that in the steady state, the rate of apoptotic cell removal is high, and this high rate is necessary for the continued clearance of the estimated one million cells that undergo apoptosis in various tissues every second in adult humans[1]. Dying cells are removed either by tissue-resident professional phagocytes (such as macrophages and immature dendritic cells (DCs)) or by neighbouring non-professional phagocytes.

In contrast to phagocytosis of bacteria and other 'danger-associated' particles, clearance of apoptotic cells is immunologically quiescent under physiological circumstances and does not involve the influx of inflammatory cells into the healthy tissues or a breakdown in immune tolerance against self-antigens. Recently, there has been a significant accumulation of knowledge on the molecular details of the apoptotic cell clearance process and on its functional relevance to disease. Such knowledge has created an exciting stage to further explore the potential therapeutic benefits of targeting the apoptotic cell clearance machinery in a variety of diseases ranging from autoimmunity to cancer.

In this Review, we introduce the key molecular features of the apoptotic cell clearance process and discuss its relevance to infection, inflammatory disease, autoimmunity, transplantation and cancer. Finally, we examine how targeting this clearance machinery could provide therapeutic benefits.

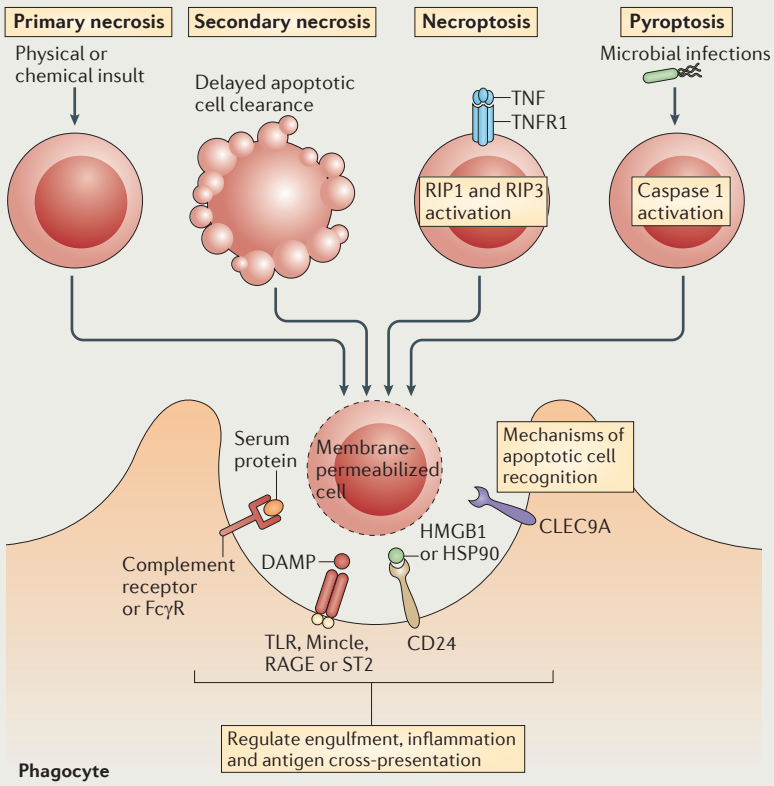
Molecular steps in apoptotic cell removal

Before their recognition by phagocytes, apoptotic cells undergo several distinct morphological changes. These changes may in turn facilitate the recognition and clearance of the apoptotic cell. An intriguing issue with respect to morphological changes during apoptosis is whether phagocytes engulf the apoptotic cells whole or in smaller 'bite-size' fragments. There is evidence for both. In most instances, professional phagocytes seem to phagocytose the targets in their entirety; this is particularly apparent in the case of macrophages or DCs that engulf apoptotic thymocytes or neutrophils[2,3]. Even fibroblasts and epithelial cells seem to engulf similarly sized dying brethren in tissues and *ex vivo*[4,5]. However, there are other cases in which a phagocyte simply cannot engulf the dying target in its entirety, possibly owing to a size difference between the phagocyte and the target.

Box 1 | **Immune recognition of membrane-permeabilized (necrotic) cells**

The plasma membrane can become permeable in response to physical and chemical insult (primary necrosis) or when uncleared apoptotic cells begin to lose membrane integrity (secondary necrosis) (see the figure). Membrane lysis can also occur through an active mechanism, when tumour necrosis factor receptor 1 (TNFR1) signalling is activated by TNF along with caspase 8 inhibition, a process known as necroptosis or programmed necrosis. Initiation of necroptosis depends on the activation of receptor-interacting protein 1 (RIP1) and RIP3 kinases[148]. Activation of caspase 1 by pathological stimuli such as microbial infection can also trigger membrane permeabilization by a form of cell death known as pyroptosis[149]. Furthermore, neutrophils and eosinophils can undergo another form of programmed cell death with the release of extracellular traps (termed neutrophil extracellular traps (NETs)) in response to pathogens and sterile inflammatory mediators[150,151] with potential antimicrobial but pro-inflammatory consequences.

A key feature of membrane lysis is the display and/or release of intracellular molecules that are otherwise hidden from the extracellular environment. Exposure of certain intracellular molecules can trigger inflammation and signal 'danger'[152] to the immune system. Such endogenous molecules (also known as damage-associated molecular patterns (DAMPs)) include: high-mobility group box 1 (HMGB1), SAP130, heat shock protein 90 (HSP90), DNA, uric acid and monosodium urate crystals, and interleukin-33 (IL-33). These endogenous molecules can be recognized variably by Toll-like receptors (TLRs), the C-type lectin Mincle, receptor for advanced glycation end-products (RAGE) and ST2 (REFS 153,154). Interestingly, interaction of HMGB1 and HSP90 with CD24 on responding cells can dampen their immunostimulatory properties to fine-tune the immune response[155]. The same molecules (such as phosphatidylserine (PtdSer)) that are exposed on membrane-permeabilized cells may also be exposed on intact apoptotic cells, so the recognition mechanisms that are used to mediate apoptotic and necrotic cell removal might overlap. Notably, in addition to direct recognition by phagocytes, many serum proteins have been found to preferentially aid the clearance of membrane-permeabilized cells through complement receptor and Fc receptor for IgG (FcγR)[156]. Furthermore, selective detection of membrane-damaged cells by receptors such as CLEC9A (C-type lectin domain family 9A) might have an important role in regulating antigen cross-presentation by CD8α⁺ dendritic cells[157,158].



For example, in inflamed adipose tissue, dying adipocytes seem to be engulfed by multiple macrophages that form 'crown-like structures' around a single adipocyte and ingest smaller fragments of the dying cell[6]. This has also been observed during the clearance of dying cells by fibroblasts in the absence of macrophages[2]. In fact, the formation of plasma membrane blebs (a common morphological feature of apoptosis) is required for the generation of smaller apoptotic cell fragments (known as apoptotic bodies). In multiple cell types, activation of RHO-associated protein kinase 1 (ROCK1) by caspase 3-mediated cleavage enhances phosphorylation of myosin light chain, which in turn promotes actomyosin contraction, membrane blebbing and the formation of apoptotic bodies[7,8] (FIG. 1). It has been unclear whether blebbing occurs *in vivo*, and some of the extensive membrane blebbing that has been observed in cultured cancer cells following apoptosis induction might be due to a lack of neighbouring phagocytes or represent late stages of cell death. However, plasma membrane blebs have recently been observed on apoptotic cells in tissues *in vivo*[9]. It remains to be determined what fraction of an apoptotic cell is cleared through the formation of such 'bite-size' fragments *in vivo* and whether the remaining 'corpse' of the cell is ingested as a larger target. Thus, in different tissues, depending on the relative sizes of the phagocyte and the target being ingested, the corpses may be taken whole or in smaller fragments. However, it is notable that in the case of substantial and excessive apoptosis, uncleared apoptotic cells and fragments can lose their membrane integrity (undergoing secondary necrosis) and are probably removed through other phagocytic mechanisms (BOX 1).

Interesting questions with respect to the morphology of dying cells include: how are apoptotic cells removed that are part of an epithelial sheet, such as epithelial cells in the gut or airways? And how is the integrity of the epithelial barrier maintained? These are not trivial issues when it is taken into account that in the gut of an adult human, an epithelial surface area roughly equivalent to a tennis court is replaced every 4 to 7 days. For cells held by attachment to the extracellular matrix, to neighbouring cells or to synthetic surfaces, detachment from the surrounding environment can be induced by caspase-mediated cleavage of components of focal adhesions[10,11] and of adherens junctions, such as E-cadherin, P-cadherin and β-catenin[12,13]. Viable neighbouring cells might replace such 'loosened' dying epithelial cells while the corpses are being removed. Alternatively, cell extrusion into the organ lumen or another tissue space[14] might allow for subsequent apoptotic cell removal by luminal phagocytes such as alveolar macrophages. It remains to be determined whether phagocytosis of apoptotic epithelial cells in different tissues involves mainly cell extrusion or other mechanisms.

Recruiting the 'right' phagocyte to prevent inflammation. It is now becoming increasingly clear that apoptotic cells at the earliest stages of death 'advertise' their presence to facilitate their own removal by phagocytes. The phagocytes are usually motile tissue-resident

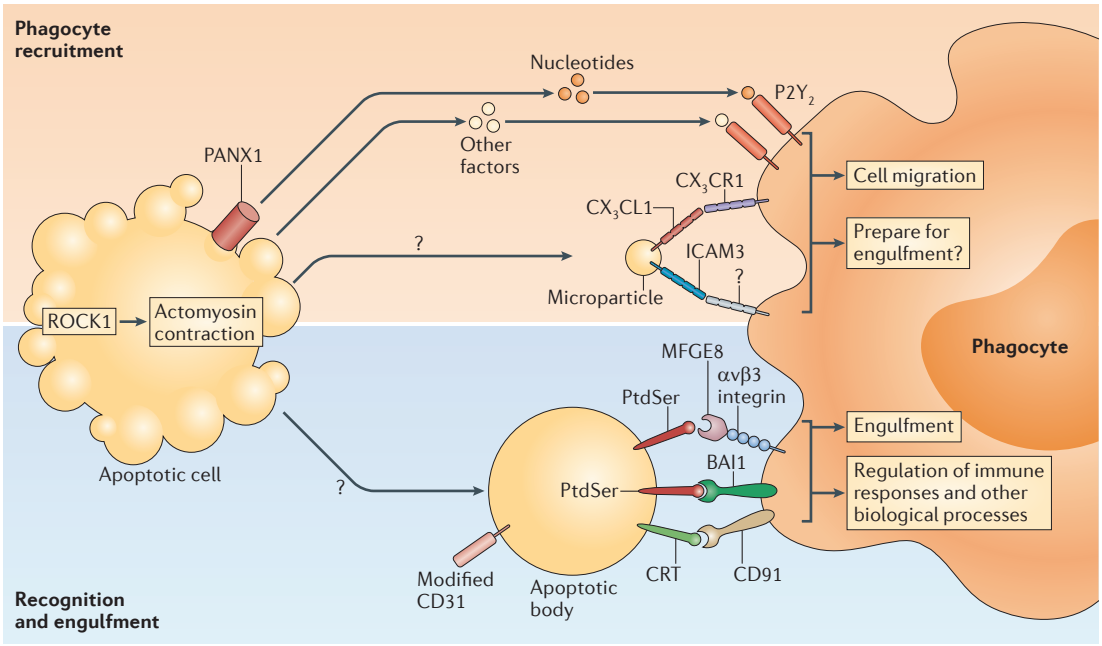


Figure 1 | **Phases of apoptotic cell clearance.** Cells undergoing apoptosis often exhibit morphological changes (for example, membrane blebbing and cellular shrinkage) to facilitate cell detachment and organelle fragmentation. Before or during the onset of apoptotic morphology, apoptotic cells also release 'find-me' signals in the form of soluble factors (for example nucleotides) or microparticle-associated molecules (including CX_3C-chemokine ligand 1 (CX_3CL1) and intercellular adhesion molecule 3 (ICAM3)) to recruit phagocytes for cell clearance. Nucleotides are released from apoptotic cells through caspase-activated pannexin 1 (PANX1) membrane channels. Whether the detection of find-me signals by phagocytes can prepare the molecular machinery necessary for engulfment in addition to cell migration warrants further investigation. On apoptotic cells or fragments of apoptotic cells (also referred to as apoptotic bodies), 'eat-me' signals (such as phosphatidylserine (PtdSer) and calreticulin (CRT)) are exposed and 'don't eat-me' signals (such as CD31) are modified to aid the the recognition by phagocytes. Phagocytes can engage eat-me signals directly through cell-surface receptors (such as brain-specific angiogenesis inhibitor 1 (BAI1) and CD91) or indirectly through bridging molecules (such as milk fat globule EGF factor 8 (MFGE8) or GAS6 (not shown)) that are in turn detected by membrane receptors (such as αVβ3 integrin or the TAM family of receptors (not shown)). Subsequent downstream signalling initiates engulfment and engulfment-associated responses from phagocytes. The mechanism underpinning the formation of apoptotic bodies and microparticles is not fully defined. CX_3CR1, CX_3C-chemokine receptor 1; $P2Y_2$, purinergic receptor $P2Y_2$; ROCK1, RHO-associated coiled-coil containing protein kinase 1.

Non-professional phagocytes
Non-professional phagocytes, such as fibroblasts, epithelial cells and endothelial cells, can engulf a variety of particles, including their dying brethren, but their primary function is not phagocytosis.

Plasma membrane blebs
Globular protrusions seen at the plasma membrane. Membrane blebs are dynamic and can occur during cell migration, cytokinesis and apoptosis.

Apoptotic bodies
Subcellular fragments released from apoptotic cells that are approximately 1–5 μm in size. Apoptotic bodies are non-uniform membrane-bound particles that contain portions of cytoplasm and fragmented organelles.

Focal adhesions
Macromolecular complexes that function as structural links between the cell and the extracellular matrix. Components of focal adhesion are also important for regulating intracellular signalling.

Adherens junctions
Intercellular macromolecular complexes that mediate cell–cell adhesion. Cadherin and catenin are key components of adherens junctions.

Cross-presentation
A process that describes the ability of antigen-presenting cells to display a peptide fragment from exogenous antigen, through MHC class I molecules, to $CD8^+$ T cells.

Organelle fragmentation
A process during apoptosis that aids the disassembly of organelles into smaller portions. Organelle fragmentation is driven by caspase-mediated cleavage of certain proteins and actomyosin contraction.

phagocytes, although in model systems, the recruitment of phagocytes directly from the circulation can also occur[15]. Apoptotic cells can attract phagocytes through the release of chemotactic factors, which are known as 'find-me' signals. These find-me signals can be soluble, or signal through submicron membrane vesicles termed apoptotic cell-derived microparticles (FIG. 1; TABLE 1).

Nucleotides such as ATP and UTP have been identified as key mediators of phagocyte recruitment towards apoptotic cells *in vitro* and *in vivo*. This process requires caspase-mediated activation of pannexin 1 (PANX1) channels to release nucleotides from apoptotic cells[16] and subsequent nucleotide detection by purinergic receptors (such as $P2Y_2$ and possibility others) on monocytes and macrophages[15]. It is notable that nucleotides such as ATP can also be secreted from cells through other active mechanisms (for example, through exocytosis, autophagy-dependent processes and autophagy-independent processes) and through passive mechanisms (for example, through membrane permeabilization). The release of ATP into the extracellular milieu can further modulate inflammation in a complex manner depending on its concentration and on how rapidly ATP is being degraded into immunosuppressive adenosine (discussed extensively in a recent review[17]). Lysophosphatidylcholine and sphingosine-1-phosphate have also been linked with monocyte recruitment to the proximity of apoptotic cells, but the *in vivo* relevance of these mediators in phagocyte recruitment remains to be defined further[18,19].

Small membrane vesicles released from apoptotic germinal centre B cells have been reported to enhance monocyte migration[20]. Consistent with this observation, certain molecules, including intercellular adhesion molecule 3 (ICAM3) and a proteolytically processed form of CX_3C-chemokine ligand 1 (CX_3CL1; also known as fractalkine), were found to associate with apoptotic cell-derived microparticles and to promote macrophage chemotaxis[21,22]. It is worth noting that phagocyte recruitment through microparticle-associated molecules could be a mechanism restricted to certain cell types that generate microparticles during apoptosis (for example, Burkitt lymphoma cells), and this area remains to be better explored.

Table 1 | **Molecular machinery of apoptotic cell recognition**

Signal	Release or exposure mechanism	Recognition mechanism	Details and comments	Refs
'Find-me' signals				
Nucleotides	PANX1	$P2Y_2$	The release of ATP and/or UTP from apoptotic cells promotes monocyte and macrophage migration *in vitro* and *in vivo*. Other P2Y family members may also facilitate detection of nucleotides by phagocytes	15,16
LPC	?	G2A	Caspase 3-mediated activation of iPLA2 is necessary to generate LPC during apoptosis. LPC augments monocyte migration *in vitro*	18
Sphingosine 1-phosphate	?	?	Purified sphingosine 1-phosphate enhances monocyte and macrophage migration *in vitro*	19
ICAM3	Microparticles	?	ICAM3 localizes to apoptotic blebs and microparticles during apoptosis. ICAM3 may also facilitate tethering of apoptotic B cells to macrophages	21,184
CX_3CL1	Microparticles	CX_3CR1	CX_3CL1–CX_3CR1 participates in the recruitment of macrophages to lymphoid follicles undergoing germinal centre reactions	22
EMAP II	?	?	The generation and release of mature EMAP II occurs during apoptosis. The ability of apoptotic cell-derived EMAP II to promote phagocyte migration has not been examined directly	185
Annexin A1	Membrane lysis	?	The release and proteolytic processing of annexin A1 by ADAM10 during secondary necrosis promotes migration of monocytes. Annexin A1 may also participate in the engulfment step of apoptotic cell clearance	186,187
'Keep-out' signal				
Lactoferrin	?	?	Lactoferrin released from apoptotic cells inhibits neutrophil migration	23
'Eat-me' signals				
PtdSer	Possibly phospholipid scramblase or aminophospholipid translocase	BAI1	BAI1 functions upstream of the ELMO1–DOCK180–RAC module to mediate apoptotic cell recognition and engulfment. BAI1 interacts with PtdSer through its thrombospondin type 1 repeats	30
		TIM1, TIM3 and TIM4	A metal ion-dependent ligand binding site located in the immunoglobulin variable domain of TIM4 mediates PtdSer binding. TIM3 may also have an important role in regulating cross-presentation of apoptotic cell-associated antigens by $CD8^+$ dendritic cells	33-35
		Stabilin 2	Stabilin 2 functions upstream of GULP and thymosin-β4 to aid apoptotic cell clearance. Stabilin 2 binds PtdSer via its epidermal growth factor-like domain repeats	31,32, 36,37
		MFGE8–αVβ3 integrin	MFGE8 is secreted by 'activated' macrophages and immature dendritic cells to promote apoptotic cell engulfment. MFGE8 interacts with PtdSer and αVβ3 integrin via its factor VIII-homologous domains and RGD motif, respectively	40
		Protein S–TAM or GAS6–TAM	Protein S and GAS6 interact with PtdSer and TAM receptors via their Gla domains and sex hormone-binding globulin domains, respectively. Usage of different TAM receptors is dependent on phagocyte and organ type	39,41, 188,189
		RAGE	RAGE is thought to function upstream of RAC1 to aid apoptotic cell recognition and uptake by alveolar macrophages	190
CRT	Possibly exocytic	CD91	Conditions that can induce both apoptosis and ER stress can facilitate pre-apoptotic exposure of CRT	42,44, 191
'Don't eat-me' signals				
CD31	N/A	CD31	Homophilic interaction of CD31 on leukocytes and macrophages promotes cell detachment. How signalling-disabled CD31 is generated on apoptotic leukocytes is unknown	192
CD46	N/A	N/A	The loss of complement regulatory protein CD46 on various cell types during apoptosis can lead to complement opsonization, which might aid recognition	49
CD47	N/A	SIRPα	Evidence suggests that CD47 on apoptotic lymphocytes could aid apoptotic cell binding to macrophages in addition to functioning as a don't eat-me signal	42,193

?, as yet unknown; ADAM10, a disintegrin and metalloproteinase domain-containing protein 10; BAI1, brain-specific angiogenesis inhibitor 1; CRT, calreticulin; CX_3CL1, CX_3C-chemokine ligand 1; CX_3CR1, CX_3C-chemokine receptor 1; DOCK180, dedicator of cytokinesis 180; ELMO1, engulfment and cell mobility 1; EMAP II, endothelial monocyte-activating polypeptide II; ER, endoplasmic reticulum; GAS6, growth arrest-specific gene 6; GULP, PTB domain-containing engulfment adaptor protein 1; ICAM3, intercellular adhesion molecule 3; iPLA2, calcium-independent phospholipase A2; LPC, lysophosphatidylcholine; MFGE8, milk fat globule EGF factor 8; N/A, not applicable; $P2Y_2$, purinergic receptor $P2Y_2$; PANX1, pannexin 1; PtdSer, phosphatidylserine; RAGE, receptor for advanced glycation end-products; RGD, arginine–glycine–aspartate motif; SIRPα, signal regulatory protein-α; TAM, Tryo3–Axl–Mer; TIM, T cell immunoglobulin mucin domain.

In addition to attracting certain phagocytes, apoptotic cells are thought to release factors, referred to as 'keep-out' signals, to exclude inflammatory cells such as neutrophils (TABLE 1). Lactoferrin, a multifunctional glycoprotein, is the only keep-out signal discovered to date[23], and its expression is upregulated by various cell types following induction of apoptosis. Lactoferrin is released by apoptotic cells, and purified lactoferrin can inhibit neutrophil chemotaxis *in vitro* and *in vivo*, possibly by dampening neutrophil activation. Importantly, although it has also been shown to limit eosinophil recruitment[24], lactoferrin had no effect on monocyte or macrophage migration towards the chemoattractant complement component C5a, demonstrating the selectivity of lactoferrin in inhibiting neutrophil and eosinophil migration[23,24]. It is important to note that there is limited information regarding the repertoire of find-me and keep-out signals released by different cell types during apoptosis. In fact, whether various find-me signals can function synergistically or additively to recruit phagocytes is not well defined. Nevertheless, it is intriguing to consider the possibility that apoptotic cells can release a unique combination of factors to control the recruitment of appropriate phagocytes for cell clearance and to limit inflammation.

Specific recognition of apoptotic cells by phagocytes. Phagocytes identify apoptotic cells among healthy viable cells on the basis of a unique combination of markers on the surface of apoptotic cells (FIG. 1; TABLE 1). Increased surface exposure of the inner-membrane lipid phosphatidylserine (PtdSer) is a common (but not exclusive) feature of apoptotic cells and functions as a key 'eat-me' signal to trigger phagocytic uptake[25]. The precise machinery that controls surface exposure of PtdSer during apoptosis is still being defined. However, recent studies suggest that both the calcium-dependent and calcium-independent activities of phospholipid scramblase disrupt the aminophospholipid asymmetry of the plasma membrane and promote PtdSer exposure during apoptosis[26–28]. It is worth noting that a substantial amount of PtdSer exposure is necessary for detection by phagocytes[29].

Several PtdSer recognition mechanisms have been identified recently (TABLE 1). PtdSer can be detected directly through membrane receptors, such as brain-specific angiogenesis inhibitor 1 (BAI1)[30], stabilin 2 (REFS 31,32) and members of the T cell immunoglobulin mucin domain (TIM) protein family (including TIM1, TIM3 and TIM4)[33–35]. After recognizing PtdSer, the seven-span transmembrane protein BAI1 can signal through the evolutionarily conserved ELMO1–DOCK180–RAC (engulfment and cell mobility 1–dedicator of cytokinesis 180–RAC) complex to facilitate cytoskeletal rearrangement for engulfment[30] (FIG. 1). Similarly, stabilin 2 can interact with PTB domain-containing engulfment adaptor protein 1 (GULP) and with thymosin-β4 to initiate apoptotic cell uptake following PtdSer binding[36,37]. TIM4 seems to function primarily as a tethering protein for PtdSer and to signal through its associated proteins to promote engulfment[38]. In addition to these bona fide PtdSer receptors, bridging molecules, including milk fat globule EGF factor 8 (MFGE8), protein S and GAS6, can bind PtdSer and are in turn recognized by their cell-surface receptors on phagocytes, such as the αVβ3 integrin and the Tryo3–Axl–Mer (TAM) family of receptors[39–41] (TABLE 1). It is intriguing that multiple PtdSer recognition mechanisms have been described for apoptotic cell clearance. It remains to be defined in mammals whether a particular mode of PtdSer recognition may be required only under specific conditions (for example, tissue development, homeostatic cell turnover or inflammation) or whether these multiple mechanisms provide a degree of redundancy. Nevertheless, the growing availability of mice deficient in PtdSer-recognition receptors and bridging molecules, as well as *in vivo* models to assess the functional consequences of apoptotic cell removal, are expected to help us to understand the need for such an array of PtdSer sensing pathways.

In addition to PtdSer, surface exposure of calreticulin (CRT) on apoptotic cells can function as another eat-me signal. Induction of cancer cells to undergo apoptosis through mechanisms that also promote endoplasmic reticulum stress (ER stress; such as anthracyclin treatment and photodynamic therapy) seems to result in rapid translocation of CRT from the ER to the plasma membrane[42–45]. Exposed CRT can subsequently be detected by CD91 (which is also known as low density lipoprotein (LDL)-receptor-related protein) on phagocytes to stimulate engulfment[42] (FIG. 1). Notably, CRT exposure seems to trigger an immunogenic response against apoptotic cell-derived antigens, rather than inducing immunological tolerance[43–45].

It is apparent that displaying certain eat-me signals alone may not be sufficient to trigger apoptotic cell engulfment[42,46]. For example, constitutive PtdSer exposure on viable lymphoma cells that express a mutant form of the scramblase TMEM16F (also known as anoctamin 6) did not promote their uptake by peritoneal macrophages or $CD8^+$ splenic DCs[46]. These observations support the idea that healthy viable cells, which can also expose PtdSer under physiological circumstances, might actively suppress phagocytic uptake by displaying 'don't eat-me' signals, such as CD31, CD46 and CD47 (TABLE 1). Engagement of CD47 (also known as integrin-associated protein) on viable cells by signal regulatory protein-α (SIRPα) on macrophages can negatively regulate engulfment[42,47,48], whereas redistribution or loss of CD47 during apoptosis may promote cell clearance[42]. Furthermore, the loss of complement regulatory protein CD46 on various cell types during apoptosis can lead to complement opsonization[49], a process that may aid their recognition by phagocytes. In addition, it remains to be fully investigated whether the exact configuration of PtdSer on the cell surface of live and apoptotic cells mediates differing signals. Collectively, exposure of a sufficient amount of eat-me signals and the loss of don't eat-me signals on the surface of apoptotic cells is necessary to trigger their removal by phagocytes.

Translating the final message. As cell death can arise under a variety of physiological and pathological conditions, including tissue development, homeostatic cell turnover, tissue injury, inflammation, tumorigenesis

Apoptotic cell-derived microparticles
Another category of subcellular fragments released from apoptotic cells that are approximately 0.1–1 μm in size. Apoptotic cell-derived microparticles and apoptotic bodies represent a spectrum of membrane-bound apoptotic vesicles characterized mainly by size and density.

Germinal centre
A lymphoid structure that arises within follicles after immunization with, or exposure to, a T cell-dependent antigen. It is specialized for facilitating the development of high-affinity, long-lived plasma cells and memory B cells.

Aminophospholipid asymmetry of the plasma membrane
The distribution of aminophospholipids (such as phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine) between the outer and inner leaflet of the plasma membrane is often asymmetrical and may differ depending on the cell type, activation status and viability. This asymmetry is actively maintained by ATP-dependent processes and compromised by activation of phospholipid scramblases.

Endoplasmic reticulum stress
(ER stress). A response by the ER that results in the disruption of protein folding and in the accumulation of unfolded proteins in the ER.

Photodynamic therapy
A treatment that uses a combination of a specific wavelength of light and a photosensitizing agent to induce the production of reactive oxygen species and cause lethal damage to the cells.

and infection, apoptotic cells might carry important and complex information for the regulation of the downstream immune response in a context-dependent manner. A key question is how apoptotic cells convey such a diverse array of immunological information. Answering this question requires the consideration of all potential variables that occur with cell death.

The first parameter to consider is the 'quality' of apoptotic cells, which determines what type of eat-me signals are being exposed to the immune system. Factors that can determine the quality of apoptotic cells include cell type, the cause of cell death and the activation status of the dying cells. As discussed above, induction of apoptosis by certain anticancer drugs can render apoptotic tumour cells pro-immunogenic, whereas apoptosis during developmental or 'homeostatic' processes is largely anti-inflammatory and immunologically silent.

In addition, the quantity of apoptotic cells may determine the magnitude of ensuing immune response. Although cell death in steady state tissues is easily and efficiently handled without inducing an immune response, large numbers of apoptotic cells, such as those observed during infection or induced by antitumour therapies, may overwhelm the engulfment capacity of local phagocytes and the uncleared cells or other components of these dying cells could induce a pro-immunogenic response. Another key parameter to consider is the apoptotic cell microenvironment, which determines what type of phagocyte is available to mediate clearance and regulate the subsequence immune response. This is particularly relevant for immune-privileged tissues such as the brain, eye and testes.

Finally, the timing of cell death and duration of apoptotic cell-derived signals may also contribute to the final immunological outcome. Thus, depending on the specific conditions in which the cell death is occurring, apoptotic cells may promote immunity or tolerance. It should also be noted that in certain instances, apoptotic cells can have a beneficial effect in tissue development and repair, as observed in myoblast fusion[9] and wound healing[50]. A better characterization of the parameters of cell death and apoptotic cell clearance that influence immune activation might help us to understand certain disease states and to develop apoptotic cell-based or cell clearance-targeting therapeutic approaches.

Targeting apoptotic cell clearance for therapy

As mentioned above, apoptotic cells are rarely detected under physiological conditions, but the presence of uncleared apoptotic cells has been linked to several different diseases that involve infection, inflammation, autoimmunity and cancer. In this section, we review the evidence that links defective cell clearance with the initiation and progression of pathology and discuss potential therapeutic implications (FIG. 2; BOX 2).

Infection. In response to an acute episode of infection or tissue injury, tissue-resident cells (both immune and parenchymal) detect pathogen-associated molecular patterns (PAMPs), including bacterial endotoxin and viral nucleic acids, as well as damage-associated molecular patterns (DAMPs), which are mainly intracellular molecules released upon cell death[51]. As a consequence, leukocytes are recruited to the site of inflammation; innate immune cells such as neutrophils are often the first cells to appear, whereas mononuclear cells and macrophages accumulate at a later stage[52]. This initial robust immune response is a beneficial one and is designed to contain and destroy invading pathogens and enhance tissue repair[53,54]. After the initial threat has been eliminated, leukocyte recruitment ceases and the already recruited cells are disposed of to restore homeostasis. Although recruited neutrophils can be cleared through transepithelial migration into the airway lumen in the context of lung inflammation[55] or through their emigration via lymphatic vessels[56], it seems that a main clearance route is by local neutrophil apoptosis and subsequent phagocytosis[57,58]. Both tissue-resident and recruited macrophages, as well as local epithelial cells, can ingest apoptotic leukocytes[59].

Following neutrophil recruitment into infected tissue, exposure to bacteria-derived products initially enhances the lifespan of neutrophils. However, the phagocytosis of pathogens, such as *Escherichia coli* or *Staphylococcus aureus*, promotes a form of apoptotic cell death of neutrophils that is termed phagocytosis-induced cell death (PICD)[60] (FIG. 2). This response is believed to be primarily protective for the host, allowing for a second round of destruction of pathogens that might remain within the engulfed apoptotic neutrophils. Incidentally, pharmacological acceleration of neutrophil apoptosis is protective in pneumococcal meningitis, resulting in an accelerated rate of recovery and reduced incidence of brain haemorrhage[61]. Small, locally acting, endogenous lipid-derived autacoids (pro-resolving lipids) that promote the removal of apoptotic cells are also involved in limiting infection-associated inflammation (BOX 2). The pro-resolving lipid resolvin E1 has recently been shown to promote PICD and thereby enhance the resolution of bacterial infection in mice[62].

However, pathogens can also make use of engulfment machinery for their benefit: phagocytosis of apoptotic neutrophils infected with the intracellular pathogen *Chlamydia pneumoniae* has been shown to result in the subsequent infection of macrophages[63]. This 'Trojan horse' strategy adopted by *C. pneumoniae* increases its virulence and replication when compared to direct infection of macrophages. Moreover, bacteria can enter a cell using cytoplasmic proteins that are also involved in engulfment of apoptotic cells; for example, IpgB1 of *Shigella flexneri* induces membrane ruffling through ELMO1 activation, promoting bacterial invasion of epithelial cells[64]. Therefore, enhancing the activity of the engulfment machinery or neutrophil apoptosis in specific infections to mediate pathogen clearance is an exciting possibility, but more investigation is needed.

Lung inflammation. The impaired or defective clearance of dying neutrophils during inflammation can lead to a prolonged inflammatory response. Although the best evidence for this has come from animal studies, such a phenomenon has also been observed in

Pathogen-associated molecular patterns
(PAMPs). Molecular signatures that are found in pathogens but not in mammalian cells. Examples include terminally mannosylated and polymannosylated compounds (which bind the mannose receptor) and various microbial components, such as bacterial lipopolysaccharide, hypomethylated DNA, flagellin and double-stranded RNA (all of which bind Toll-like receptors).

Damage-associated molecular patterns
(DAMPs). As a result of cellular stress, cellular damage and non-physiological cell death, DAMPs are released from the degraded stroma (for example, hyaluronate), from the nucleus (for example, high-mobility group box 1 protein), from the cytosol (for example, ATP, uric acid, S100 calcium-binding proteins and heat-shock proteins) and from mitochondria (formylated peptides and mitochondrial DNA). Such DAMPs are thought to elicit both local and systemic inflammatory responses.

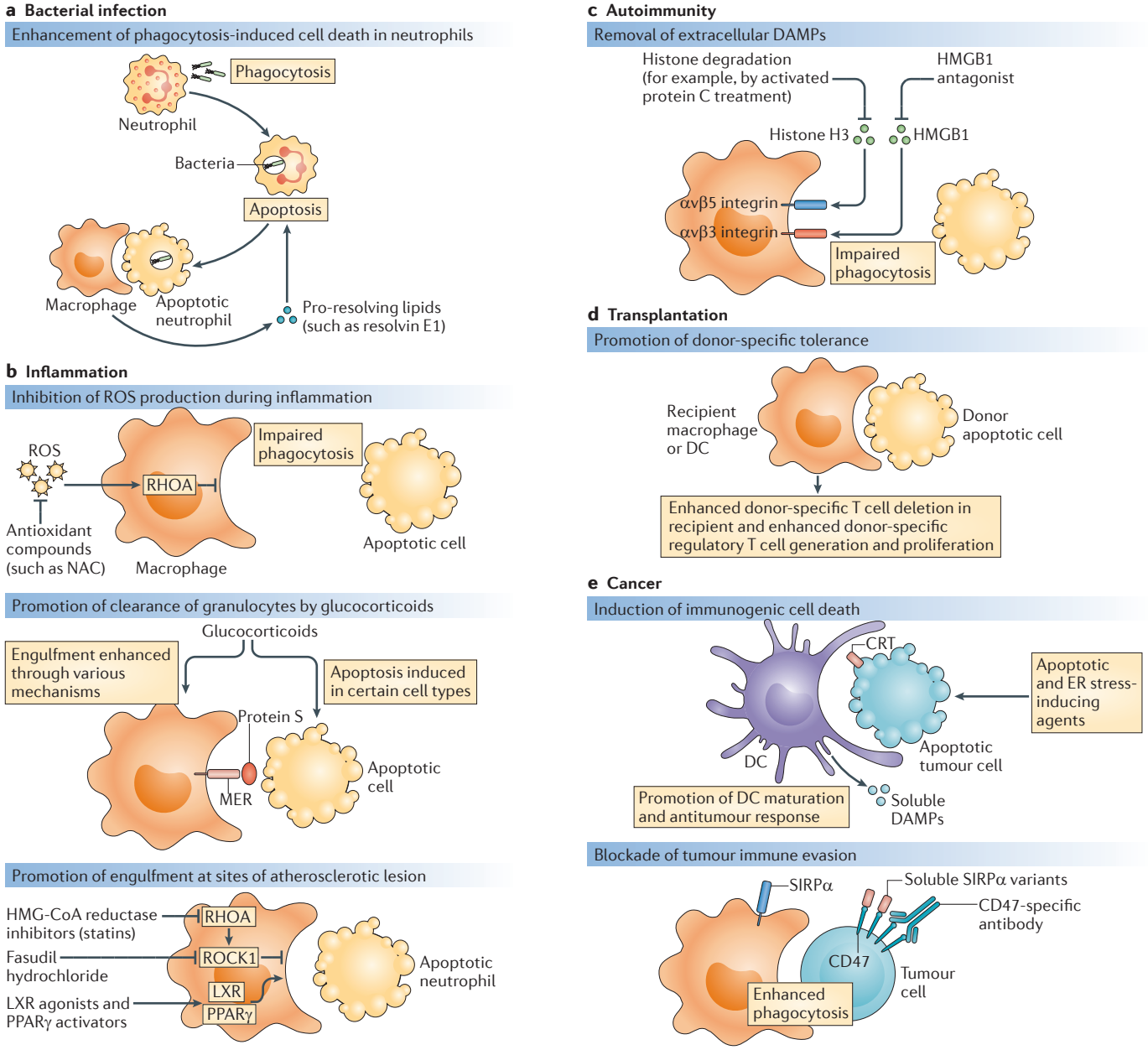


Figure 2 | **Potential approaches for targeting the apoptotic cell clearance process for therapeutic benefits. a** | Bacterial infection. Following phagocytosis of invading bacteria, neutrophils frequently undergo phagocytosis-induced cell death with subsequent engulfment by surrounding phagocytes, providing a second round of pathogen destruction. The engulfing phagocytes also increase production of pro-resolving lipid mediator release (for example, resolvin E1) with enhanced host-directed bacterial killing. **b** | Inflammation. Reactive oxygen species (ROS) that are produced at sites of inflammation impair phagocytosis through the activation of RHOA within phagocytes. Scavenging ROS (for example, by using *N*-acetylcysteine (NAC)) enhances apoptotic cell clearance. Glucocorticoids can potentially augment eosinophil clearance by promoting both eosinophil apoptosis and cell clearance through a protein S–MER-dependent pathway. Impaired engulfment in atherosclerosis is, in part, mediated by increased activity of RHOA and its downstream mediator RHO-associated coiled-coil containing protein kinase 1 (ROCK1), both of which are negative regulators of apoptotic cell engulfment. RHOA inhibition by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) or ROCK inhibition by fasudil hydrochloride seems to have a beneficial effect in atherosclerosis, possibly by regulating engulfment. The LXR (liver X receptor) and PPARγ (peroxisome proliferator-activated receptor-γ) nuclear receptors are both positive regulators of engulfment, with pharmacological activation leading to protection against atherosclerosis and inflammation. **c** | Autoimmunity. At sites of inflammation, extracellular damage-associated molecular patterns (DAMPs), such as histones and high-mobility group box 1 (HMGB1), negatively regulate apoptotic cell engulfment by binding to αvβ5 integrin and αvβ3 integrin, respectively, on the surface of phagocytes. Strategies to degrade DAMPs (for example through the degradation of histone H3 by activated protein C) can improve apoptotic cell clearance. **d** | Transplantation. The recognition and uptake of donor apoptotic cells by recipient dendritic cells (DCs) and macrophages can promote donor-specific tolerance by the generation and/or expansion of regulatory T cell populations in the recipients and limit allograft rejection. **e** | Cancer. Induction of tumour cell death accompanied with calreticulin (CRT) exposure and the release of DAMPs can promote DC-mediated engulfment and DC maturation to initiate an antitumour immune response. In addition, targeting tumour cells with CD47-specific blocking antibodies or soluble signal regulatory protein-α (SIRPα) variants inhibits CD47–SIRPα interaction and facilitates tumour cell removal by macrophages. ER, endoplasmic reticulum.

Box 2 | **Endogenous controllers of apoptotic cell clearance**

During the spontaneous resolution of an episode of inflammation, locally produced molecules modulate apoptotic cell clearance. These include the specialized pro-resolving lipid mediators lipoxins (which are generated from arachidonic acid), resolvins and protectins (which are generated from omega-3 fatty acids)[159]. Although these different classes of pro-resolving lipids are distinct in both their production and biological effects, they all reduce neutrophil recruitment and enhance clearance of apoptotic cells.

Apoptotic neutrophils enhance the production of pro-resolving lipid mediators by macrophages during their engulfment[160]. The benefits of these molecules have been shown in animal models of inflammation including asthma[161], lung injury[62,162] and colitis[163]. Furthermore, emerging evidence has demonstrated that they also contribute to antimicrobial defence during bacterial infection[164]. There is also evidence that pro-resolving lipid production may be deficient in human inflammatory disease[165], although this is an area that requires further study. Despite the locally acting and short-lived nature of these lipid mediators, structural analogues with longer half-lives have been developed, and pro-resolving lipid mediators are undergoing early clinical trials in humans (ClinicalTrials.gov identifiers: NCT01675570 and NCT01639846).

Lysophosphatidylserine (LysoPS), a lipid exposed on apoptotic cells (particularly neutrophils) in an NADPH oxidase-dependent manner, has been shown to enhance macrophage engulfment[166,167]. This may partly explain why patients with chronic granulomatous disease (CGD) who lack a functional NADPH oxidase have defects in apoptotic cell clearance and a hyper-inflammatory phenotype with a propensity for autoimmune disease[168].

Tissue-resident macrophages express 12/15-lipoxygenase that can generate oxidized phosphatidylethanolamine (OxPE) on their cell membranes[169]. OxPE has been shown to bind the soluble apoptotic cell-bridging molecule milk fat globule EGF factor 8 (MFGE8) and thus prevent the uptake of apoptotic cells by recruited inflammatory monocytes[169]. This binding and sequestering of MFGE8 by OxPE does not inhibit the engulfment of apoptotic cells by tissue-resident macrophages, which predominantly recognize apoptotic cells through the phosphatidylserine (PtdSer) receptor T cell immunoglobulin domain and mucin domain protein 4 (TIM4). Lack of 12/15-lipoxygenase results in apoptotic cell clearance by inflammatory monocytes or macrophages, with the subsequent presentation of apoptotic cell-derived intracellular antigens and development of autoimmunity with glomerulonephritis[169]. Whether defects in LysoPS production or in the control of 12/15-lipoxygenase activity are defective in human disease is currently unknown, but the targeting and mimicking of endogenous controllers of apoptotic cell clearance is an attractive therapeutic avenue.

Chronic obstructive pulmonary disease
(COPD). A group of diseases characterized by the pathological limitation of airflow in the airway, including chronic bronchitis and emphysema. COPD is most often caused by tobacco smoking but can also be caused by other airborne irritants, such as coal dust, and occasionally by genetic abnormalities, such as α1 -antitrypsin deficiency.

Pulmonary fibrosis
A heterogenous group of disorders characterized by diffuse abnormalities of the pulmonary interstitium, with increased and variable inflammation, and fibrosis. Frequently of unknown aetiology, pulmonary fibrosis can also be related to autoimmune disease and secondary to medications.

Cystic fibrosis
An autosomal recessive genetic condition secondary to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR; a chloride channel). This leads to a multisystem disorder with lung, gastrointestinal, endocrine and fertility complications. Chronic infection of the lungs ensues, leading to significant morbidity and mortality.

Efferocytosis
The phagocytic clearance of apoptotic cells.

human disease, including chronic obstructive pulmonary disease (COPD)[65], pulmonary fibrosis[66] and cystic fibrosis[67]. The mechanism underlying impaired phagocytosis in inflammation involves, in part, the production of reactive oxygen species (ROS) by neutrophils (FIG. 2). ROS activate the GTPase RHOA (a negative regulator of efferocytosis) in surrounding phagocytes, thereby reducing apoptotic cell engulfment by neighbouring cells[68–70]. Interestingly, antioxidants such as the thiol compound *N*-acetylcysteine (NAC) promote clearance of apoptotic cells by macrophages during lipopolysaccharide (LPS)-mediated lung inflammation in mice by inhibiting both ROS production and RHOA activity, and NAC can also enhance production of the anti-inflammatory cytokine transforming growth factor-β (TGFβ)[71]. However, antioxidant therapy in humans with acute lung injury and acute respiratory distress syndrome has thus far provided no convincing evidence of efficacy[72]. Perhaps the drugs fail to reach the relevant phagocytes, or perhaps such therapies need to be targeted to specific subgroups of patients with a defect in efferocytosis.

Although alveolar macrophages from adult patients with asthma of mild to moderate severity have normal phagocytic capacity, those from patients with severe asthma are defective in clearing apoptotic cells[73]. Similarly, alveolar macrophages from children with poorly controlled asthma have defective phagocytosis[74]. The molecular events causing defective phagocytosis in patients with severe asthma are not yet understood, but it is relevant to note that corticosteroids, the mainstay of treatment in asthma, not only induce eosinophil apoptosis[75] but also enhance their engulfment by monocyte-derived macrophages *in vitro*[76]. This corticosteroid-induced enhanced clearance depends on the binding of protein S to apoptotic cells and the upregulation of tyrosine-protein kinase MER (a member of the TAM family) on the surface of macrophages[77] (FIG. 2). Furthermore, enhanced clearance of apoptotic eosinophils by macrophages has been observed in asthmatic humans after steroid therapy[78]. Steroid treatment seems to be less effective in neutrophil-dominated lung inflammatory disorders, and the ability of steroids to induce neutrophil apoptosis seems to be context dependent[79,80]. In addition to alveolar macrophages and lung-associated DCs, airway epithelial cells have recently been reported to engulf neighbouring apoptotic cells, and a defect in this process increases the production of pro-inflammatory mediators and exacerbates airway inflammation[5]. With this increased evidence of defective apoptotic cell clearance in lung inflammatory diseases, combined with knowledge of the mechanisms behind the therapeutic benefits of commonly used anti-inflammatory medications such as corticosteroids, it is hoped that novel approaches for targeting inflammatory diseases (within the lung and in other tissues) are on the horizon.

Atherosclerosis. Atherosclerosis is one of the leading causes of death in Western societies, and its pathogenesis involves chronic inflammation of the vascular wall, predominantly as a result of the accumulation of mononuclear immune cells[81]. Monocytes and macrophages have a crucial role in the initiation and progression of atherosclerosis. Although there are resident macrophages in the arterial wall, the recruitment of $LY6C^+$ inflammatory monocytes and $LY6C^-$ 'patrolling' monocytes, and the differentiation of these cells into

macrophages and monocyte-derived DCs is thought to critically influence atherosclerosis[82]. After taking up various oxidized lipids in the intima, lipid-laden macrophages undergo apoptosis and can be engulfed by surrounding macrophages[82]. In the early stages of atherosclerosis, apoptosis in the vascular walls seems to be counterbalanced by rapid and efficient engulfment[82]. However, in mature atherosclerotic lesions (known as plaques), there is reduced clearance of apoptotic cells and progression to secondary necrosis (BOX 1), which coincides with plaque lesion expansion and an increased risk of rupture[83]. Plaque rupture leads directly to acute coronary syndromes and stroke in humans.

This reduction in apoptotic cell clearance observed in mature plaques seems central to the pathological process of plaque progression[84], as defects in the phagocytic components such as MER, MFGE8 or the complement component C1q results in the accumulation of apoptotic debris within plaques and accelerates atherosclerosis[85–88]. Conversely, induction of apoptosis within plaques by a physiological stimulus (for example, through TNF-related apoptosis-inducing ligand (TRAIL)), has been shown to be beneficial and atheroprotective[89], which is thought to be due to increased anti-inflammatory signalling within this microenvironment. Therefore, defective macrophage engulfment, and in turn a more pro-inflammatory state, seems to drive accelerated atherosclerosis. Importantly, healthy phagocytes release anti-inflammatory cytokines that dampen inflammation following apoptotic cell clearance[82] and may help to control atherosclerosis progression.

Another key question is why human macrophages *in situ* lose the ability to rapidly clear dead cells. Although the reasons for this are incompletely understood and are likely to be multifactorial, several possible mechanisms have been suggested. Oxidized lipoproteins, which are present in plaques *in vivo*, inhibit efferocytosis *in vitro* by binding to CD14 (REF. 90). In addition, the activity of RHO kinase is reported to be increased in atherosclerotic lesions[91]. Interestingly, the widely used 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (also known as statins), which are principally used as cholesterol-lowering agents in atherosclerosis and vascular disease, have long been thought to have additional anti-inflammatory effects that are partly due to enhancement of the phagocytic activity of macrophages[92]. The mechanism underlying enhanced phagocytosis induced by statins involves RHOA inhibition[93] (FIG. 2). RHOA activates ROCK1, which has an inhibitory effect on engulfment. Interestingly, the ROCK inhibitor fasudil hydrochloride inhibits both the early development and late progression of atherosclerosis in mice[94], although these beneficial effects have not yet been linked to enhanced efferocytosis. Statins and ROCK inhibitors also display anti-inflammatory effects in other non-vascular preclinical models of inflammation, including acute lung injury[95], bleomycin-induced pulmonary fibrosis[96] and inflammatory arthritis[97], although a direct link between these effects and phagocytosis of dying cells is not yet established.

The uptake of apolipoprotein B-containing lipoproteins by macrophages that accumulate within the vascular walls, which leads to foam cell formation, is a key early event in atherosclerosis[98,99]. Improved cholesterol efflux from foam cells can revert this stage of atherosclerosis, leading to macrophage egress and a reduction in lesion size[100]. It was previously shown that when macrophages engage apoptotic cells (but not necrotic cells), cholesterol efflux is stimulated from the engulfing macrophages[101]. The enhanced cholesterol efflux by macrophages occurs through upregulation of mRNA and protein for the cholesterol transporter ABCA1 (REF. 101). ABCA1 is an important molecule in macrophage cholesterol efflux and transports free cholesterol from within the cells to lipid-poor apolipoprotein A1 that is then modified in the plasma for transport to the liver and excretion[102,103]. Loss of ABCA1 promotes atherogenesis, whereas overexpression of ABCA1 reverses the disease[104,105]. Recent reports suggest that ABCA1 upregulation and signalling downstream of ABCA1 (after binding to apolipoprotein A1) can also dampen macrophage inflammatory responses[81,106,107]. Thus, 'foamy' macrophages undergoing necrosis in late-stage lesions might fail to upregulate ABCA1 expression, thereby preventing the cholesterol efflux and ABCA1-mediated immunosuppressive effects[81,106,107]. The precise phagocytic receptor (or receptors) that induces the upregulation of ABCA1 on apoptotic cell recognition and thus promotes cholesterol efflux from the phagocyte has not yet been defined.

Other downstream modulators of the phagocyte response to ingested apoptotic cells include liver X receptors (LXRs) and peroxisome proliferator-activated receptors (PPARs), which are nuclear receptors that can act as positive regulators of engulfment by upregulating MER expression[108] (FIG. 2). LXR activation by synthetic agonists has been demonstrated to have beneficial effects in animal models of atherosclerosis[109]. In addition, activators of PPARs have already been approved for clinical use in the treatment of diabetes, and have been shown to enhance murine macrophage efferocytosis[110] and reduce progression of atherosclerosis in humans in a glucose homeostasis-independent manner[111]. Therefore, such treatments may have additional beneficial effects via promotion of apoptotic cell clearance.

Autoimmunity. Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disorder with a variable clinical presentation; it commonly affects the skin, lungs, kidneys and central nervous system. It is characterized by the presence of autoantibodies that are specific for nuclear components and, frequently, by the presence of DNA and nucleosomes in the circulation[112]. In patients with SLE, there is increased spontaneous appearance of apoptotic cells within lymph nodes and blood, and accumulation of apoptotic cells within the skin following exposure to ultraviolet radiation[113,114]. The increase in apoptotic cells observed in SLE is thought to reflect an impaired ability of SLE phagocytes to engulf dead cells, rather than an intrinsic

Intima
The innermost layer of an artery, which consists of loose connective tissue and is covered by a monolayer of endothelium. Atherosclerotic plaques form within the intima.

C1q
A complement protein and a component of the classical complement pathway. C1q is involved in diverse functions including immune function, autoimmunity and facilitates apoptotic cell clearance.

Statins
A family of inhibitors targeting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, an enzyme that catalyses the conversion of HMG-CoA to L-mevalonate. These molecules are mainly used as cholesterol-lowering drugs, but they also have immunoregulatory and anti-inflammatory properties. L-mevalonate and its metabolites are implicated in cholesterol synthesis and other intracellular pathways.

Foam cell
A macrophage in the arterial wall that ingests oxidized low-density lipoprotein and assumes a foamy appearance. These cells secrete various substances contributing to plaque growth and inflammation.

alteration in the apoptotic programme[115]. Mice that lack MFGE8 accumulate apoptotic lymphocytes within lymph nodes and develop an SLE-like disease that involves autoantibody formation, splenomegaly and glomerulonephritis[116]. Genetic polymorphisms and aberrant splicing of MFGE8 have been reported in a small subset of patients with SLE, suggesting that this pathway of apoptotic cell clearance might be dysregulated in some patients[117,118]. Such impaired clearance of apoptotic cells eventually leads to secondary necrosis, which allows intracellular antigens, normally compartmentalized within an apoptotic cell, to gain access to the extracellular environment. This presumably increases the risk of such antigens being recognized as non-self, with production of autoantibodies and consequent autoimmunity. Complement proteins also have a key role in apoptotic cell clearance and the development of autoimmunity, and deficiencies in complement components, particularly C1q, are highly associated with SLE[119,120].

It is notable that not all defects in apoptotic cell clearance seem to result in autoimmunity: the absence of CD14 or mannose binding lectin (MBL) in mice leads to defective apoptotic cell engulfment and their accumulation in tissues but does not have any pro-inflammatory or autoimmune consequences[121,122]. Whether apoptotic cells in these deficient mice can still provide a downstream immunomodulatory signal to prevent autoimmunity, despite not being engulfed, requires further investigation. If apoptotic cell engagement alone by a phagocyte can be beneficial in ameliorating autoimmunity, harnessing those features could offer a therapeutic modality even in the context of continued defective apoptotic cell clearance.

Rheumatoid arthritis is a chronic systemic inflammatory disease associated with progressive joint destruction. It is a systemic autoimmune disease with most individuals having circulating autoantibodies against citrullinated peptides. There is little direct evidence that human inflammatory arthritis is caused by defects in cell clearance[123,124], but at sites of inflammation the extracellular debris (which includes oxidized lipids and intracellular components such as high-mobility group box 1 (HMGB1), histone H3 and histone H4) acts as a negative regulator of efferocytosis[125]. Histone H3 binds to macrophages, most likely to αvβ5 integrins, which decreases uptake *in vitro* and *in vivo*. This reduced engulfment by histones can be reversed by administration of activated protein C, which causes degradation of histones[125] (FIG. 2). Similarly, HMGB1 reduces phagocytosis by binding to and masking PtdSer on apoptotic neutrophils and by binding αvβ3 integrins on phagocytes[126] (FIG. 2). Furthermore, increasing the levels of the TAM receptor-agonists protein S and GAS6 (REF. 127) or using LXR agonists[128] and PPARγ activators[129] has therapeutic benefits in mouse models of inflammatory arthritis. It remains to be seen whether such agents have benefits in human disease, and the results of a recently concluded human trial of PPARγ agonists in rheumatoid arthritis have yet to be reported (ClinicalTrials.gov identifier: NCT00554853).

Transplantation. Prescription of immunosuppressive drugs to patients following transplantation is often necessary to delay or prevent allograft rejection. However, such immunosuppressive medications exhibit numerous side effects and may not be effective for long-term allograft survival. Apoptotic cells carry self-antigens and actively dampen immunity, and apoptotic cell-based therapy has been developed to limit allograft rejection by promoting immunological tolerance towards donor organs, tissues or cells[130] (BOX 3). Inoculation of apoptotic cells from the donor in transplant recipients, before or during transfusion or transplantation, seems to improve donor cell engraftment and solid allograft survival in various mouse models[131–134] (FIG. 2). Depending on the experimental system, it was shown that recipient DCs and/or macrophages are necessary to mediate the apoptotic cell-induced tolerogenic effects on engraftment[134,135]. Masking the PtdSer on apoptotic cells failed to provide the same benefit, suggesting that recognition and uptake of donor apoptotic cells is necessary to induce allograft tolerance[132]. Mechanistically, the uptake of donor apoptotic cells by splenic DCs was found to promote the generation and/or proliferation of $CD4^+FOXP3^+$ regulatory T cells and the deletion of alloreactive $CD4^+$ T cells, representing a potential mechanism to reduce the risk of transplant rejection[133]. The potential use of apoptotic cell-based therapy to induce immune tolerance towards certain antigens has implications beyond transplantation, particularly in the treatment of autoimmune diseases (BOX 3).

Cancer. High levels of cell death can occur within a tumour milieu, and the mechanisms through which dying tumour cells are cleared can profoundly influence tumour-specific immunity. Thus, manipulation of the immunological context of dying cell removal has great potential for the control of tumour progression and generation of an antitumour response[136]. One possible approach to promote antitumour immunity is by counteracting the immunosuppressive properties of apoptotic cells. Consistent with this idea, interfering with PtdSer-mediated recognition of dying cells by masking PtdSer with annexin V favours an antitumour response, possibly by delaying apoptotic cell clearance and causing bias with respect to the type of phagocyte (for example, DCs) that mediates cell clearance[137]. However, it is important to note that blocking apoptotic cell engulfment may promote sterile inflammation through the release and exposure of DAMPs by uncleared secondary necrotic cells (BOX 1). Chronic inflammation that results from this approach might conversely favour tumour growth[138,139], as well as autoimmunity[115], and needs to be considered carefully.

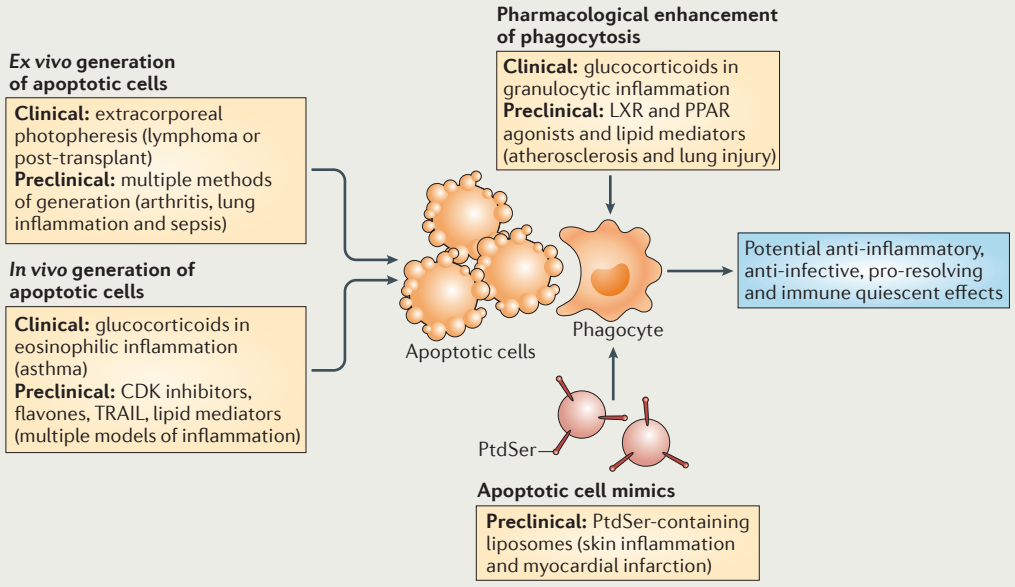
An alternative approach to promote antitumour immunity is to trigger an immunogenic form of tumour cell death through specific cell-death inducers. The ability of certain chemotherapeutic drugs such as doxorubicin (an anthracycline) to augment antitumour immunity through a caspase-, DC- and $CD8^+$ T cell-dependent mechanism[140] was found to depend on the molecular machinery for dying cell clearance[44,45]. Anthracyclines seem to promote exposure of the eat-me signal CRT on tumour cells, and blockade of

Box 3 | **Apoptotic cells as a potential therapeutic intervention**

Apoptotic cells or apoptotic cell mimics have immunomodulatory functions, and their administration could be used as a therapeutic intervention (see the figure). Extracorporeal photopheresis, in which leukocytes are made apoptotic *ex vivo* before systemic re-administration, is already an accepted treatment for cutaneous T cell lymphoma in humans and has shown benefits in transplant rejection, graft-versus-host disease and autoimmune disorders[170].

Although the molecular events underlying the potential immune-regulating function of apoptotic cells are less clear, transforming growth factor-β (TGFβ)-dependent proliferation of regulatory T cells, as well as changes in macrophage phenotype, have been implicated in apoptotic cell-mediated immune modulation[134,171]. The administration of cells that are made apoptotic *ex vivo* has been shown to reduce both the acute and chronic phases of inflammatory arthritis in rodents[172] by reducing the levels of tumour necrosis factor (TNF) (which negatively regulates apoptotic cell clearance)[173] and by enhancing the production of TGFβ and the generation of regulatory T cells[172]. Local administration of apoptotic cells has also been used to attenuate both bleomycin- and lipopolysaccharide (LPS)-induced lung inflammation; apoptotic cell delivery resulted in reduced neutrophil recruitment into the lung, enhanced phagocytosis by alveolar macrophages, reduced pro-inflammatory cytokine production and increased TGFβ production[174,175]. The infusion of apoptotic cells 24 hours after the initiation of sepsis has also been shown to protect against lethality in a mouse model of sepsis; apoptotic cell delivery led to reduced levels of pro-inflammatory cytokines and reduced neutrophil recruitment into organs[176]. At least part of the beneficial effect in the sepsis model is mediated by the direct binding of LPS by apoptotic cells, which led to the recognition and clearance of LPS-covered apoptotic cells by macrophages in an anti-inflammatory manner[176].

However, the therapeutic use of apoptotic cells needs to be carefully considered in cases in which the capacity for apoptotic cell engulfment is reduced *in vivo*, as administered cells may progress into secondary necrosis, which could exacerbate inflammation or autoimmunity. Notably, macrophages that ingest necrotic cells cause increased T cell proliferation[177]. Moreover, the repeated administration of apoptotic cells can lead to autoimmunity[178]. Whether apoptotic cell mimics, such as phosphatidylserine (PtdSer)-containing liposomes, can deliver the benefits of apoptotic cells without risking autoimmunity awaits further investigation, but this strategy has already been used to improve skin oedema and post-myocardial infarct repair in mice[179,180]. In addition, strategies that generate apoptotic cells *in situ* in models of inflammation have shown potential: therapeutic agents including cyclin-dependent kinase (CDK) inhibitors[181], flavones[182] or the death receptor ligand TNF-related apoptosis-inducing ligand (TRAIL)[183] have all demonstrated benefits in models of inflammation. Furthermore, the combined delivery of apoptotic cells with enhancers of phagocytosis may be required for full therapeutic efficacy to prevent secondary necrosis of apoptotic cells. LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor.



CRT exposure on anthracycline-treated tumour cells can markedly reduce DC-mediated cell clearance and antitumour immunity[44] (FIG. 2). Importantly, supplying exogenous CRT to dying tumour cells that cannot display endogenous CRT enhanced their phagocytosis by DCs and their immunogenicity[44]. In addition to CRT, other DAMPs that are released by dying tumour cells, such as HSP90 and HMGB1, can promote antitumour immunity through a DC-dependent process[141,142].

Cancer cells often hijack a variety of normal cellular processes to enable survival and expansion in an organism. Recently, the ability of tumour cells to upregulate the don't eat-me signal CD47 to evade recognition and engulfment by phagocytes was found in many mouse models of myeloid leukaemia, as well as in patients with myeloid proliferative diseases (including acute myeloid leukaemia and myeloid blast crisis phase chronic myeloid leukaemia)[143]. Importantly, higher expression of

CD47 in patients with acute myeloid leukaemia, non-Hodgkin lymphoma, ovarian cancer, glioma and glioblastoma correlated with poor prognosis[143–145], indicating a link between CD47 upregulation and tumorigenicity. Consistent with the function of CD47 as a don't eat-me signal through interaction with macrophage SIRPα, ectopic expression of CD47 in a $CD47^{low}$ acute myeloid leukaemia cell line was reported to increase tumour cell survival by limiting their engulfment by macrophages[143]. Importantly, CD47 blockade using a CD47-specific antibody in mice that had received $CD47^{hi}$ tumour cells from human patients reduced tumour engraftment, growth and metastasis, thereby indicating therapeutic potential for CD47-targeting in cancer[144–147] (FIG. 2). Recently, a combination therapy using tumour-specific monoclonal antibodies (for example, rituximab and trastuzumab) and soluble SIRPα variants that can antagonize CD47 function exhibited a synergistic effect in promoting the engulfment of tumour cells by macrophages and the regression of tumour growth in mouse models[147].

Taken together, the evidence suggests that the manipulation of the apoptotic cell clearance process by delaying apoptotic cell recognition and removal, inducing immunogenic cell death and targeting the cell clearance machinery that has been hijacked by tumour cells can effectively promote antitumour immunity. However, challenges remain in validating these novel therapeutic approaches clinically. Moreover, whether other aspects of the apoptotic cell clearance process, such as phagocyte recruitment and the formation of apoptotic bodies and microparticles, can be modulated to control tumour progression warrants further investigation.

Concluding remarks

It has been suggested that prompt and efficient clearance of apoptotic cells is the ultimate goal of the apoptotic programme, as well as a key process that can prevent inflammation and maintain self-tolerance under physiological conditions. Accumulating evidence suggests that clearance of apoptotic cells is impaired in multiple human disease processes, with mounting evidence indicating that the defect in apoptotic cell clearance is directly involved in driving disease pathogenesis in several different contexts. Furthermore, over the past several years, a number of notable discoveries have been made in terms of the molecules and mechanisms that regulate apoptotic cell clearance in a range of species, from model organisms to humans.

What is particularly revealing is that several steps within the process can be beneficial: the molecules exposed on the apoptotic cells themselves seem to provide important differentiation signals; the phagocytic recognition step induces several anti-inflammatory mediators that dampen the immune response; the actual corpse internalization process seems to help limit certain infections; and the engulfment process can be made pro-immunogenic under specific conditions, depending on the type of apoptosis induction and the type of phagocyte. This combined knowledge opens new avenues in therapeutic intervention for both dampening inflammation in specific autoimmune or inflammatory disease states and promoting effective immune responses against tumour-derived antigens. The next challenge for the field is in harnessing the benefits of the apoptotic cell clearance process for human therapies.

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Competing interests statement
The authors declare no competing interests.

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