Corpse clearance defines the meaning of cell death

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While philosophers seek the meaning of life, cell biologists are becoming ever more interested in the meaning of death. Apoptosis marks unwanted cells with 'eat me' signals that direct recognition, engulfment and degradation by phagocytes. Far from being the end of the story, these clearance events allow scavenger cells to confer meaning upon cell death. But if the phagocytic 'spin doctors' receive or transmit the wrong messages, trouble ensues.

Since the seminal description of cell death by apoptosis[1] it has been clear that the final phase of this programme of cell deletion *in vivo* is swift and safe phagocytosis of intact 'unwanted' cells[2,3]. For many years the significance of this event has been underestimated. New data indicate that phagocyte clearance of cells dying by apoptosis is much more than mere waste disposal. Instead, the engulfment of dying cells by phagocytes may define the meaning of cell death in higher organisms, particularly when clearance is achieved by 'professional' scavengers of the macrophage line rather than by neighbouring cells acting as 'semi-professional' phagocytes. Depending on the context, the removal of apoptotic cells by phagocytes might suppress inflammation, modulate the macrophage-directed deletion of host cells or invading parasites and critically regulate immune responses (Fig. 1). How can such meaning be conferred on the clearance of dying cells by phagocytes?

Clearance of unwanted self

'Eat me' signals

Two approaches have helped to define 'eat me' signals displayed by apoptotic cells and the phagocyte receptors that recognize such surface changes and mediate the engulfment of dying cells (Fig. 2). First, a basic system for signalling engulfment has been defined by the study of genetic defects in the clearance of cell corpses arising during development of the nematode *Caenorhabditis elegans*[4–7] (Fig. 3). Second, the characterization of inhibitors that specifically block the phagocyte recognition of apoptotic cells *in vitro* has implicated a repertoire of mammalian cell-surface and extracellular 'bridging' molecules (Fig. 4) for which roles *in vivo* are now being confirmed in deletion mutants.

Cells undergoing apoptosis can display a number of 'eat-me' flags. Some are relatively well characterized, such as the exposure of phosphatidylserine (PtdSer) normally restricted to the inner-membrane leaflet of the dying cell[8] (as discussed further below), or changes in surface sugars detected by phagocyte lectins[9]. Other 'eat-me' markers are more poorly defined, such as sites that bind adhesive 'bridging' molecules present in extracellular fluid. These include C1q, the first component of complement, for which a bridging role *in vivo* has been confirmed by elegant studies demonstrating the defective clearance of apoptotic cells in deletion-mutant mice[10,11]. Potential bridging roles for other components of the complement cascade such as iC3b (ref. 12), the abundant serum protein β_2 glycoprotein I (β_2GPI)[13] and thrombospondin[2], also need clarification. Moreover, we must characterize how phagocyte scavenger receptors such as CD68 (refs 14,15) recognize apoptotic cell-surface sites that can be masked by antibodies against oxidized low-density lipoproteins[16] and how the immunoglobulin superfamily member ICAM-3 (intercellular adhesion molecule-3) can function as an 'eat-me' flag[17].

The phagocyte's reaction

Although we have a much clearer picture of the mammalian phagocyte receptors capable of mediating the engulfment of dying cells (Fig. 4), we do not yet know what contribution is made to the clearance task by each molecule. Existing data suggest that macrophages might 'tether' dying cells by using phagocyte surface CD14 (ref. 18) or β_2 integrins[12] (which bind the potential bridging complement fragment iC3b), before engaging receptors that drive phagocytosis. Indeed, new data[19] demonstrate that the phagocyte integrin $\alpha_v\beta_5$ may direct the cytoskeletal changes necessary for the phagocyte surface to envelop apoptotic cells by recruiting the CrkII–DOCK 180–Rac1 signalling complex that was first implicated by mutations in the respective *C. elegans* homologues CED-2, CED-5 and CED-10 (Fig. 3), which not only inhibit the clearance of cell corpses but also affect the migration of gonadal distal tip cells in the nematode[4,7]. Similarly, the mammalian class B scavenger CD36 receptor might interact with the homologue of CED-6 (ref. 20), a signalling adaptor protein[5]. Although the identity of CED-1 is not yet clear, we speculate that this *C. elegans* molecule might be some form of scavenger receptor (Fig. 3).

We also need to clarify the contribution made by subtle and dynamic reorganization of the phagocyte membrane, which is dependent on lipid fluxes similar to those occurring in the dying 'prey'. In mammalian cells these lipid transport events, which include the exposure of PtdSer, are directed by the ABC1 transporter[21,22], a homologue of the *C. elegans* protein CED-7, which must function in both scavenger cell and target for efficient engulfment[6] (Fig. 3). Perhaps these phagocyte membrane changes, potentially driven either by apoptotic cell ligation of phagocyte receptors or by the release of soluble signals from dying cells, explain why

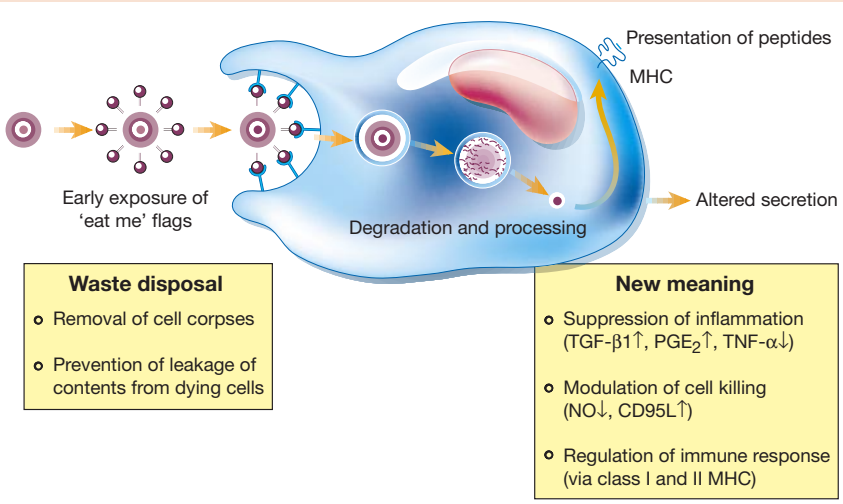

Figure 1 The meaning coded into the phagocytic clearance of cells dying by apoptosis. Until recently, engulfment of apoptotic cells was viewed as protective waste disposal (left). However, recent data reviewed in this article indicate a new meaning (right) determined by phagocyte responses that modulate inflammation, control tissue remodelling by phagocyte-directed cell killing and regulate immune responses. CD95L,CD95 ligand; MHC, major histocompatibility complex; NO, nitric oxide.

ubiquitously expressed cell-surface molecules such as lectins or integrins (like the $\alpha_v\beta_3$ vitronectin receptor) can acquire a specific role in the uptake of apoptotic cells by a wide range of phagocytes, both professional and semi-professional[2,3,9].

Nevertheless, other phagocytic receptor recognition mechanisms might have a prominent role only in particular tissues (for example, defective apoptotic cell clearance in C1q-null mice is particularly evident in the kidney[10]) or in particular cells. Thus, the expression of CD36 is restricted to macrophages and a few other cell types and yet an important role in the clearance of dying cells during development is demonstrated by defects in a *Drosophila melanogaster* mutant deficient in the CD36 homologue Croquemort[23]. Similarly, although healthy mice deficient in the class A scavenger receptor do not demonstrate an obvious defect in the clearance of apoptotic cells from the thymus and other organs[24], unpublished work (G. Thomas, N. Platt, S. Gordon and J.S.) points to a defect *in vivo* in the ingestion of apoptotic leukocytes by macrophages during peritoneal inflammation.

Our current understanding of the molecular mechanisms mediating the clearance of apoptotic cells therefore remains poor. The apparent redundancy of these mechanisms could be consistent with the probable importance for health of safely clearing apoptotic cells, but might also reflect how little we know about the circumstances *in vivo* in which various mechanisms are engaged. Indeed, the plethora of 'eat-me' signals, bridging molecules and phagocyte receptors provides a rich substrate from which the clearance event could acquire additional meaning. The potential significance programmed into these molecular mechanisms was suggested by a simple experiment *in vitro*[25]. Macrophages are crucial both for the clearance of apoptotic cells generated in injured tissue and for host defence against infection by bacteria or protozoa. Normally the ingestion of particles of similar size to these invaders triggers macrophages to secrete molecular mediators capable of initiating protective but potentially injurious inflammatory responses. But the ingestion of large numbers of apoptotic cells failed to elicit this macrophage release of pro-inflammatory mediators[25]. However, such a release was observed if experimental conditions were deliberately changed so that the recognition mechanisms for 'quiet clearance' were replaced by bridging immunoglobulin and macrophage Fc receptors[25]. Thus, mechanisms allowing phagocytes to recognize apoptotic cells as 'unwanted self' are special in that they are uncoupled from inflammatory responses. As we discuss in the next section, these molecular mechanisms might have a much wider significance in health and disease.

Suppression of inflammation

Although inflammatory responses are vital for host defence against infection, when persistent they also underlie important diseases such as asthma or rheumatoid arthritis. Dangerous immune cells can be quietly cleared from inflamed sites by undergoing apoptosis followed by engulfment by phagocytes, promoting the resolution of acute inflammation[2]. Moreover, the uptake of apoptotic cells actively suppresses the secretion from activated macrophages of pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α)[26,27]. Safe clearance might therefore be doubly beneficial in inflammatory responses, preventing the secondary necrosis of apoptotic cells, with associated uncontrolled release of injurious contents, and 'calming' pro-inflammatory macrophages.

How is anti-inflammatory meaning conferred on the phagocytic clearance of apoptotic cells? 'Resetting' of activated macrophages can be mimicked by the ligation of macrophage receptors mediating the engulfment of apoptotic cells, notably CD36 (ref. 26), its 'bridging' ligand thrombospondin[26] and a newly discovered receptor for PtdSer exposed by apoptotic cells[28]. Indeed, receptor-triggered release of the anti-inflammatory and immunosuppressive cytokine transforming growth factor-β1 (TGF-β1) by macrophages ingesting apoptotic cells might be crucial in mediating the autocrine or paracrine suppression of macrophage-directed inflammation[26–28]. Macrophages are nevertheless activated to secrete pro-inflammatory mediators by the ingestion of white blood cells undergoing secondary necrosis after apoptosis, but not by intact apoptotic cells[29]. Therefore, whether clearance has an anti-inflammatory meaning might be determined by the state of the dying cell, the phagocyte receptors engaged and the downstream signalling pathways activated, which remain poorly understood.

Moreover, the anti-inflammatory action of phagocyte clearance of apoptotic cells might be perturbed in disease processes. For example, anti-phospholipid autoantibodies that recognize and bind PtdSer exposed by apoptotic cells can be found in patients with

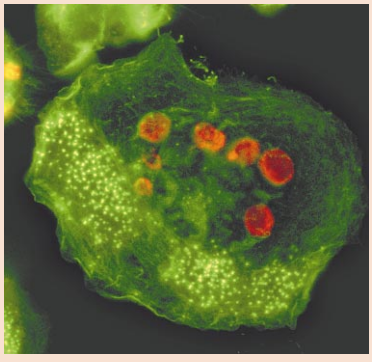

Figure 2 A human monocyte-derived macrophage ingests multiple apoptotic bodies. Jurkat T-cell targets were labelled with 5-(and 6)-carboxytetramethylrhodamine succinimidyl ester and irradiated to induce apoptosis. The macrophages were stained with fluorescein isothyocyanate-conjugated phalloidin to identify actin filaments. (Photograph courtesy of M. Janes and P. Henson).

persistent and relapsing inflammatory disorders such as systemic lupus erythematosus (SLE). Such autoantibodies can coat apoptotic cells so that they are bound by macrophage Fc receptors with the result that TNF-α release is promoted rather than suppressed[30], threatening conversion of the anti-inflammatory clearance of dying cells into a pro-inflammatory event.

Control of macrophage-directed cell death

Macrophages are not merely scavengers of dying cells; they can also direct the death of unwanted cells during tissue remodelling. For example, elegant experiments on macrophage depletion and repletion have demonstrated a key role in the deletion of surplus blood vessels in the developing rodent eye[31]. Macrophages recruited from the blood can dock onto and direct apoptosis in unwanted microvascular endothelial cells. Furthermore, activated macrophages can also delete resident cells during the remodelling of inflamed sites in adult mammals, inducing apoptosis in neighbouring cells by mechanisms including nitric oxide release[32].

The capacity for macrophages to direct cell death is regulated by the uptake of apoptotic cells. For example, the cytolysis of tumour cells by activated macrophages is inhibited by the ingestion of apoptotic but not necrotic cells[33]. This might explain the surprising cohabitation of macrophages and malignant cells in many tumours. Blockade of the ingestion of cancer cells undergoing apoptosis by macrophages in the tumour might therefore be a new therapeutic approach in malignancy, disengaging an undesirable brake on macrophage cytocidal capacity. Moreover, blocking the anti-cytocidal response of phagocytes taking up apoptotic cells could provide a new approach towards the treatment of diseases caused by protozoans. These organisms can exploit the suppressive effects of the ingestion of apoptotic cells to evade macrophage-directed killing of parasites. For example, the protozoan parasite *Trypanosoma cruzi* causes the cardiac illness Chagas's disease, in which T cells die by activation-induced apoptosis. By mechanisms including the ligation of macrophage $\alpha_v\beta_3$, the local release of TGF-β1 and prostaglandin E_2 (PGE_2), and the generation of polyamines necessary for parasite growth consequent on the induction of macrophage ornithine decarboxylase activity, the uptake of apoptotic T cells inhibits the generation of protective nitric oxide by macrophages and promotes the invasion of macrophages by *T. cruzi*[34]. But inhibiting PGE_2 release from phagocytic macrophages with cyclo-oxygenase inhibitors such as aspirin or indomethacin not only protects macrophages from the induction of ornithine decarboxylase activity and infection by parasites *in vitro*, but also markedly decreases parasitaemia in mice infected with *T. cruzi*[34].

We therefore already know that inhibiting the suppressive effects of apoptotic cell ingestion after the macrophage-directed death of 'unwanted' cancer or parasite cells could have therapeutic use. Similar principles could be important in manipulating the remodelling of tissues injured by inflammatory and vascular diseases. But caution is required because there might be dual meaning in the ingestion of apoptotic cells by macrophages at sites of tissue remodelling. In some circumstances, far from suppressing macrophage cytocidal capacity, the uptake of apoptotic cells can trigger the release of the death-inducing cytokine CD95 ligand (Apo-1/Fas ligand) by macrophages, resulting in the apoptosis of bystander cells[35].

Regulation of immune responses

Glimpses of further consequences of the clearance of dying cells by phagocytes came from the observations that bone-marrow-derived dendritic cells could ingest apoptotic cells[36] and could present antigen derived from ingested apoptotic cells to T cells, thus initiating immune responses mediated by cytotoxic lymphocytes[37,38]. Dendritic cells are specialized for the presentation of ingested antigen to lymphocytes but do so most efficiently if they subsequently receive a maturation or 'danger' signal such as an exposure to microorganisms[39]. The phagocytosis of dying cells by dendritic cells is an important candidate mechanism for the promotion of immune responses to organisms capable of inducing apoptosis in host cells. Dendritic cells can also present antigen derived from ingested apoptotic cells to helper T cells[39]. Exactly how peptides from ingested cells are presented on major histocompatibility complex (MHC) molecules by dendritic cells needs to be clarified, but dendritic cells use the $\alpha_v\beta_5$ integrin rather than $\alpha_v\beta_3$ in the engulfment of apoptotic cells, which could prove to be significant[19,40].

Nevertheless, the presentation of apoptotic-cell-derived antigens by dendritic cells must be subject to exquisite controls, as this threatens to incite immunity against self components[41], particularly because protein cleavage within dying cells might generate 'neo-autoantigens'[42]. Indeed, the engulfment of apoptotic cells by dendritic cells can be observed in health. For example, migratory dendritic cells transport apoptotic intestinal epithelial cells to T-cell areas of mesenteric lymph nodes in apparently healthy rats[43]. Circumstantial evidence[39,44] suggests that in the absence of maturation signals, the uptake by dendritic cells of self components packaged in apoptotic cells might reinforce tolerance to self tissue so that when tissue injury does occur, T cells likely to respond to self antigens have been anergized or deleted. This fascinating idea needs to be tested, but the immune system seems able to detect two classes of meaning in the phagocytosis of apoptotic cells by dendritic cells: a message to respond to non-self coupled with a reminder not to attack self[39].

Available data indicate that a 'pro-immune meaning' can be conferred on the phagocytosis of dying cells by dendritic cells when

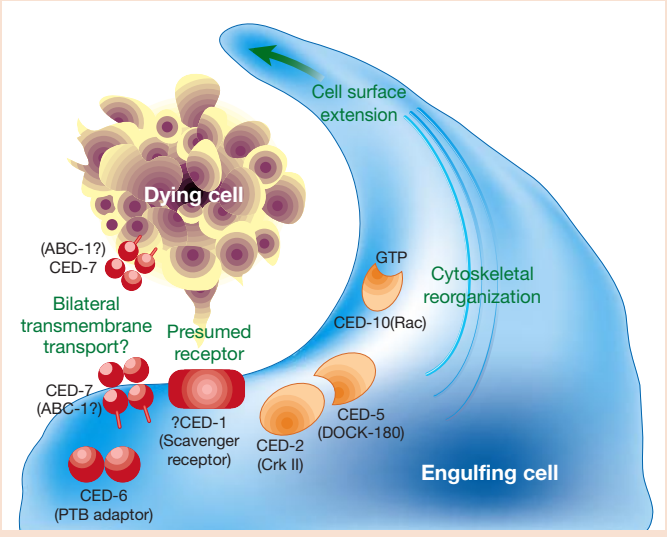

Figure 3 Signalling the engulfment of dying cells in *Caenorhabditis elegans*. Mutations in six genes are known to affect the engulfment of cell corpses by non-professional neighbouring cells in this nematode. CED-2, CED-5 and CED-10 intracellular proteins signal in a manner comparable to their respective mammalian homologues CrkII, DOCK 180 and Rac, mediating the cytoskeletal reorganization and extension of the engulfing cell surface around the dying cell. CED-7, homologous with mammalian phagocyte ABC-1, acts in both dying and engulfing cells[6], possibly in transmembrane lipid transport. We speculate that CED-1, yet to be characterized, is analogous to mammalian scavenger receptors; CED-7 and CED-1 probably promote engulfment by interacting with the signalling adaptor protein CED-6. PTB, phosphotyrosine binding.

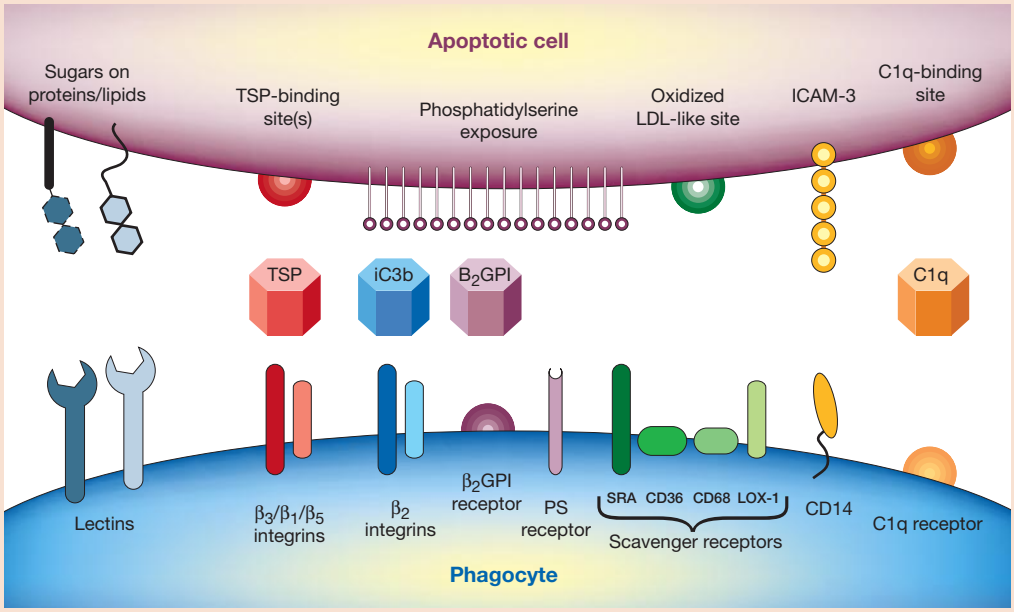

Figure 4 The phagocyte recognition array in the mammalian clearance of apoptotic cells. Inhibitor studies in assays of the ingestion of apoptotic cells *in vitro* by phagocytes have revealed a variety of candidate molecules, many of which have incompletely understood roles or uncharacterized binding partners, as discussed in the text. A repertoire of 'eat me' signals (top) interact with receptors on the phagocyte (bottom), either directly or via serum-derived bridging molecules (middle). LDL, low-density lipoprotein; SRA, class A scavenger receptor; TSP, thrombospondin.

there is defective clearance of apoptotic cells. For example, humans deficient in C1q almost invariably develop SLE. C1q-knockout mice are also highly susceptible to the development of a SLE-like disorder[10]. Inflamed tissues in these mice contain many free cells undergoing death by apoptosis, consistent with demonstrable defects in the clearance of apoptotic cells by macrophages[11]. Therefore, in C1q-deficient individuals, secondary necrosis of apoptotic cells escaping clearance could provide 'danger' signals that might switch the presentation by dendritic cells of self peptides derived from ingested apoptotic cells from 'tolerogenic' to 'pro-immune'[39,45]. This might explain why SLE is characterized by autoimmunity directed against antigens that normally reside within cells. Agents that promote the safe clearance of dying cells by phagocytes, such as glucocorticoids[46] and lipoxins[47], could be exploited in therapies.

Deciphering lipid-coded clearance

Decoding still further the meaning of the clearance of dying cells by phagocytes will provide new insights into the pathogenesis and treatment of inflammatory, autoimmune, malignant and infective conditions. The recent characterization of a complete system for PtdSer-directed phagocytosis of apoptotic cells indicates that surface lipids on dying cells might determine the meaning of clearance. Exposure of PtdSer, mediated by the poorly understood inhibition of an aminophospholipid translocase and the activation of a lipid scramblase (which is itself dependent on the entry of Ca^{2+} ions and the phosphorylation of scramblase), reveals an 'eat me' flag that could direct a range of phagocyte responses[9,48,49]. A newly identified stereospecific PtdSer receptor expressed by professional and amateur phagocytes seems to be coupled to anti-inflammatory clearance, stimulating macrophage TGF-β1 secretion and inhibiting lipopolysaccharide-induced TNF-α release[28]. However, although other receptors have broader lipid specificity, exposure to PtdSer might also promote the direct ligation of apoptotic cells by CD36 (ref. 50), CD68 (ref. 14), CD14 (ref. 18) and LOX-1 (ref. 15) on the phagocyte surface, with as yet unknown consequences. Further complexity is added by the possible bridging of PtdSer to other phagocyte receptors by soluble proteins such as β_2GPI[13] and opsonic complement fragments such as iC3b[12]. Therefore, even the single cipher of PtdSer exposure could encode a range of meanings into clearance of dying cells.

Conclusions and further challenges

The clearance of apoptotic cells by phagocytes initially attracted interest as the final act in programmed cell death and an intriguing problem in the discrimination of self from unwanted self. The genetics of *C. elegans* has provided a basic understanding of the signalling of engulfment, and studies of mutations affecting the clearance of dying cells by professional phagocytes in *Drosophila* will help to unravel the complexity inferred from inhibitor studies in mammalian systems. However, the recent revelation that the clearance of apoptotic cells by phagocytes defines meaning for cell death indicates that studies in mammals *in vivo* are urgently required to address regulatory roles in inflammation, immune responses and tissue remodelling. Dead men may tell no tales, but dead cells certainly do, the phagocyte having the last word. □

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