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# The disposal of dying cells in living tissues

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**Cells continuously die and disappear from the midst of living tissues. However, some of their constituents survive. DNA is horizontally transferred to phagocytic cells, and apoptotic cell antigens shape the immune repertoire. When massive apoptosis occurs, which overwhelms tissue scavenger cells, or when the function of phagocytes abates, dying cells escape clearance *in vivo*. Remnant dying cells come to phagocytes disguised: factors capable to envelop their membranes pervade the entire organism, or are generated in given tissues. Some are constitutively present, while other are generated during early or late phases of the inflammatory response, possibly to face the further burden of the dead inflammatory cells. This camouflage influences the disposal of the corpses: decoying molecules either bridge the corpse to the phagocyte or hide it. Furthermore, factors associated to the plasma membrane of the apoptotic cell shape the signals the phagocyte releases *in situ*. Finally, molecules contained or released by the dying cell alter the apprehension by the phagocyte of its prey, influencing its immunogenicity.**

*Keywords:* apoptosis; autoimmunity; C1q; pentraxins; phagocytosis.

## Introduction

The phagocytosis of apoptotic cells has been the topic of several excellent and comprehensive reviews.[1–11] Because of the interest of our laboratory in the fate of the internalised cell corpses and in immune-mediated systemic diseases, the present review will focus on recent studies on the *in vivo* clearance of cell corpses. These studies offer new perspectives to design rational strategies for intervention in autoimmune and neoplastic patients.

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## Receptors and ligands in the clearance of dying cells

*The Sultan said: "There's evidence abundant To prove this unbelieving dog redundant." To whom the Grand Vizier, with mien impressive, Replied: "His head, at least, appears excessive."*

*Habeeb Suleiman*

Over the last few years, receptors for apoptotic cells have been identified on the surface of the phagocyte. They include integrins, scavenger receptors, lectins, a recently identified phosphatidylserine (PS) receptor, and the CD14 lipopolysaccharide receptor. *In vitro*, even the simultaneous blockade of multiple interactions does not abrogate phagocytosis.[7] This suggests that phagocytes use more than one receptor-based recognition mechanism. The co-operation of independent, low affinity receptors may be required for rapid uptake and to reduce escape possibilities.[10,12,13]

Phylogenetically, redundancy is not novel. Seven independent genes, belonging to two distinct groups, are required for the efficient disposal of apoptotic cells in the nematode *Caenorhabditis elegans*.[10,14,15] Animals carrying one mutation from each group of genes show a clear-cut increase in the number of uncleared, persistent dead cell corpses. Most genes are not involved in the recognition of the corpses, but are required for the ensuing engulfment. CED-1, which has homologies with a potential scavenger receptor, is the only trans-membrane molecule so far directly implicated in the recognition and engulfment of dying cells.[16] However, CED-1 null mutants have a partially conserved ability to remove dead cells, suggesting the existence of back-up systems. *C. elegans* contains, among the mammalian receptors identified so far, the ortholog of the recently characterised PS receptor only.[17] The involvement of this receptor in the recognition of nematode cell corpses has not yet been proven.[18]

The hemocytes of *Drosophila melanogaster* remove cells undergoing apoptosis during embryogenesis.[19] They specifically express Croquemort, a CD36-related receptor, and use it to remove apoptotic cells.[20] However, also in *Drosophila*, Croquemort is not the only receptor involved

in the clearance of apoptotic cells: croquemort-deficient fly hemocytes still recognise and remove apoptotic cells, although with a substantially lower efficiency.

Redundancy in mammals is more extreme. The deletion of candidate receptors not only does not abrogate phagocytosis, but also is apparently ineffectual. Platt and co-workers showed that murine macrophages rely on the class A scavenger receptor (SRA) to recognise and uptake apoptotic thymocytes.[21] However, mice with a genetic deletion of the receptor cleared apoptotic cells *in vivo* with normal efficiency.[12] Thrombospondin 1 (TSP1) is necessary for clearance ensuing the recognition of apoptotic cells via the $\alpha v\beta 3$ vitronectin receptor integrin.[22,23] However, TSP1 deficient mice do not display any obvious defect in the clearance of apoptotic cells.[24] The same occurs for mice deficient of the $\beta 2$-Glycoprotein I plasma cofactor, ($\beta 2$-GPI), the ligand required for activation of the putative $\beta 2$-GPI receptor on the phagocyte membrane,[25] which are phenotypically normal.[26] Apparently, the whole array of phagocytic receptors expressed exclusively by macrophages is redundant: embryos null for the transcription factor PU.1, which do not have macrophages, still get rid of unwanted dead cell corpses, relying on "amateur" mesenchimal neighbour cells.[27]

In contrast, the genetic deletion of molecules unrelated to phagocytic receptors jeopardises the clearance of apoptotic cells *in vivo*, indicating a non-redundant role in the disposal of the corpses. Not only cell associated molecules, like the ABC-1 transporter or members of the Tyro/Axl/Mer tyrosine kinase receptors (TAM), are crucial for *in vivo* disposal of dying cells, but also soluble molecules, including the first fractions of the classical complement activation pathway, C1q and C4. Pentraxins, like the serum amyloid P component, are also involved.[28]

## Other phagocytic partners

Hamon and co-workers reported the accumulation of dead cells in the limbs of mice bearing a genetic deletion of the ATP-binding-cassette transporter 1 (ABC1)[29] (Table 1). The phenotype described in ABC1 $-/-$ mutants reminds the pattern observed in *C. elegans* single mutants, in which the delayed phagocytosis results in transient accumulation of dying cells without detectable physiological abnormalities.[30,31]

In healthy organisms the early exposure of "eat me" signals triggers immediate recognition, and phagocytes swiftly engulf the dying cells before apoptotic features are apparent.[1] In contrast, the apoptotic cells in ABC1 null mice have the chance to complete the apoptotic program *in vivo* and display features of advanced apoptosis, a phenomenon exceedingly rare *in vivo*. Peritoneal macrophages

**Table 1**. Non-redundant factors required for the *in vivo* clearance of dying cells

| Factor | Reference | In vivo evidence |
|---|---|---|
| Cell associated factors | | |
| ABC1 | [29] | 1. Apoptosis at the interdigital webs of limb buds. |
| | | 2. Decreased *i.p* phagocytosis of apoptotic thymocytes in thioglycollate elicited mice. |
| WAS protein | [42] | Decreased *i.p* phagocytosis of apoptotic Jurkat cells in thioglycollate elicited mice. |
| TAM receptors | [38] | Apoptosis in hippocampus, cerebellum, neocortex, prostate, liver, vessels, retina, spleen, gonads. |
| | [33] | 1. Apoptosis in the thymus of dexamethasone treated mice. |
| | | 2. Decreased *i.p* phagocytosis of apoptotic thymocytes in thioglycollate elicited mice. |
| | | 3. Decreased *i.v.* clearance of apoptotic lymphocytes |
| Soluble factors | | |
| C1q | [45] | Apoptosis in the kidneys of adult mice. |
| | [46] | 1. Decreased *i.p* phagocytosis of apoptotic Jurkat cells in thioglycollate elicited mice. |
| | | 2. Decreased *i.p* phagocytosis of apoptotic thymocytes in thioglycollate elicited mice. |
| | | 3. Decreased *i.p* phagocytosis of apoptotic thymocytes in untreated mice. |
| C4 | [46] | 1. Decreased *i.p* phagocytosis of apoptotic Jurkat cells in thioglycollate elicited mice. |
| | | 2. Decreased *i.p* phagocytosis of apoptotic thymocytes in thioglycollate elicited mice. |

from ABC1 −/− mice also have a reduced ability to engulf autologous apoptotic thymocytes *in vivo*. The results were confirmed by phagocytosis assays *in vitro*.[29] The *in vitro* phagocytic ability of ABC1 −/− macrophages is comparable to macrophages from SRA −/− mice, a phenotype that does not correlate to defective phagocytosis *in vivo*.[12] However, ABC1 plays a symmetric role in the lipid redistribution of both the phagocyte and the apoptotic cell, a feature also described for the CED-7 hortolog of ABC1 in *C. elegans*.[32]

The *in vivo* consequences of a defective clearance of the corpses were carefully analysed by Scott *et al*.[33] These authors generated mice functionally knock out of the Mer receptor tyrosine kinase (see also[34]). Mer belongs to a family of receptor tyrosine kinases, the TAM receptors, expressed in the neural, lymphoid, vascular and reproductive systems.

Mer is the only sub-family member expressed by monocyte-derived professional phagocytes.[34] Indeed the growth arrest specific gene 6 product (Gas6), a ligand for the TAM receptors partially homologue to protein S, favours the phagocytic clearance of PS exposing substrates.[35] Gas6 binds to PS exposing apoptotic cells, and the efficiency of the clearance of Gas6-coated apoptotic cells increased 20-fold.[36] Some evidence suggests that TAM receptors and in particular Mer, play a role in the negative regulation of inflammatory and immune responses.[37] The thymi of functional knock out mice of the Mer receptor contain only sparse apoptotic cells.[33] Upon injection of adult mice with dexamethasone, a seven fold increase in remnant apoptotic bodies was evident. The accumulation of apoptotic cells was not due to increased apoptosis, but to defective clearance by macrophages.[33] Defective clearance was confirmed by *i.p.* injection of apoptotic thymocytes and by *in vitro* assays. The defective clearance of apoptotic cells associated in clear-cut systemic autoimmunity, even in animals that did not receive any pharmacological treatment (discussed below).

Mice lacking more than one TAM receptor (Tyro3-Axl, Tyro3-Mer and in particular Tyro3-Axl-Mer) show striking features of apoptosis and cell degeneration in most adult tissues. Apoptotic cells accumulate in the spleen, hippocampus, cerebellum, neo-cortex of the brain, prostatic epithelium, liver parenchyma, granulosa cells associating with growing ovarian follicles, testes and blood vessels walls.[38] These mutants as well display clear-cut evidences of spontaneous autoimmunity[39] (also discussed below).

Macrophages from Mer-deficient animals selective fail to phagocytose apoptotic cells.[33] Downstream defects may involve more general regulators of the phagocytic event. The Wiskott-Aldrich Syndrome protein (WASp) is expressed in hematopoietic cells, and is involved in the transduction of signals from membrane receptors to the actin-based cytoskeleton.[40] Phagocytes from WAS patients have a reduced ability to internalise IgG opsonized particles.[41] Macrophages from WASp −/− mice bind to apoptotic cells efficiently, suggesting that WASp does not directly contribute to the recognition of dying cells. However, macrophages from WASp-deficient mice have a reduced ability to internalise apoptotic cells and IgG-opsonized sheep red blood cells.[42] Furthermore, WASp −/− thioglycollate-elicited inflammatory peritoneal macrophages fail to clear apoptotic xenogeneic leukaemia cells *in vivo*.[42]

Mice bearing a genetic deletion of the first component of the complement system, C1q, highlight the link between the clearance of dying and the immune homeostasis. This molecule indeed selectively binds to the membranes of keratinocytes undergoing apoptosis *in vitro*[43] and *in vivo*.[44] Apoptotic glomerular cells accumulate in the kidneys of C1q deficient animals.[45] A consistent number of animals (25%) also develop glomerulonephritis. Thioglycollate-elicited inflammatory peritoneal macrophages from C1q and C4 deficient mice fail to clear *in vivo* syngeneic apoptotic thymocytes.[46] The addition of the purified complement fraction corrects the defect. Resident peritoneal macrophages of C1q deficient, but not of C4 deficient, mice also display a delayed clearance of syngeneic apoptotic thymocytes.[46]

Therefore, the phagocytic clearance of apoptotic cells in the kidneys and in the peritoneum requires C1q. A complex interaction involving C1q, the mannose binding lectin, the C1q receptor calreticulin and the heat shock protein receptor CD91 on the phagocyte surface is involved in the initiation of the uptake of dying cells.[47] Not all tissues however depend on C1q. C1q binds to keratinocytes undergoing apoptosis after ultraviolet exposure of wild-type animals. However, C1q-deficient mice dispose of apoptotic keratinocytes efficiently, and do not develop autoantibodies (see Section 4).[44]

Early complement fractions are probably not the only soluble inflammatory factors that regulate the disposal of dying cells *in vivo*. Indirect evidence implies pentraxins. Pentraxins are acute phase proteins usually characterised by cyclic pentameric structure that are conserved during the phylogenesis.[48,49] Short pentraxins, like the C reactive protein (CRP) or the serum amyloid P component (SAP), are produced in the liver in response to inflammatory mediators. PTX3 is the prototypic long pentraxin, structurally related to, but distinct from, CRP and SAP. Primary pro-inflammatory factors induce the release of PTX3 in peripheral tissues by endothelial cells and monocytes.[50–54]

The function of pentraxins includes amplification of innate resistance against microbial infections and regulation of the scavenging of DNA released from dying cells.[55] However, binding of SAP enables microbes to evade phagocytosis by neutrophils, and treatment with drugs that inhibit SAP binding prolong the survival of

mice injected with Gram– bacteria.[56] The data indicate that SAP-coated organisms evade recognition *in vivo* and become more pathogenic, suggesting for SAP an antiopsonic role.

Both the classical short pentraxins (SAP and CRP) and the prototypic long pentraxin (PTX3) bind to cells undergoing apoptosis.[57–60] The ligand(s) for pentraxins on the apoptotic cell has not yet been identified. It localises to the membrane and well-characterised ligands like chromatin, small nuclear ribonucleoproteins or C1q have been excluded. In all *in vitro* systems (Jurkat leukaemia cells for CRP and SAP and Jurkat cells, activated human lymphocytes and PMN for PTX3), pentraxins preferentially recognise late apoptotic cells.[58–60] In contrast, TSP1, $\beta$2-GPI and Annexin V recognise cells displaying features of early apoptotic cells and with features of advanced apoptosis[23,61,62] (Table 2). To the best of our knowledge, among the factors that bind to apoptotic cells, only pentraxins and C1q share the selective recognition of late apoptotic cells (and *our unpublished results*).[63]

Scavenger phagocytes swiftly recognise and internalise early apoptotic cells. Bridging molecules like TSP1, and possibly $\beta$2-GPI, are required for this process (discussed in).[5] Diverse pathophysiological conditions, including acute inflammation, cause the synchronous death of parenchyma and infiltrating cells. Normal clearance mechanisms dispose of most cell corpses. However, uningested dying cells can persist. They release their content into the tissue environment, causing direct tissue damage.[1] For example, elastase and serine proteases released by apoptotic PMNs that escaped phagocytosis are responsible for the release of inflammatory factors by macrophages challenged with late apoptotic cells.[64]

Released moieties elicit the functional maturation of the most potent antigen presenting cells, the dendritic cells (DCs).[65] Accordingly, the cytoplasm of apoptotic cells is enriched of an adjuvant activity that favours the generation of cytotoxic T lymphocytes in response to particulate antigens.[66] Therefore, dying cells that escaped phagocytosis in mammals (1) cause inflammation and (2) favour the initiation of immune responses in the absence of pathogens. *C. elegans* lack professional scavenger cells and an organised immune system. Un-ingested cell corpses persist in double mutant *C. elegans* nematodes (see above) without apparent disturbance to the organism homeostasis.[1,10,13,18] Therefore, persistent cell corpses disturb preferentially the immune system. Persistent corpses evolve *in vivo* towards late apoptosis, and inflammatory factors, including C1q or pentraxins specifically bind to them. This event possibly offers a second chance for safe clearance *in vivo*.

**Table 2**. Factors binding to apoptotic cell membranes

| Factor | Reference | Early | Late |
|---|---|---|---|
| TSP | [62] | + | +++ |
| Pentraxins | | | |
| SAP | [60] | + | +++ |
| CRP | [59] | +/− | +++ |
| | (PRQ & AAM unpubl.) | + | +++ |
| PTX3 | [58] | +/− | +++ |
| $\beta$2-GPI | [61] | ++ | ++ |
| C1q | [63] | +/− | ++ |
| AnxV | [123, 124] | +++ | + |
| Autoantibodies | | | |
| aPL | [125, 126] | +++ | +++ |
| Anti-Ro/La | [127] | +++ | +++ |
| ANCA | [128] | + | +++ |

## Consequences of the clearance failure. Autoimmunity

Well-characterised *in vivo* models are now available, in which the clearance of apoptotic cells is deficient (Table 1). These models allow a relatively unbiased appreciation of the consequences *in vivo* of inadequate clearance of dying cells. Dying cells contains both antigens and adjuvants sufficient to inititate an autoimmune response.[65,67,68] Without an efficient disposal system, autoimmunity occurs.

Indeed, animals bearing deletion of C1q or of the TAM receptors develop striking features of systemic autoimmunity. C1q −/− animals spontaneously develop antinuclear antibodies and immune-mediated glomerulonephritis.[45] Both features are prominent in the prototypic systemic autoimmune disease, Systemic Lupus Erythematosus (SLE). The genetic deficiency of C1q almost invariably associates in humans with the development of SLE.[69] More than 50% of five-month-old Mer deficient mice spontaneously develop anti-DNA antibodies.[33] Triple TAM receptor deficient animals develop severe lymphoproliferation with anti-dsDNA anti-phospholipids (aPL) antibodies. APL also represent a frequent and clinically relevant feature of SLE patients.[70] APL antibodies associate in triple deficient mice, like in human patients, with recurrent thrombosis and pregnancy failures.[37,39] Autoimmune features were not investigated in ABC1 deficient mice (G. Chimini, personal communication), while WASp deficiency is a model for the human WAS, in which 40% of the patients, although immunodeficient, develop autoimmune features.[71]

Therefore, uncleared apoptosis associates to autoimmunity *in vivo*. This contention is supported also by unrelated experimental approaches. Synchronised cellular destruction by apoptosis *in vivo* facilitates antigen presentation of self antigen to T cells.[72,73] The administration of large

numbers of apoptotic thymocytes, sufficient to overwhelm the scavenging abilities of the animal, causes the transient development of anti-nuclear and aPL antibodies.[74] Therefore, autoimmunity probably frequently initiates in response to deregulated cell death. However, in most cases it aborts. The stimulus that triggered apoptosis[75,76] and genetically determined influences favour the continuance of the autoimmune response and the development of severe clinical involvement.

In animals prone to autoimmunity, like the NZW/NZW F1 mice, apoptotic cells cause a dramatically accelerated over SLE-like disease, with early death of the animals due to renal involvement (A. Bondanza, A. A. Manfredi, P. Rovere Querini, unpublished observations). Intriguingly, while intercurrent microbial infection can stimulate normal acute-phase SAP production, which is a major acute-phase pentraxin in mice, the levels of SAP do not rise in these animals.[77]

Only few SLE patients have characterised genetic defects that directly impinge on the clearance of apoptotic cells. However, several data suggest a link with initiation and maintenance of autoimmunity in SLE. A defect in the ability of macrophages from SLE patients to phagocytose apoptotic cells has been described.[78] The defective clearance associates with the accumulation of apoptotic cells in the blood and in the tissues of SLE patients. Furthermore, products selectively released by apoptotic cells that escaped phagocytosis, like nucleosomes, are detectable in a fair percentage of SLE patients. We refer readers to recent reviews on the topic.[79–90]

Different mechanisms contribute to limit the noxious effect of un-internalised apoptotic cells. Tissue transglutaminase, which exerts a finely tuned role in the regulation of the apoptotic machinery, is responsible for the protein crosslinks that prevent the leakage of intracellular constituents from late apoptotic cells.[91,92]

Phagocytes involved in the clearing of dying cells release immunosuppressive cytokines, including IL-10 and TGF-$\beta$1[4,11,93]. Therefore, in normal conditions, apoptotic cells that escaped phagocytosis are "sealed", and the environment is enriched in immunosuppressive cytokines. Inflammatory factors, including the granulocyte macrophage colony stimulating factor, promote the phagocytic ability of leukocytes, enabling them to clear dying cells more efficiently.[94,95] Furthermore, during acute inflammation pentraxins are generated locally, like PTX3, or systemically, like SAP and CRP.

PTX3 contributes to the molecular camouflage of uningested apoptotic cells, hindering their phagocytosis by antigen presenting DCs.[58] CRP bound to apoptotic cells facilitates the binding of C1q and their phagocytic clearance by TGF-$\beta$ releasing macrophages.[59] Finally, factors produced specifically in different tissues contribute to the disposal of apoptotic cells. This is the case of the surfactant protein A, a member of the collectin protein family (like the CD91 molecule) identified in the lungs, which enhances the phagocytosis of apoptotic cells by macrophages.[96]

## Consequences of the clearance failure. Tumor immunity

Autoimmune responses are probably common. However, only in subjects with an appropriate genetic background autoimmunity lasts. The control on immune responses elicited by dying tumor cells is apparently less stringent. Tumor cells frequently dye by apoptosis as a result of the unbalance between pro- anti anti-apoptotic factors or as the consequence of anti-neoplastic treatments.[97–99] Scavenger phagocytes interspersed in the tumor masses or, more often, surrounding cells phagocytose the corpses.

DNA is horizontally transferred from apoptotic cells to recipient cells after phagocytosis.[100–102] When phagocytes lack the p53-dependent censorship, the DNA from the apoptotic cell, including whole chromosomes, is propagated. The pathway is particularly efficient for the horizontal transfer of genes that confer to recipient cells a selective advantage. Therefore the phagocytosis of apoptotic tumor cells contributes to genetic instability and diversity within tumors.[100]

When cells with an intact p53 system phagocytose apoptotic tumor cells the transfer of oncogenes is not detectable. In contrast, intracellular antigens of dying tumor cells survive the phagocytic process and are "cross-presented" to tumor-specific T cells[2,103–117]; reviewed in.[65,73,118] High numbers of lymphoma cells dying by apoptosis *in vivo* recruit in normal mice a long-lasting anti-neoplastic immune response, endowed with memory and specificity.[2] Agents that interfere with the immunosuppressive clearance of apoptotic lymphoma cells substantially enhance the immunogenicity of the tumor (submitted for publication).

The access to antigen presenting cells influences the immunogenicity of uningested apoptotic cells *in vivo*, since they compete with more efficient or more represented phagocytes. This step is bypassed challenging DCs *in vitro* with dying tumor cells. Accordingly, DCs loaded with apoptotic tumor cells initiate in diverse systems immune responses against the antigens expressed by dying cells. DC that phagocytosed dying tumor cells are therefore an attractive target to achieve anti-neoplastic immunisation.[119,120]

Other less characterised factors also contribute to increase (or to quench) tumor immunogenicity. Feng *et al*. reported that the expression of heat shock proteins (HSP) enhances the immunogenicity of apoptotic leukaemia cells.[116] This is in keeping with the observation that DCs that phagocytosed HSP-expressing apoptotic leukaemia cells trigger a more efficient anti-neoplastic immune response.[116]

Both apoptosis induction and the intracellular expression of HSP (but not their release into the microenvironment) are necessary for anti-neoplastic immunisation. HSP are often expressed *in vivo* when extensive cell death takes place[121] and play a role in chaperoning and sustaining the immunogenicity of relevant antigens.[122] Further studies are warranted to verify whether this factor contributes to the immunogenicity of dying cells that escaped phagocytosis *in vivo*.

## Conclusions

The phagocytosis of dying cells prevents in higher organisms the initiation of immune responses against intracellular antigens. This finding has profound implications with respect to immune homeostasis and may allow to design therapies: (1) to interfere with the maintenance of autoimmune response, or with the initiation of graft rejection (2) to exploit the immune response against growing cancers. A more sophisticated understanding of the molecular events involved is thus necessary.

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