

Review

From regulation of dying cell engulfment to development of anti-cancer therapy

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Cell death and efficient engulfment of dying cells ensure tissue homeostasis and is involved in pathogenesis. Clearance of dying cells is a complex and dynamic process coordinated by interplay between ligands on dying cell, bridging molecules, and receptors on engulfing cells. In this review, we will discuss recent advances and significance of molecular changes on the surface of dying cells implicated in their recognition and clearance as well as factors released by dying cells that attract macrophages to the site of cell death. It is now becoming apparent that phagocytes use a specific set of mechanisms to discriminate between live and dead cells, and this phenomenon will be illustrated here. Next, we will discuss potential mechanisms by which removal of dying cells could modulate immune responses of phagocytes, in particular of macrophages. Finally, we will address possible strategies for manipulating the immunogenicity of dying cells in experimental cancer therapies. *Cell Death and Differentiation* (2008) 15, 29–38; doi:10.1038/sj.cdd.4402271; published online 16 November 2007

'While I thought that I was learning how to live, I have been learning how to die.'

Leonardo da Vinci

Activation of the innate immune system is the first line of defense against infection and the effects of localized injury or trauma. This activation is aimed at eliminating the infectious agent and restoring homeostasis. However, in the human body close to 500 billion cells die each day, and they are either shed off directly from body surfaces or continuously removed by a remarkably efficient phagocytic system without causing inflammation or scars. In the late 19th century, the immunologist Ilya Metchnikoff first observed the process of phagocytosis and made a link between cellular immunity and cells 'eating' other cells. Since then it has been established that phagocytosis has a key role in immunological processes. Rapid recognition and clearance of dying cells by phagocytes plays pivotal roles in development, maintenance of tissue homeostasis, control of immune responses, and resolution of inflammation. Dying cells can be cleared by professional phagocytes, such as macrophages and immature dendritic cells (DCs), or by nonprofessional phagocytes, such as endothelial cells, fibroblasts, smooth cells, and epithelial cells. Important insight into the genetic pathways implicated in clearance of dying cells came from studies on *Caenorhabditis elegans*. The engulfment genes fall into two partially redundant pathways (reviewed in detail in Mangahas

and Zhou¹) that converge at a common effector, ced-10 (mammalian homolog Rac-1). One pathway is composed of the proteins ced-2, ced-5, and ced-12 (mammalian homologues are CrkII, Dock180, and ELMO, respectively), and its upstream components are GTPase, RhoG/MIG-2, and TRIO/UNC-73. The second pathway includes membrane proteins ced-1, ced-7, ced-6, an adaptor protein, and dynamin 1.^{1,2}

Clearance of dying cells is a complex process in which many surface molecules, adaptors, and chemotactic molecules are involved, and it is controlled at multiple levels. This review covers recent advances in understanding the molecular interactions involved in recognition and clearance of stressed/dying/dead cells, and the effect of their interaction on the immune system. We also discuss repelling signals that protect living cells from being engulfed. We will briefly consider how exposure of certain molecules on the surface of dying cells, such as phosphatidylserine (PS) and calreticulin, can be used for raising immunogenicity of tumor cells,^{3,4} and how these findings could be applied for designing novel experimental anti-cancer treatments.

Positive Regulators of uptake Dying Cell

In order to guarantee instant recognition and uptake by phagocytes, apoptotic cells undergo early membrane modifications. The best characterized change in the surface of apoptotic cells that facilitates their recognition is the loss of

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Abbreviations: CR, complement receptor; DC, dendritic cell; eIF2 α , eukaryotic initiation factors 2- α ; ER, endoplasmic reticulum; Gas6, growth arrest-specific 6; HMGB-1, high mobility group box 1; IFN- γ , interferon- γ ; IL, interleukin; JNK, c-Jun N-terminal kinases; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mer, myeloid epithelial reproductive tyrosine kinase; MFG-E8, milk fat globule protein E8; NF- κ B, nuclear factor- κ B; PAF, platelet activating factor; PP1, protein phosphatase; PS, phosphatidylserine; ROS, reactive oxygen species; SIRP α , signal regulator protein- α ; SOCS, suppressors of cytokine signaling; STAT1, signal transducer and activator of transcription 1; TGF- β , transforming growth factor- β ; TLR, Toll-like receptor; TNF, tumor necrosis factor

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phospholipid asymmetry and translocation of PS from the inner to the outer leaflet of the lipid bilayer, which occurs very early during the apoptotic process.⁵ Although the precise mechanisms involved in initiating the movement of PS to the external surface of the membrane are still unclear, some possibilities have been suggested, including a coordinate increase in phospholipid flip-flop (flip refers to inward and flop to outward movement) due to inactivation of the aminophospholipid translocase.⁶ Recently, a novel molecular mechanism of PS exposure has been discovered in *C. elegans*, by using an RNAi reverse genetic approach and a transgenic strain expressing a green fluorescent protein::Annexin-V reporter. Zullig *et al.*⁷ identified a *tat-1* gene as a possible aminophospholipid transporter gene, because knocking down this gene abrogated PS exposure on apoptotic cells and reduced clearance of dead cell corpses. Recognition of PS is mediated by a broad range of extracellular bridge molecules⁸ (summarized in Figure 1), such as β 2-glycoprotein, milk fat globule protein E8 (MFG-E8), protein S, growth arrest-specific 6 (Gas6), and thrombospondin. Oxidation of proteins and lipids at the apoptotic cell surface is also important for engulfment. Oxidized PS has higher specificity than non-oxidized PS for bridging molecules such as MFG-E8.⁹ Moreover, clearance of apoptotic cells *in vivo* also occurs through interaction of CD36, a heavily glycosylated multi-ligand receptor belonging to the class B scavenger receptor group, with membrane-associated oxidized PS.¹⁰ A new and possibly clinically relevant

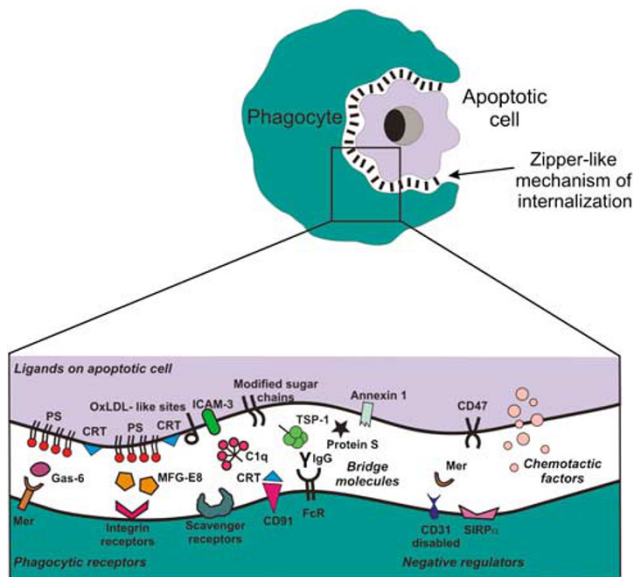


Figure 1 Overview of phagocyte receptors, bridging molecules, ligands on apoptotic cells, and chemotactic factors involved in regulating the recognition and clearance of apoptotic cells (partial list). Clearance of apoptotic cells is stimulated by various molecules displayed on the surface of phagocytes and on dying cells. Apoptotic cells are engulfed by a zipper-like mechanism. C1q, complement protein C1q; CD47, integrin-associated protein; CD91, α_2 macroglobulin receptor; chemotactic factors (LPC, tRNA synthase, annexin-I); FcR, Fc fragment of immunoglobulin G receptor; Gas6, growth arrest-specific gene 6; ICAM-3 (CD50), intercellular adhesion molecule-3; integrin receptors (vitronectin receptor $\alpha v\beta 3/5$, CR3 $\alpha m\beta 2$, CR4 $\alpha x\beta 2$); Mer, myeloid epithelial reproductive tyrosine kinase; MFG-E8, milk fat globule protein E8; PS, phosphatidylserine; scavenger receptors (SR-A, LOX-1, CD68, CD36, CD14); SIRP α , signal regulatory protein- α ; TSP-1, thrombospondin-1

homeostatic function for PS exposed on apoptotic cells has recently been proposed. Exposure of PS on UV-induced apoptotic Jurkat T lymphocytes was necessary and sufficient to stimulate the efflux of cholesterol from J774 murine macrophages and human blood monocyte-derived macrophages.¹¹ Failure to stimulate cholesterol efflux by the macrophages during the uptake of necrotic cells (but not during apoptotic cell uptake) could lead to progression of atherosclerosis during late-stage atherosclerotic lesions, which contain apoptotic cells and a core of necrotic cells.¹¹ A new alternative treatment for atherosclerosis in the future may be the addition of either artificial targets that mimic apoptotic cells, such as PS-containing liposomes, or the triggering of specific engulfment receptors on phagocytes, promoting cholesterol efflux from phagocytes.¹¹ In addition, it has been reported that statins, potent cholesterol-lowering agents, could increase clearance of apoptotic cells *in vitro* and *in vivo*, supporting the notion that efficient clearance of apoptotic cells in the atherosclerotic lesion is an important regulatory mechanism.¹²

PS on the surface of apoptotic cells also colocalizes with other molecules, including calreticulin¹³ (Figure 2) and annexin-I.¹⁴ However, it is not yet clear whether this colocalization is just a topological issue or involves a functional interaction providing cooperative stimuli for engulfment. Calreticulin was identified in 1974 as a soluble protein from the lumen of the endoplasmic reticulum (ER). The multifunctional calreticulin binds (buffers) Ca^{2+} in the lumen of the ER with high affinity, participates in the folding of newly synthesized glycoproteins, preventing their aggregation,¹⁵ and also performs other cellular functions outside the ER, such as cellular adhesion, and gene expression.¹⁵ Calreticulin is also present on the surface of most cell types, and its level on the surface increases during apoptosis. The precise mechanism initiating externalization of calreticulin to the surface of the plasma membrane is still unclear. It is possible that the chaperone calreticulin is transported together with membrane proteins, as in the case of the major histocompatibility complex class I,¹⁶ or that its KDEL ER targeting sequence is proteolytically removed. In this respect, it has been shown that calreticulin is a substrate for ced-3, and that it could be cleaved also by human caspases during apoptosis.¹⁷ Another aspect is the blebbing of apoptotic cells, implying an increase in the membrane surface area provided from the cell's internal membrane stores, such as the ER. In support of this notion, Franz *et al.*¹⁸ showed that apoptotic cells during cellular shrinkage expose calnexin, an ER integral membrane protein. Interestingly, the ER of phagocytes also supplies additional membrane required for phagocytosis,¹⁹ indicating a possible homotypic interaction between surfaces of dying cells and phagocytes. Several studies underline the importance of calreticulin exposure during phagocytosis in several models. For example, in *Dictyostelium* cells, calreticulin and calnexin are important for yeast particle phagocytosis.²⁰ Moreover, calreticulin has been identified as a marker for engulfment of apoptotic cells by *Drosophila* phagocytes.²¹ Since calreticulin is detected on the surface of apoptotic cells as well as on phagocytes,²² several modes of action for calreticulin have been proposed to explain its role in engulfment.²³ When exposed on the surface of target cells

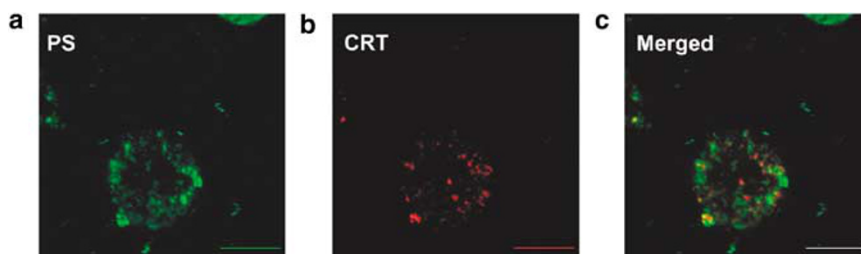


Figure 2 Fluorescence microscopy showing patchy distribution of phosphatidylserine labeled with annexin-V (a) and calreticulin (b). (c) is a merged image of (a) and (b) (1st antibody: rabbit anti-calreticulin polyclonal antibody, SPA-600, Stressgen Bioreagents; 2nd antibody: Alexa Fluor-488 goat anti-rabbit IgG, Invitrogen) during apoptosis of L929sAhFas cells after anti-Fas treatment. Scale bars = 10 μ m

during apoptosis, it can directly activate CD91²⁴ on the surface of phagocytes. In contrast, calreticulin on the surface of phagocytes could promote engulfment of apoptotic cells through involvement of bridge molecules, such as collectins and adiponectin. Calreticulin bound to CD91 on the phagocyte surface could induce engulfment of dying cells by interacting with collagenous tail regions of the collectins, which bind to the surface of apoptotic cells via their globular heads.^{23,25} The collagenous tails of collectins stimulate pro-inflammatory cytokine production by binding to calreticulin/CD91.²⁵ Adiponectin, an abundant circulating adipocyte-derived cytokine that is decreased in obese individuals, can opsonize apoptotic cells and promote engulfment through interaction with calreticulin and CD91 on the phagocyte surface.²⁶ Interestingly, calreticulin even mediates uptake of viable cells if the interactions between CD47 on the viable target cell and signal regulator protein- α (SIRP α), on the phagocyte are disrupted.¹³ The former is an integrin-associated protein, and the latter is a heavily glycosylated transmembrane protein with an immunoreceptor tyrosine-based inhibition motif domain.

Annexin-I, a member of a family of 13 proteins, binds negatively charged phospholipids in a Ca²⁺-dependent manner, and is recruited from the cytosol and exported to the outer plasma membrane leaflet of apoptotic cells, where it colocalizes with PS and becomes involved in the efficient clearance of apoptotic cells.¹⁴ Moreover, Fan *et al.*²⁷ reported that phagocytosis of apoptotic lymphocytes by macrophages was inhibited by pretreatment of either target cells or phagocytes with antibodies to annexin-I and annexin-II, indicating that annexins bridge phagocytes and apoptotic targets during recognition and phagocytosis.

Importantly, apoptosis is accompanied not only by exposure of the above-mentioned molecules, but also by changes in cell surface glycoconjugates.²⁸ During the course of apoptotic cell death, the levels of α -D-mannose and β -D-galactotose increase, whereas the levels of glycoproteins containing α 2,3-sialic acid decrease.²⁹ Moreover, another study showed that N-acetylglucosamine-, mannose-, and fucose-containing epitopes are exposed on cells undergoing apoptosis.³⁰ During the late phase of apoptotic cell death, further membrane alterations contribute to additional bridging molecules, depending on the stage and type of cell death. For example, C1q, mannose-binding lectin, pentraxin-3, C3, C4, C-reactive protein (an acute phase protein), and thrombospondin bind predominantly to late apoptotic or secondary necrotic cells.⁸

Great progress has been achieved during the last decade in unraveling molecules on the surface of apoptotic cells, but it is still not clear how non-apoptotic cells, namely necrotic and autophagic cells, are recognized by macrophages. We showed that apoptotic cells are engulfed by a zipper-like mechanism of phagocytosis (Figure 1), whereas necrotic cells are internalized by a macropinocytotic mechanism, such that parts of the cell are co-ingested together with extracellular fluid.^{8,31,32} In contrast with the initial paradigm, PS exposure mediates recognition and engulfment not only of apoptotic cells, but also of necrotic cells^{33,34} and of cells dying from autophagic cell death,³⁵ indicating that PS-mediated clearance may be a general mechanism irrespective of the way a cell dies. Accordingly, several macrophage receptor systems known to be involved in the engulfment of apoptotic cells also contribute to the uptake of necrotic cells. The thrombospondin-CD36- α v β 3 complex and CD14 are involved in the engulfment of heat-induced necrotic peripheral blood lymphocytes by human monocyte-derived macrophages.³⁶ In that study, simultaneous inhibition of the thrombospondin-CD36- α v β 3 system and PS reduced but did not block necrotic cell clearance, suggesting that other molecules may be involved. Obviously, knowledge of the precise molecular mechanisms of recognition and phagocytic uptake of non-apoptotic cells and its functional consequences is limited, and many more interesting and challenging findings are expected.

Negative Regulation of Dying Cell Uptake

It is remarkable that some living cells also expose PS, such as activated B cells and T lymphocytes, but they are apparently protected from engulfment.⁸ One of the mechanisms for this protection is related to repelling signals mediated through CD31 (platelet endothelial cell adhesion molecule). CD31 on viable cells homotypically interacts with its identical counterpart on phagocytes, promoting detachment, and preventing engulfment of the viable cells by an active and temperature-dependent mechanism.³⁷ Disabling this mechanism on viable apoptotic cells allows them to be ingested. Another mechanism of negative regulation of dying cell clearance is disruption of the interaction between CD47 on the target cell and SIRP α (SHPS-1), a heavily glycosylated transmembrane protein on the engulfing cell.¹³ Interestingly, apoptotic neutrophils negatively regulate their own uptake through an integrin-dependent process, which can be mimicked by ligation of α v β 3, α 6 β 1, and α 1 β 2.³⁸ Therefore, it is conceivable that living

cells continuously prove their viability by expressing 'repelling' signals to protect themselves from being removed by phagocytes (summarized in Figure 1).

The absence of this 'repelling' mechanism may explain the phenomenon of the so-called cellular cannibalism when cancer cells 'feed' by engulfing living cells in conditions of low nutrient supply. It has been reported that human metastatic melanoma cells but not primary melanoma cells can engulf living autologous melanoma-specific CD8⁺ T cells.³⁹ The underlying mechanisms of recognition and engulfment implicated in the uptake of live cells are currently unknown, and it will be important to understand in molecular terms how some cancer cells can overcome repelling signals displayed by their live targets.

Obviously, engulfment of dying cells must be restricted to sites at which it is required. It must also be controlled and finished quickly, because an intense phagocytic response is associated with the production of reactive oxygen species (ROS) and tissue injury.⁴⁰ For that reason, uptake of dying cells is modulated by cell-cell contacts as well as by soluble factors released by phagocytes and target cells. The clearance of apoptotic cells is reduced by pretreating mature macrophages (but not immature macrophages) with tumor necrosis factor (TNF), an effect that is possibly related to the generation of ROS, which act through activation of the GTPase Rho.⁴¹ Clearance of apoptotic cells apparently is inhibited by activated RhoA.⁴² This implies that generation of ROS in phagocytes has a negative effect on clearance, in contrast to the positive role of oxidized lipids on recognition and uptake.⁴³ Although a molecular link between an altered redox state and activation of GTPase Rho is unknown, a recent study reported that redox agents, including the superoxide anion and nitrogen dioxide, can react with a conserved GXXXGK(S/T)C motif in a number of Rho GTPases.⁴¹

A soluble form of the myeloid epithelial reproductive (Mer) receptor tyrosine kinase, produced by cleavage of the membrane-bound Mer protein by a metalloproteinase, is released from macrophages in a constitutive manner.⁴⁴ As a decoy receptor for Gas6, soluble Mer prevented Gas6-mediated stimulation of membrane-bound Mer, resulting in defective apoptotic cell uptake by macrophages.⁴⁴ These studies emphasize that dying cell clearance is regulated at multiple levels. Cells maintain a living state by constant regulatory survival signals to avoid default apoptosis induction, and they may also require continuously active mechanisms protecting them from phagocytosis. Therefore, a molecular understanding of the mechanisms of modulation of dying cell clearance may allow the development of therapeutic approaches based on compulsory and controlled phagocytosis of viable cancer cells, without the need to induce cell death in order to stimulate phagocytosis.

Factors Released by Dying Cells

There is growing evidence that phagocytic clearance is regulated by endogenously produced mediators that are released by dying cells (Figure 1 and Table 1). These cytosolic factors induce migration of phagocytes to sites of cell death

and facilitate phagocytosis. In this respect, it has been demonstrated that caspase-3-mediated activation of phospholipase A₂ leads to processing of phosphatidylcholine into lysophosphatidylcholine (LPC) and generation of arachidonic acid.⁴⁶ Subsequently, LPC is released by apoptotic cells, and it acts as a chemoattractant for monocytes and macrophages to sites of apoptosis.⁴⁶ Another elegant report showed that inner ectodermal cells in embryonic bodies lacking the essential autophagic gene *Beclin1* or *Atg5* underwent apoptosis normally but were not engulfed by neighboring cells, because they fail to express PS and secrete lower levels of LPC.⁵⁶ That study demonstrated that autophagy, beyond its survival and cell death function, is also essential in the exposure of 'eat-me' signals on apoptotic cells, suggesting that autophagosome formation during apoptosis may be one mechanism to expose phagocytosis signals on the surface of dying cells.

The release of membrane microvesicles containing modified and oxidized lipids from the cell undergoing apoptotic death also serves as a source of chemoattractants.⁴⁵ In addition to these membrane-mediated mechanisms, several cellular proteins released from apoptotic cells, including p43 aminoacyl tRNA synthetases⁵⁷ and a ribosomal protein dimer, mediate monocyte attraction.⁵⁰ The exposure of calreticulin on the surface of apoptotic cells, its ability to stimulate phagocytes,¹³ and its possible release⁵⁸ indicate that its potential chemoattractant activity should be examined. Efficient and rapid clearance of apoptotic cells requires signals that not only attract phagocytes but also promote engulfment. Annexin-I acts as a pro-phagocytic factor that is released by apoptotic cells, promoting phagocytosis of apoptotic polymorphonuclear neutrophils by macrophages.⁴⁹ Importantly, factors released from dying cells could modulate the immune responses. Recently, thrombospondin-1, a calcium-binding protein released from the engulfing cell, was also identified as a protein that is synthesized *de novo* when apoptosis is induced in monocytes, and that tolerizes immature DCs.⁴⁸ Necrotic cells release massive amount of high mobility group box 1 (HMGB-1) protein, which incites inflammatory responses of macrophages through pathways mediated by Toll-like receptor 2 (TLR2) and TLR4.^{8,52} Whether HMGB-1 protein is specifically released by necrotic cells but not by apoptotic or secondary necrotic cells remains controversial. Apparently, in apoptosis and secondary necrosis HMGB-1 remains bound on the DNA.⁵² Besides HMGB-1 release, necrotic cells possess other ways to stimulate inflammatory responses. We have demonstrated that during necrotic cell death the translation machinery continues until the very end of the cell death process, whereas apoptosis is associated with rapid inhibition of translation.⁵⁹ Moreover, cells dying by necrosis actively secrete inflammatory cytokines such as interleukin (IL)-6, and are characterized by nuclear factor- κ B (NF- κ B) and p38 mitogen-activated protein kinase (MAPK) activation, whereas these events are not present in the same cell types when dying by apoptosis.⁵⁵ More work is required to identify the effects of different triggers of cell death on the production of chemoattractive signals and to understand their roles in maintenance of homeostasis or induction of inflammation.

Table 1 Released factors from dying/dead cells and their effects

Released factors	Type of cell death	Function	Reference
Microvesicles	Apoptosis	Chemotactic, pro- and anti-inflammatory, cell death	45
Lysophosphatidylcholine	Apoptosis	Chemotactic	46
H ₂ O ₂	Apoptosis	Cell death	47
Trombospondin-1	Apoptosis	Tolerogenic	48
Annexin-I	Apoptosis	Stimulation of engulfment	49
Ribosomal protein S19 dimer	Apoptosis	Chemotactic	50
HSP70, HSP90, gp 96, calreticulin	Necrosis	Pro-inflammatory	51
HMGB-1	Necrosis	Pro-inflammatory	52
Uric acid	Necrosis	Pro-inflammatory	53
mRNA	Necrosis	Pro-inflammatory	54
IL-6	Necrosis	Pro-inflammatory	55

HMGB-1, high mobility group box 1; HSP, heat shock proteins; IL-6, interleukin 6

Immunological Effects of Dying Cell Clearance

Early studies found that clearance of apoptotic cells induces an anti-inflammatory reaction (summarized in Table 2) leading to production of transforming growth factor- β (TGF- β), prostaglandin E₂, and platelet activating factor (PAF),⁶⁰ which have direct autocrine and paracrine effects on pro-inflammatory cytokine production. One of the leading models places TGF- β as a central anti-inflammatory modulator during apoptosis⁶⁰ acting by inhibiting p38 MAPK phosphorylation and NF- κ B activation, and subsequent cytokine production.⁷³ Furthermore, stimulation of the production of 15-lipoxygenase and 15-hydroxyicosatetraenoic acid, and inhibition of thromboxane synthase, thromboxanes, 5-lipoxygenase, sulfido-peptide leukotrienes, nitric-oxide synthase, and nitric oxide is dependent on TGF- β production upon apoptotic cell uptake.⁶⁹ It has been well established that under different *in vitro* conditions apoptotic cells suppress the secretion of pro-inflammatory mediators, such as TNF, IL-1, and IL-12 by macrophages stimulated with lipopolysaccharide (LPS). Moreover, it has been demonstrated that apoptotic cells inhibit the type-I interferon (IFN)-mediated induction of chemokine CXCL10.⁷² Apoptotic cells also inhibit IFN- γ -mediated signals by attenuating signal transducer and activator of transcription 1 (STAT1) activation, and downstream gene expression by inducing suppressors of cytokine signaling 1 (SOCS1) and SOCS3, which are negative regulators of the Janus kinase-STAT pathway.⁷²

The anti-inflammatory effects of apoptotic cells are mediated by autocrine and paracrine means through cytokines, but it has been demonstrated that apoptotic cells also exert their anti-inflammatory effect directly by binding to macrophages, independently of phagocytosis or the involvement of soluble factors (Table 2).⁶⁷ Contact of activated macrophages with apoptotic cells or their treatment with PS is sufficient to strongly inhibit their production of IL-12.⁷⁴ Apoptotic and necrotic cells, upon recognition by macrophages, have different effects on MAPK signaling.⁷¹ Early apoptotic cells were found to be as effective as late apoptotic cells (secondary necrotic) in inhibiting extracellular signal-regulated kinase 1/2 activity and stimulating c-Jun N-terminal kinases 1/2 (JNK1/2) and p38. By contrast, necrotic cells had no detectable effect on JNK and p38.⁷¹ Moreover, it was recently proposed that TNF release by LPS-stimulated

macrophages is suppressed by TGF- β only if the macrophages have first contacted apoptotic cells; thus, bystander macrophages appear to be refractory to TGF- β released by engulfing macrophages.⁶⁸ This observation may explain how macrophages balance pro- and anti-inflammatory responses in an *in vivo* situation where apoptotic cells are associated with inflammation and infection.⁷⁵ Indeed, macrophages reconcile two opposing responses: avoidance of unnecessary inflammatory responses to dying cells and the need for an appropriate response to pathogens. It is conceivable that macrophages engaging apoptotic cells suppress in a cell autonomous way their own inflammatory responses, but will nevertheless still allow newly recruited macrophages to function independently by responding normally to pathogens or pro-inflammatory stimuli.

In vitro, macrophages could be polarized to a pro-inflammatory (M1) or anti-inflammatory (M2) profile when exposed to IFN- γ and LPS, or to IL-4 and IL-10, respectively.⁷⁶ These pretreatments lead to the development of distinct phenotypes and physiological activities in these macrophages. M1 represents classically activated macrophages that have increased their production of pro-inflammatory cytokines (TNF, IL-1, IL-6, IL-12), inducible nitric oxide synthase and ROS, and increased their ability to present antigen.⁷⁶ Thus, these classically activated M1 macrophages are potent effector cells that destroy infectious microorganisms and tumor cells, and are therefore called 'killer' macrophages. In contrast, M2 macrophages result from an alternative activation characterized by increased expression of mannose receptor, dectin 1, and arginase, and generation of ornithine and polyamines.⁷⁶ M2 macrophages, in contrast to M1, promote angiogenesis, tissue remodeling, and repair, and are therefore called 'healer' macrophages. Alternatively activated macrophages (M2) are found during the healing phase of acute inflammatory reactions in chronic inflammatory diseases, such as rheumatoid arthritis and psoriasis.⁷⁷ It is conceivable that 'healing' macrophages are more involved in recognition of dying cells and tissue repair, while 'killer' macrophages are more engaged in infection and inflammation. Recently, the capacity of several types of phagocytes (M1 versus M2) to take up apoptotic cells was compared. 'Healing' macrophages (M2) were four times more effective than 'killer' macrophages (M1) at taking up early apoptotic cells than late apoptotic or necrotic cells.⁷⁸

Table 2 Summary of phagocytes responses during clearance of dying/dead/stressed cells

Target cells	Type of cell death and inducer	Stage of cell death	Phagocytes	Phagocyte response	Autocrine/paracrine or contact-dependent phagocyte responses	Reference
Human neutrophils, Jurkat human T cells	Apoptosis by incubation in culture for 24 h or UV treatment	Apoptosis (>99% trypan blue ⁻)	LPS or zymosan stimulated human monocyte-derived macrophages	Apoptotic cells: TGF- β PG E ₂ , PAF \uparrow TNF- α , IL-1 β , GM-CSF, IL-10, leukotriene C ₄ , thromboxane B ₂ \downarrow	Autocrine/paracrine	60
MEFs, thymocytes	Apoptosis by gamma irradiation; Necrosis by dounce-lysis, freeze-thawing	Apoptosis (annexin-V ⁺ /PI ⁻)	Murine bone marrow-derived DC	Necrotic MEFs and thymocytes: NF- κ B, KC (mRNA and protein), MIP-2 (mRNA), VEGF (mRNA) \uparrow Apoptotic thymocytes: no \uparrow KC	ND	61
Murine P388 cells	Apoptosis by etoposide treatment; necrosis by freeze-thawing	Apoptosis (annexin-V ⁺ /PI ⁻)	PMA-treated human THP-1 cells	Apoptotic cells: mRNA IL-1 α , IL-8, MIF \uparrow but on protein level only IL-8 \uparrow Necrotic cells IL-8 \uparrow on mRNA and protein level	Autocrine/paracrine (as shown by transwell system)	62
Murine splenocytes, thymocytes, and Jurkat human T cells	Apoptosis by gamma irradiation, hydrocortisone, staurosporine; necrosis by heating and freeze thawing	Apoptosis (annexin-V ⁺ /PI ⁻)	Murine peritoneal and bone marrow-derived macrophages	Apoptotic cells: Akt \uparrow , ERK1/2 \downarrow Necrotic cells: ERK1/2 \uparrow	Contact-dependent (colchicines resistant)	63
Human neutrophils	Apoptosis by incubation in culture for 24 h	Apoptosis (>80% annexin-V ⁺ and >90% EB ⁻ and AO ⁻)	Human LPS-activated monocytes	Apoptotic cells: IL-10, TGF- β \uparrow and TNF- α , IL-1 β \downarrow	ND	64
Murine CTLL-2 cells	IL-2 deprivation	Very early (annexin-V ⁻) and early apoptosis (annexin-V ⁺ /PI ⁻) cells	Phorbol myristate acetate-treated human THP-1 cells	Very early apoptotic cells: silent removal (no mRNA \uparrow of IL-1 β , IL-10, TGF- β) Early apoptotic cells: mRNA of IL-1 β , IL-10, TGF- β , IL-8 \uparrow	ND	65
Murine hepatocytes	Apoptosis by UV treatment	ND	Murine Kupffer cells	mRNA of TNF- α , Fas ligand \uparrow	ND	66
Murine DO11.10, human HeLa, CHO cell	Apoptosis by actinomycin D treatment, cyclochemide; necrosis by heat treatment	Apoptosis (annexin-V ⁺ /PI ⁻)	LPS stimulated RAW 264.7 murine macrophage cell line	Apoptotic cells: mRNA of IL-6, TNF- α , NF- κ B, AP1 \downarrow and on protein level TNF- α , IL-6 \downarrow Necrotic cells: mRNA of IL-6, TNF- α , NF- κ B, AP1 \uparrow and on protein level TNF- α , IL-6 \uparrow	Contact-dependent (cytochalasin D resistant)	67
Murine L929sAhFas cells	Apoptosis by anti-Fas antibodies, Fas ligand treatment; necrosis by mTNF treatment	Apoptosis (annexin-V ⁺ /PI ⁻); necrosis (annexin-V ⁺ /PI ⁺)	LPS stimulated Mf4/4 and J774 murine macrophage cell lines	Apoptotic and necrotic cells: no changes on mRNA (TGF- β , TNF- α , IL-6, IFN- β) and protein level (TNF- α , IL-6)	ND	34
Human neutrophils	Apoptosis by incubation in culture for 17 h	Apoptosis (>99% trypan blue ⁻)	Murine LPS stimulated bone marrow derived-macrophages	Apoptotic cells: TNF- α , IL-12, IL-10 \downarrow	Contact -dependent (as shown by transwell system)	68
Jurkat human T cells	Apoptosis by UV irradiation	>80% trypan blue ⁻	Murine LPS activated peritoneal macrophages and RAW 264 macrophage cell line	Apoptotic cells: PG F ₂ , PG F _{1α} \uparrow AC, COX-2, 15-LO, 15-hydroxyeicosatetraenoic acid \uparrow LPS activated: TNF- α , NO, iNOS, thromboxane (TXA ₂) \downarrow	Autocrine/paracrine	69
Murine CTLL-2 cells, thymocytes	Early and late apoptosis by IL-2 deprivation; necrosis by freeze-thawing; X-rays	Early (annexin-V ⁺ /PI ⁻) and late apoptosis (annexin-V ⁺ /PI ⁺)	Murine peritoneal resident macrophages	Early apoptotic cells: NO \uparrow Late apoptotic and necrotic cells: IL-8, MIP-2 \uparrow	ND	70
Jurkat human T cells and kidney	Apoptosis by staurosporine	Early (annexin-V ⁺ /PI ⁻) and late	Murine bone marrow-derived macrophages	Early and late apoptotic cells: ERK1/2 \downarrow , JNK1/2, p38 \uparrow	Contact-dependent	71

Table 2 (Continued)

Target cells	Type of cell death and inducer	Stage of cell death	Phagocytes	Phagocyte response	Autocrine/paracrine or contact-dependent phagocyte responses	Reference
proximal tubule epithelial cell lines	treatment; necrosis by heat treatment	apoptosis (annexin-V ⁺ PI ⁺)		Necrotic cells: ERK1/2 ↑		
Human Jurkat T cells	Apoptosis by anti-Fas treatment	Apoptosis (>70% annexin-V ⁺)	Human LPS- and IFN- γ - activated monocyte-derived macrophages	Apoptotic cells on LPS-activated: TNF- α , IL-6, CXCL10 ↓; no effect on I κ B α degradation, p38 MAPK, JNK, and p42/44 ERK phosphorylation; STAT1, STAT3 phosphorylation ↓ Apoptotic cells on IFN- γ activated: STAT1 tyrosine phosphorylation, CXCL10, CXCL9 ↓	ND	72

AC, arachidonic acid; AO, acridine orange; AP-1, activator protein 1; COX, cyclooxygenase; DC, dendritic cell; EB, ethidium bromide; ERK1/2, extracellular signal-regulated kinase 1/2; GM-SCF, granulocyte-macrophage colony stimulating factor; IFN- γ , interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinases; KC, keratinocyte-derived chemokine; LO, lipoxygenase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MIF, macrophage migration inhibitory factor; MIP-2, macrophage inflammatory protein-2; ND, not determined; NF- κ B, nuclear factor-kappa B; NO, nitric oxide; PAF, platelet activating factor; PAF, platelet-activating factor; PG, prostaglandin; PI, propidium iodide; PMA, phorbol myristate acetate; SOCS, suppressors of cytokine signaling; STAT1, signal transducer and activator of transcription 1; TGF- β , transforming growth factor beta; TNF tumor necrosis factor; VEGF, vascular endothelial growth factor. Stage of cell death was determined by PS exposure (annexin-V) and integrity of plasma membrane (exclusion of PI, EB, AO, or trypan blue). The initial paradigm of immunoregulation by dying cells was mainly ascribed to autocrine and paracrine acting cytokine produced by phagocytes, following recognition and/or phagocytosis of dying cells. However, recent studies indicate that dying/dead/stressed cells can exert their immunomodulatory effects directly upon binding to the phagocytes, independently of subsequent engulfment and soluble factor involvement (contact-dependent)

In contrast to the numerous studies describing the anti-inflammatory consequences of apoptotic cell clearance, several reports indicate that uptake of apoptotic cells can be immunologically silent with absence of anti- and pro-inflammatory effects,⁶⁵ or else it can have pro-inflammatory consequences.^{75,79} These discrepancies may be explained by the activation or differentiation state of macrophages (discussed above), the source and activation state of target cells, the type of cell death stimuli, and the presence of TLR ligands resulting in differential recognition mechanisms and immunological consequences. Some bridging molecules, such as surfactant proteins A and D, have a dual function depending on binding orientation and on the receptor system that is triggered.²⁵ Through their globular heads, SP-A and SP-D bind SIRP α , resulting in block of pro-inflammatory mediator production, but their collagenous tail stimulates pro-inflammatory responses by binding to calreticulin/CD91.²⁵ The efficiency of presenting antigens from phagocytosed cargo can also depend on the presence of TLR ligands within the cargo. Phagocytosis of apoptotic cells with simultaneous stimulation of bone marrow-derived DCs by LPS does not lead to presentation of the antigens derived from apoptotic cells.⁸⁰ However, antigens derived from apoptotic cells are successfully processed if dying cells are pretreated with LPS, which is controlled by TLR in a phagosome autonomous manner.⁸⁰ Recently it has been shown that apoptotic cell preparations of allogeneic origin containing phytohaemagglutinin or anti-CD3/CD28 activated T cells, but not resting T cells, are able to induce activation of DCs and presentation of alloantigens.⁸¹

Recognition and internalization^{8,31,32} of necrotic cells differs from that of apoptotic cells, and promotes pro-inflammatory macrophage responses.⁸² Ucker and colleagues elaborated further the differences between apoptotic and necrotic cell clearance with respect to inflammatory responses.^{67,71} They showed that the inhibitory effect of apoptotic targets, at all stages of apoptotic cells death, irrespective of cell membrane integrity, is dominant over the stimulatory effect of necrotic targets.^{67,71} It is conceivable that differential responses of macrophages to apoptotic and necrotic cells are triggered by distinct receptor-mediated recognition, while similar responses are triggered by the shared machinery of phagocytosis. In that respect, we demonstrated that in both apoptotic and necrotic cell death in the same cell type, clearance occurs in a PS-dependent manner, and in both cases does not activate NF- κ B (DV Krysko and P Vandenaabeele, unpublished data) or cytokine induction.³⁴ The challenge for future research is to identify the nature of tolerogenic signals and their receptors. The therapeutic modulation of these signals will be important for treatment of autoimmune diseases or immunotherapy of cancer. Studying the individual receptor systems and their combinations involved in phagocytosis of dying cells is a challenging task. In an elegant study using apoptotic cell surrogate systems, Skoberne *et al.*⁸³ addressed the individual roles of the α v β 5 and complement receptors (CRs) in phagocytosis by DCs and induction of immunity. The authors showed that CR3 and CR4, though substantially less efficient than α v β 5 at internalizing apoptotic cells, initiate signals that make DCs tolerogenic. On the other

hand, internalization of apoptotic cells via $\alpha v\beta 5$ does not invoke tolerance.

Exploitation of Molecules on the Surface of Dying Cells for Cancer Immunotherapy

Since most chemotherapeutic drugs and radiation elicit apoptotic cell death, clearance of apoptotic cells may maintain an anti-inflammatory milieu in the extracellular environment, contributing to an immunosuppressive network in a primary tumor site, and thereby promoting further tumorigenesis. As outlined above, an immunosuppressive milieu is generated by the induction and release of, for example, TGF- β and PAF. In this sense it is important to understand how we can modulate the immunogenicity of dying cells, for example by shielding immunosuppressive molecules that are exposed on the surface of dying cells. One of these molecules is PS, which is not only exposed on the surface of apoptotic and necrotic cells, but also on tumor cell-associated endothelial cells as a result of oxidative stress and activating cytokines.⁸⁴ Noteworthy, a monoclonal antibody (3G4) directed against anionic aminophospholipids, principally PS, reduces the growth of xenogenic and syngenic tumors by 50–90%.⁸⁴ The anti-tumor effect of 3G4 is partly mediated by damaging the tumor vasculature.⁸⁴ Because 3G4 substantially increases the engulfment of PS-expressing cells by bone marrow-derived mouse macrophages *in vitro* in an Fc-dependent fashion, it is likely that the mechanism of 3G4 cytotoxicity *in vivo* is mediated by macrophages and involves an Fc receptor-mediated inflammatory response and respiratory burst.⁸⁵ Another approach to restore immunogenicity of tumor cells is to block phagocytic clearance of apoptotic tumor cells using the PS-binding protein annexin-V. Injection of annexin-V-coupled tumor cells in combination with experimental radiotherapy resulted in a strong anti-tumor effect,³ with 90% of the mice rejecting the tumor after challenge compared to 25% of control animals. Moreover, *in vivo* clearance of annexin-V-coupled tumor cells by thioglycollate-elicited macrophages was decreased. Annexin-V not only inhibits phagocytosis by shielding PS, but also by interfering with PS-associated receptors and ligands.⁸⁶ Recently, Jinushi *et al.*⁸⁷ proposed a novel immunoregulatory effect of MFG-E8-mediated uptake of apoptotic cells. MFG-E8 is a bridging molecule that stimulates phagocytosis of apoptotic cells through specific binding to PS on apoptotic cells via COOH-terminal factor VIII homologous domain and to $\alpha v\beta 3$ integrin expressed on phagocytes via an NH₂-terminal EGF-like domain.⁸⁸ They have shown that coexpression of MFG-E8 inhibits vaccination activity of irradiated GM-CSF-secreting tumor cells in the B16 melanoma model, whereas an MFG-E8 mutant potentiates GM-CSF-induced tumor destruction.⁸⁷ Interestingly, this MFG-E8 mutant retains the capacity to bind PS on apoptotic cells but contains a modified integrin-binding domain that inhibits phagocytosis of apoptotic cells.^{87,88} A possible explanation for this anti-tumor effect may be that the MFG-E8 mutant inhibits the clearance of irradiated apoptotic tumor cells by masking PS on apoptotic cells, which then become secondary necrotic cells thereby enhancing immunogenicity. These findings suggest that shielding of PS on apoptotic cells by mutant MFG-E8 or annexin-V together with the adjuvant

activity of GM-CSF may provide a novel combined anti-tumor strategy.

Obeid *et al.*⁴ have shown that anthracycline compounds are unique among approximately 20 apoptosis-inducing agents in their ability to induce immunogenic apoptotic cell death. The immunogenicity of apoptotic cells apparently depends on the exposure of calreticulin on the plasma membrane of dying cells, and on the ability to enforce the early phosphorylation of eukaryotic initiation factor 2- α (eIF2 α), an event leading to inhibition of translation initiation.⁵⁹ Interestingly, the authors showed that strong calreticulin exposure can be induced by knockdown of GADD34 or of the catalytic subunit of protein phosphatase (PP1), which together form the PP1/GADD34 complex involved in the dephosphorylation of eIF2 α , or using inhibitors of PP1/GADD34, such as tautomycin, calyculin A (inhibitors of the catalytic subunit of PP1), and salubrinal (inhibitor of the PP1/GADD34 complex). These treatments were insufficient to increase immunogenicity, indicating that calreticulin exposure is not enough on its own.⁵⁹ However, when combined with non-immunogenic chemotherapeutic drugs, these inhibitors significantly increased immunogenicity.⁵⁹ This finding raises the possibility that combined delivery of calreticulin and chemotherapeutic drugs may provide a novel way to increase the therapeutic effect of drugs that induce non-immunogenic cell death. How calreticulin induces immunogenicity is not clear. As discussed above, calreticulin is also expressed on the surface of phagocytes (immature DC, macrophages, and monocytes) where it can bind NY-ESO-1 protein, a non-mutated, highly immunogenic cancer/testis antigen, resulting in its cross-presentation and contributing to spontaneous immune responses against tumor-associated antigens.⁸⁹ Although administration of annexin-V and calreticulin is a promising therapy, their side effects should be kept in mind.⁹⁰ An N-terminal fragment of calreticulin (vasostatin) has potent antiangiogenic effects.⁹⁰ Moreover, calreticulin profoundly affects wound healing by recruiting cells essential for repair, and thereby stimulating cell growth and increasing extracellular matrix production.⁹⁰

These results demonstrate that the type of stimulus is extremely important not only for the type of cell death, but also for initiating subroutines that result in differential immunomodulatory and phagocytic effects. Identification of these complex interactions between dying cells and phagocytes may lead to new experimental therapies involving modulation of molecules exposed on the surface of dying cells, as in principle has been elegantly demonstrated by using calreticulin in experimental cancer therapy.⁵⁹

Conclusion

The words written by Leonardo da Vinci 'While I thought that I was learning how to live, I have been learning how to die' reflect the paradigmatic shift that occurred in cell biology during the last decade. Only one decade ago cancer cell biology was explained mainly in terms of increased proliferation, cell survival, and energy metabolism. However, the insights from the last decade increasingly support the view that cellular life includes active modulation of cell death pathways, mechanisms for removal of dying cells, and the intricate relationship with the innate immune system. Many

questions remain regarding what determines the difference between immunomodulatory aspects of apoptotic *versus* necrotic cells, the apoptotic subroutines, or cells dying due to other forms of cellular stress. Insights into the molecular mechanisms of cell death, phagocytosis, and their immunomodulatory features will lead to novel experimental immunotherapies for cancer and autoimmune diseases, and is therefore a challenging research area.

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