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# Programmed cell clearance: Molecular regulation of the elimination of apoptotic cell corpses and its role in the resolution of inflammation

Bengt Fadeel [a,*], Ding Xue [b], Valerian Kagan [c]

[a] Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
[b] Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO, USA
[c] Center for Free Radical and Antioxidant Health, Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA, USA

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ABSTRACT

Programmed cell clearance is a physiological process of elimination of apoptotic cell corpses. Recent studies have disclosed several ligand–receptor interactions that dictate the recognition or non-recognition of cells by macrophages and other phagocytes. The externalization of the anionic phospholipid, phosphatidylserine is effectively recognized by specific receptors on professional phagocytes and facilitates the clearance of apoptotic cells. Macrophage disposal of cells at sites of inflammation is believed to play an important role in the resolution of the inflammatory process, and recent studies have suggested a role for the NADPH oxidase in the process of macrophage elimination of activated neutrophils. The present review will focus on the molecular regulation of programmed cell clearance, and discuss the role of cell elimination in the resolution of inflammation.



## 1. Introduction

During normal development and tissue turnover, cells are programmed to undergo apoptosis and cell corpses are subsequently removed by professional phagocytes (macrophages) or neighboring cells. The recognition and engulfment of apoptotic cell corpses, a complex and dynamic process which we refer to as *programmed cell clearance*, is thought to prevent chronic inflammation through the disposal of dying cells prior to the leakage from these cells of noxious constituents into surrounding tissues [1]. Moreover, rapid engulfment of apoptotic cells, orchestrated through the interaction of numerous recognition or eat-me signals, bridging molecules, and engulfment receptors, is believed to prevent inadvertent immune responses to self antigen present within or on the surface of dying cells. Indeed, the immune system is equipped with pattern recognition receptors to distinguish not only between foreign pathogens (non-self) and normal healthy tissues (self) but also to enable the discrimination between healthy viable cells (self) and dying cells (altered self) during the course of tissue remodeling or tissue injury [2,3].

## 2. PS signaling

A common feature of all eukaryotic membranes is the non-random (asymmetric) distribution of different lipid species in the lipid bilayer. One of the best known examples is the plasma membrane with its asymmetric distribution of aminophospholipids – phosphatidylserine (PS) and phosphatidylethanolamine (PE) – resulting in exclusive abundance or high prevalence of these phospholipids in the inner leaflet of the membrane in viable cells and their absence from the outer leaflet [4]. The lack of these lipids, particularly PS, on the cell surface creates remarkable opportunities for sensitive and specific PS signaling. Indeed, externalization of PS is effectively recognized by phagocytes as an eat-me signal and facilitates the disposal of apoptotic cells [1]. Maintenance of the PS gradient across the plasma membrane is mediated by aminophospholipid translocase activity and this represents a significant energy expenditure for the cell. Egress of PS is also not a spontaneous process but is regulated by enzymatic mechanisms, including phospholipid scramblase activity. Recent studies in the nematode have revealed a novel mechanism of activation of the phospholipid scramblase, as discussed below. Additionally, oxidation reactions leading to accumulation of oxidatively modified forms of PS may stimulate transmembrane diffusion of PS and its oxidized forms (PS-OX) during apoptosis. Notably, both PS and PS-OX, as well as other phospholipids such as oxidized phosphatidylcholine (PC-OX) are recognized by specific phagocyte receptors and contribute to the clearance of dying cells [5–7].

The role of PS as a recognition signal for macrophages was first described for aging or damaged erythrocytes [1]. Intriguingly, more recent studies have shown that macrophage engulfment of nuclei expelled from erythroid precursors also is a PS-dependent event [8]. In addition, activated platelets are known to display PS, and

* Corresponding author. Address: Division of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, 171 77 Stockholm, Sweden. Fax: +46 8 34 38 49.
*E-mail address:* bengt.fadeel@ki.se (B. Fadeel).

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this serves to provide a pro-coagulant surface during the process of blood clotting [1]. PS externalization on the surface of apoptotic cells was first described by Fadok et al. [9] and was subsequently shown to be a general feature of apoptosis regardless of the initiating stimulus [10,11]. However, there are a few exceptions to this general rule, as reported previously in this journal [12]. Importantly, we and others have shown that apoptotic cells that do not express PS fail to undergo efficient engulfment by macrophages, whereas the clearance defect can be restored by repleting the plasma membrane of target cells with exogenous PS [13,14]. Furthermore, Chang et al. [15] provided evidence that oxidation-specific epitopes may mediate macrophage recognition of pre-necrotic, dying cells, and our studies have shown that specific oxidation of PS during apoptosis contributes to the recognition and engulfment of apoptotic cells by macrophages [14,16,17]. We also demonstrated the involvement of extra-mitochondrial cytochrome c in the selective catalysis of PS oxidation during apoptosis [18]. Hence, oxidation of lipids during apoptosis is not merely an unavoidable and deleterious consequence of the apoptotic process but plays a specific signaling role in the clearance of cell corpses by neighboring phagocytes.

## 3. Recognizing PS

Many potential receptors have been implicated in the recognition of dying cells, including the so-called PS receptor (PSR), various members of the integrin receptor and scavenger receptor families, as well as TIM-4 (T cell immunoglobulin- and mucin-domain-containing molecule-4) and the related protein, TIM-1, and several other molecules [1]. Interestingly, a recent study identified PS-OX and, to a lesser extent, PC-OX as specific signals for the scavenger receptor, CD36 [19]. Moreover, we previously reported that the bridging molecule, MFG-E8 interacts preferentially with oxidized PS [20].

Importantly, studies in mice that are deficient for PSR [21,22] or the PS-binding protein, milk fat globule-epidermal growth factor 8 (MFG-E8) [23] have revealed the occurrence of numerous unengulfed apoptotic cells in various tissues and the occurrence of lupus-like autoimmune disease, thus providing compelling evidence that programmed cell clearance mediated via the recognition of PS on apoptotic cells is required for maintenance of tissue homeostasis. MFG-E8 has also been reported to diminish the severity of tissue fibrosis in mice by binding and targeting collagen for uptake by macrophages [24].

## 4. Lessons from the worm

Two partially redundant pathways are implicated in the engulfment of apoptotic cells in *Caenhorhabditis elegans* [1]. The first pathway consists of CED-2, CED-5, CED-10, and CED-12 (CED, cell death abnormal), while the second pathway involves CED-1, CED-6, and CED-7. A PSR homolog (PSR-1) was identified in *C. elegans* and shown to act upstream of the engulfment genes, CED-2, CED-5, CED-10 and CED-12 [25]; loss-of-function mutations in PSR-1 resulted in a moderate impairment of cell corpse clearance in the worm. More recent studies have demonstrated that the aminophospholipid translocase, TAT-1 is required for maintenance of phospholipid asymmetry in living cells in the worm [26]. Furthermore, externalization of PS was found to occur through WAH-1 (worm homolog of AIF)-dependent activation of the phospholipid scramblase, SCRM-1, a homolog of the mammalian phospholipid scramblases [27]. Interestingly, previous studies demonstrated that the microinjection of apoptosis-inducing factor (AIF) into the cytosol of human cell lines induces dissipation of the mitochondrial trans-membrane potential and PS externalization on the cell surface [28]. Taken together, these studies provide evidence for a critical role of mitochondria in PS externalization during apoptosis.

## 5. Cardiolipin asymmetry

Another example of phospholipid asymmetry during apoptosis is the non-random distribution of the mitochondria-specific phospholipid, cardiolipin (CL). There are at least two possible roles of CL asymmetry in mitochondrial signaling. The first one is a recently established pathway for CL trans-membrane migration and formation of a complex with cytochrome c in mitochondria, leading to the release of pro-apoptotic factors into the cytosol [29]. In this case, collapse of CL trans-membrane asymmetry is a critical prerequisite for its binding to cytochrome c to activate the latter into a catalytically competent peroxidase. In addition, we hypothesized that the asymmetric distribution of CL could act as a mitochondrial eat-me signal in autophagy signaling. Autophagy is an evolutionarily conserved mechanism used by cells for the continuous turnover of damaged and obsolete macromolecules and organelles and may serve as a mechanism of adaptation to stress, whereas in other circumstances, it constitutes an alternative pathway of programmed cell death [30]. The existence of mitochondrial autophagy (mitophagy) implies that there should be specific mitochondrial signals that are involved in triggering of the autophagic machinery. Indeed, recent studies in yeast have revealed a receptor, Atg32 that is anchored in the mitochondrial outer membrane and directs autophagosome formation to mitochondria [31,32]. These studies also indicated that the induction of Atg32-dependent mitophagy is mediated by oxidative stress. Our recent experiments in mammalian cells have shown that knockdown of CL by targeting the CL synthase, the key enzyme in CL de novo synthesis, resulted in markedly compromised apoptosis and a concomitant enhancement of mitophagy (Kagan, unpublished observations). Further studies are warranted to define the potential roles of CL externalization/oxidation in apoptosis and autophagic disposal and recycling of organelles.

## 6. Role in inflammation

Chronic granulomatous disease (CGD) is a rare hereditary condition characterized by mutations in different subunits of the NADPH oxidase in phagocytic cells. Patients with CGD suffer from recurrent, severe bacterial and fungal infections and formation of chronic tissue granulomas. We reported previously that neutrophils from CGD patients are able to undergo normal constitutive apoptosis but are defective for ROS-dependent exposure of PS [33]. Furthermore, our recent studies have shown that macrophages from CGD patients or normal donor macrophages pre-incubated with a pharmacological inhibitor of NADPH oxidase have a diminished capacity for phagocytosis of PS-positive target cells [34]. Impaired macrophage clearance of apoptotic cells has also been reported in $gp91^{phox}$-deficient mice, a common model of CGD [35]. Taken together, these studies suggest that programmed cell clearance is dependent on a functional NADPH oxidase not only in the target cell, but also in macrophages (Fig. 1). Notably, the NADPH oxidase plays a dual role in superoxide production and the regulation of intracellular pH. We hypothesized that the failure to regulate intracellular pH may underlie the impaired capacity of macrophages from CGD patients to engulf apoptotic target cells. Our recent studies using normal donor macrophages have demonstrated that pre-incubation of macrophages at alkaline pH significantly suppressed the engulfment of apoptotic cells while pre-incubation at acidic pH, on the other hand, resulted in an increase in macrophage clearance of cell corpses (Fadeel, unpublished observations). Further studies are needed to unravel the

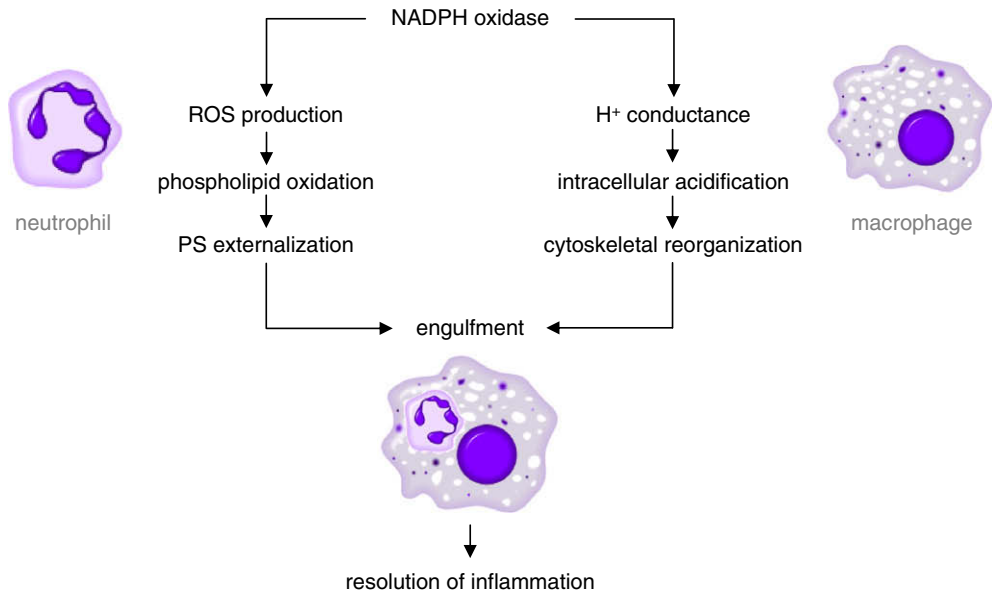


**Fig. 1.** Hypothetical diagram illustrating the dual role of the NADPH oxidase in programmed cell clearance. Macrophage recognition and clearance of activated neutrophils at sites of inflammation is believed to play a critical role in the resolution of inflammation. Our studies have shown that the NADPH oxidase is required for PS oxidation and externalization in activated human neutrophils [33]. The absence of PS exposure in neutrophils that lack expression of a functional oxidase results in inefficient clearance of these cells by professional phagocytes. In addition, the NADPH oxidase was shown to play a role in the engulfing cell during programmed cell clearance [34]. The molecular pathways governed by the NADPH oxidase in macrophages remain to be understood, but one may speculate that the NADPH oxidase is required to produce reactive oxygen species (ROS) in the target cell whereas in the engulfing cell, the regulation of intracellular pH is important. Further studies are warranted to test this hypothesis.

role of NADPH oxidase in macrophages for engulfment of apoptotic cells.

Interestingly, the NADPH oxidase also plays a role in anti-bacterial autophagy [36], thus linking the oxidative and non-oxidative killing activities of the NADPH oxidase in phagocytes.

Neutrophils were previously shown to possess two different pathways of PS exposure: a caspase-dependent pathway associated with induction of apoptosis, and a ROS-dependent pathway that is engaged in activated cells [33]. More recent studies have identified a third pathway of PS externalization in primary human neutrophils that is neither caspase-dependent nor ROS-dependent but is induced by neighboring phagocytes [37]. In a related study, we noted that activated macrophages were able to cause inhibition of aminophospholipid translocation in the plasma membrane of neighboring target cells via a nitrosative stress-dependent mechanism, resulting in PS exposure on the surface of the latter cells and their subsequent engulfment [38]. These studies suggest that activated macrophages may engage in a PS-dependent feedback mechanism that potentially could serve to limit excessive inflammatory responses. The in vivo relevance of this pathway awaits further testing.

## 7. Future perspectives

Programmed cell clearance is a genetically regulated pathway for the recognition and internalization of apoptotic cell corpses. PS-dependent signaling has been shown to play an important role in this process. Importantly, programmed cell clearance is not merely a passive process of waste disposal. Uptake of apoptotic cells by professional phagocytes has important immunological consequences. For instance, recent studies have suggested that phagocytosis of apoptotic (altered self) infected (non-self) cells is critical for the induction of a subset of T cells known as T helper 17 ($T_h17$) cells, which have been shown to play an important role in host defense against infections, and in tissue inflammation during autoimmunity [39]. Furthermore, very recent studies have revealed that elevated surface expression of CD47 on leukemic stem cells enables these cells to avoid phagocytosis and is an indicator of poor prognosis for patients with acute myeloid leukemia [40]. The molecular dissection of programmed cell clearance may thus yield novel targets for therapeutic intervention in inflammation and in autoimmune disease, as well as in cancer.

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