Elimination of the Unnecessary: Intra- and Extracellular Signaling by Anionic Phospholipids

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Abstract

High fidelity of biological systems is frequently achieved by duplication of the essential intracellular machineries or, removal of the entire cell, which becomes unnecessary or even harmful in altered physiological environments. Carefully controlled removal of these cells, without damaging normal cells, requires precise signaling, and is critical to maintaining homeostasis. This review describes how two anionic phospholipids - phosphatidylserine (PS) and cardiolipin (CL) - residing in distinct compartments of the cell, signal removal of "the unnecessary" using several uniform principles. One of these principles is realized by collapse of inherent transmembrane asymmetry and the externalization of the signal on the outer membrane surface - mitochondria for CL and the plasma membrane for PS – to trigger mitophagy and phagocytosis, respectively. Release from damaged cells of intracellular structures with externalized CL or externalized PS triggers their elimination by phagocytosis. Another of these principles is realized by oxidation of polyunsaturated species of CL and PS. Highly specific oxidation of CL by cytochrome c serves as a signal for mitochondria-dependent apoptosis, while oxidation of externalized PS improves its effectiveness to trigger phagocytosis of effete cells.

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Keywords

"Funny how the unnecessary can seem so important, expanding, contracting, cleaning itself. Whatever it is, it won't let us in. It folds inside itself like a dying star, in a way it's superior, as original as every murder."

From: "The Unnecessary" by Karen Murai, The Paris Review No. 114 (Spring 1990)

Introduction

The subject of "the unnecessary" has been metaphorically and also constructively discussed and used by artists, politicians, and economists with different goals and in different contexts. Perhaps one of the most illuminating examples of this is a well-known principle in Pablo Picasso's creative life namely that "art is the elimination of the unnecessary", which he so exquisitely and skillfully applied to his work. We ask, what may be the relevance of this principle to biological processes?

The reliability of biological processes is achieved, to a large extent, through the initial generation of excessive amounts of biological material, including organelles and cells, which upon successful completion of their specific functions become unnecessary. Removal of the unnecessary in a timely manner is essential to prevent the unnecessary from becoming undesired or even harmful. This principle is well illustrated during development, such as in the elimination of the tail in the tadpole during metamorphosis [1] and tissue remodeling of a vertebrate limb bud during development [2], wherein unnecessary cells and tissue are removed through the process of apoptosis. Elimination of unnecessary cells also occurs in inflammation, wherein specialized cells recruited to the inflammation site must be cleared after successful completion of their job. Other examples include, mammary gland involution and ovarian follicle atresia, which are characterized by removal of milk-producing epithelial cells [3] and immature ovarian follicles [4] respectively, through apoptosis. The importance of clearance is underscored by observations that its inhibition results in severe developmental defects, organismal lethality [5–7], autoimmune disease [8, 9], or promotion of tumorigenesis [10]. Thus, robust and reliable mechanisms that direct timely removal of unwanted cells are essential, not just for maintenance of homeostasis, but also for circumventing undesired pathophysiological consequences. Given the remarkable importance of eliminating of the unnecessary in biology, in this brief review, we will consider recent advancements in our understanding of the role that two anionic phospholipids – phosphatidylserine (PS) and cardiolipin (CL) – play in the signaling and clearance of "unnecessary" organelles and cells.

Synthesis and localization of PS and CL

There are at least two different ways in which PS and CL participate in different types of elimination signaling: a) by trans-membrane re-distribution and subsequent appearance on

the cell/organelle surfaces thus enabling recognition by specialized receptors and b) by oxidation of polyunsaturated acyl chains of the phospholipid molecules leading to modification of the membranes including their barrier functions. Intriguingly, PS and CL are compartmentalized in cells such that CL is localized almost exclusively in the inner mitochondrial membrane (IMM) whereas PS is essentially absent from the mitochondria [11, 12]. It is tempting to speculate that this separation is an evolutionary attainment to utilize and optimize two spatially distinct yet related membrane signaling functions realized in mitochondria and extra-mitochondrial compartments, respectively.

While CL and PS have distinct locations in the cell, their biogenesis occurs at contiguous sites. PS synthesis occurs in the endoplasmic reticulum (ER), specifically in ER domains proximal to the mitochondria, known as the mitochondria-associated membranes (MAM) [12, 13]. The synthesis of CL is well documented and is described in detail in several recent reviews [11, 14–17].

Following its initial synthesis, CL undergoes acyl chain "remodeling" to generate tissue and organ specific CL molecular species. This secondary stage of synthesis is catalyzed by enzymes including i) a CL-specific phospholipase(s) e.g. Cld1p in yeast cells that are needed to make monolyso-CL (MLCL) [18]; ii) An acyl transferase (tafazzin) that is located in the intermembrane space (IMS) of mitochondria which can reversibly exchange acyl chains between phospholipids and MLCL.[19–23]; iii) An MLCL acyltransferase-1 (MLCLAT-1) that is located in the matrix of the mitochondria, described as a splice variant of trifunctional protein [14, 24–26] and iv) An acyl-CoA:lyso-CL-acyltransferase-1 (ALCAT-1) that is reported to be beyond the outer side of the mitochondrial outer membrane (OMM) in the mitochondria associated membranes (MAMS) of the endoplasmic reticulum (ER) [14, 27, 28]. How and where these enzymes participate in the final synthesis of CL will dictate the location of CL in and around the mitochondria. After remodeling, most of the CL is located in the IMM, specifically concentrated on the inner leaflet of the IMM. CL has multiple binding affinities for the numerous mitochondrial proteins, including mitochondrial respiratory super-complexes [29–33] and respiratory components including cytochrome c [34] and is also believed to aid in the organization and stabilization of the highly curved IMM membrane structure [15, 35]. The diversified association with a multitude of mitochondrial proteins and its reliance on the mitochondrial membrane potential for stability [36, 37] defines CL's role as a coordinator of numerous mitochondrial functions, including their critical roles as a signal for mitophagy and as an activator of the NLRP3 inflammasome (see below).

The synthesis of PS occurs via two separate synthetic pathways in mammalian cells and is distinct from the yeast PS synthetic pathway [12, 38]. In yeast, PS synthesis occurs via a CDP-diacylglycerol precursor which is also the precursor of CL in both yeast and mammalian CL synthesis. [15, 39–42]. The yeast CL biosynthetic pathway is conserved in mammals, but the biosynthesis of PS in mammalian cells has evolved using two separate and distinct PS biosynthetic pathways (see [38] for a recent review).

Once synthesized, PS travels to the mitochondria, where it is decarboxylated to phosphatidylethanolamine (PE), catalyzed by the IMM enzyme, PS decarboxylase.

Knocking out this enzyme in mice resulted in embryonic lethality. Morphological analysis revealed highly abnormal and fragmented mitochondria [43]. It is likely that the accumulation of mitochondrial PS, rather than decreased levels of PE, caused the lethal phenotype. In line with this, knocking out the Psd1 gene in yeast, which codes for the yeast mitochondrial PS decarboxylase, significantly impaired cell viability [43]. Thus, mitochondria might be considered to be "PS averse", likely due to the interference of PS with the mitophagic and apoptotic signaling by CL.

PS externalization is an engulfment signal

When phagocytosis was first discovered and described by I. Mechnikov in 1883 [44], the molecular dynamics of signaling and the role of PS externalization was unknown. It took more than a century that witnessed a multitude of fundamental discoveries in biology before it was established that one of the hallmarks of the activation of apoptosis and initiation of phagocytosis is the appearance of PS on the outer leaflet of the apoptotic cell membrane [45, 46]. The externalized PS is a recognition signal for specific receptors on the professional phagocytes (including macrophages) which initiates the engulfment of target injured or dying cells [47]. Studies using model systems demonstrate that the presence of externalized PS alone is sufficient to activate phagocytosis. For example, PS-coated single-walled carbon nanotubes are readily phagocytized when compared with non-coated controls [48]. Likewise, integration of PS into the outer surface of viable cells, that are not apoptotic, promoted phagocytosis by macrophages [45, 49].

Externalization of PS is a near-universal feature of apoptosis and is commonly involved in recognition and elimination of apoptotic cell corpses (Figure 1). Of particular importance is the PS-dependent phagocytosis of neutrophils by macrophages, a process that defines the transition from a pro-inflammatory to an anti-inflammatory state [50]. Normally, PS is asymmetrically distributed across the plasma membrane – being located predominantly in the inner leaflet along with phosphatidylethanolamine [51, 52]. The maintenance of this asymmetry is controlled by an ATP-dependent aminophospholipid translocase, also called a flippase [53]. During apoptosis this asymmetry collapses resulting in PS exposure on the external face of the cell membrane. Two possible mechanisms for this re-shuffling of PS are proposed. The activation of a calcium dependent scramblase can rapidly catalyze the inversion of phospholipids that are asymmetrically located on the plasma membrane [54]. The rate of scramblase activity (>10,000 phospholipids per second) far exceeds the ATP dependent flippase activity of (~1–100 ATP per second). Inactivation of the flippase concomitant with scramblase activation, has been proposed as the most efficient way to achieve PS exposure on the outer membrane leaflet [54, 55]. Experimentally, this concept was demonstrated by nitrosative stress under non-apoptotic conditions, wherein selective inhibition of the aminophospholipid translocase (flippase) resulted in PS externalization and recognition of these non-apoptotic cells by macrophages [56] – a phenomenon referred to as "buried alive" [57]. This underscores the uniqueness and sufficiency of PS externalization as an engulfment signal for professional phagocytes.

Importantly, there are concentration thresholds of PS on the surface, needed for the recognition of apoptotic cells, in order to initiate phagocytosis [58]. The existence of a

threshold mechanism is essential for the prevention of excessive phagocytosis of non-apoptotic cells which may transiently present externalize PS on their surface [47, 58, #24, 59, 60].

PS oxidation promotes apoptotic cell clearance

During inflammation, massive production of reactive oxygen species (ROS) creates pro-oxidant environments potentially favoring PS oxidation [61], yet for phagocytosis, externalized PS is not required to be oxidized. However, presenting externalized, oxidized PS strongly enhances the effectiveness of phagocytosis [62]. Cells grown in cultures on standard media do not contain significant amounts of oxidizable PS, yet apoptosis and macrophage phagocytosis can be triggered. Metabolic incorporation of oxidizable polyunsaturated fatty acids (PUFA) such as linoleic acid, (LA) results in the appearance of PS containing PUFA acyl chain(s). During apoptosis, activation of the redox machinery in these cells stimulates the formation of oxidized PS species (PSox) and appearance of PSox on the outer leaflet of the plasma membrane. This is accompanied by a significantly higher clearance of apoptotic cells realized via both increased number of phagocytosis-positive cells and the increased content of engulfed target cells per phagocyte [62].

Structural variations between oxidized and non-oxidized PS raise the possibility of species specific PS-receptor interactions. Indeed, eukaryotic cells have amassed a rich repertoire of distinct receptors on different types of professional phagocytes for recognizing apoptotic cells via PS binding. These include, scavenger receptors A & B, CD36, vitronectin, PS-receptor, CXCL16, MerMFG-E8, Tim-4, BAI-1, lipoprotein receptor–related protein (LRP) and TREM2 [63–68]. The "language" of engulfment is further diversified and enhanced by soluble, PS-binding proteins, including thrombospondin-1, milk fat globule-EGF factor 8 (MFG-E8), growth arrest-specific 6 (GAS-6), and β2-glycoprotein I [47, 69–71]. Such a multiplicity in receptor expression likely reflects ligand specific signaling responses as has been suggested by the differential binding preference of PS for CXCL16, TIM-4 and CD36 and of PSox for BAI-1 and GAS-6 [62]. It is also conceivable that an engulfment "synapse" is formed between the phagocyte and its apoptotic prey requiring more than one ligand-receptor couple [72].

Numerous recent reviews have provided information on macrophage recognition of cells undergoing apoptosis [53, 73]. Evolutionary conservation of receptor multiplicity further underscores that eliminating the unnecessary is vital to homeostasis [74]. While it is commonly accepted that the phagocytosis of apoptotic cells triggers the resolution of inflammation [75, 76], the actual meaning of each individual signal and combinations of different molecular signals in pro-/anti-inflammatory conditions has not been deciphered. With the development of redox (phospho)lipidomics the identification of the role of individual PS and oxidized PS species as "orchestral players" of inflammation can be unraveled [77].

CL asymmetry is a signal for mitophagy

From a signaling perspective, externalization of PS on the cell membrane, is similar to CL externalization on the mitochondrial surface (Figure 1). Factors that determine the need for mitophagy may include maintenance of an adequate membrane potential, oxidation of proteins and lipids and the ability of mitochondria to undergo biogenesis. Mitochondrial externalization of CL serves as an elimination sign initiating mitophagy in analogy to PS externalization which signals phagocytosis, yet the molecular details are very different. Externalized CL can bind to microtubule-associated protein 1 light chain 3 (LC3) which is part of the autophagy machinery [78]. The externalization mechanisms for CL and PS are also very different, but the signaling principal is the same. To appear on the mitochondrial surface, CL has to transgress the IMM, the intermembrane space and finally the OMM. The initiation of CL asymmetry collapse happens when mitophagy is warranted and initiated. Assuming that most of CL in the IMM is not "free", but rather engaged in different types of lipid-protein and protein-protein interactions [79], one can wonder how CL gets released from its complexes to traverse from the IMM inner side to the outer side of the OIM and becomes a mitophagy beacon. Obviously, the dissipation of mitochondrial membrane potential is the trigger and the driving force of this process. The details of the machinery engaged in the mitophageal translocations of CL are not fully understood. There are several "suspects" for the CL redistribution in the IMM. Given the essentiality of mitochondrial depolarization and potential involvement of redox-driven mechanisms, uncoupling proteins seem to be interesting candidates [80, 81]. The inter-membrane space translocation may be particularly challenging in terms of energetic expenditures. Interestingly, a mitochondrial isoform of nucleoside diphosphate kinase (NDPKD) has been demonstrated as the driving force of this process in mitophagy [82, 83]. The participants of the CL translocating machinery in the OMM are also not definitively identified although mitochondrial scramblase proteins (e.g., scramblase 3) are possibly involved [84, 85].

Intriguingly, while redox mechanisms are believed to act as almost universal triggers of mitochondrial damage and mitophagy, there is no compelling requisite for CL oxidation for mitophagy, which is in sharp contrast to the obligatory dependency of CL oxidation for apoptosis (see below). This may reflect substantial differences in the execution pathways and physiological roles of mitophagy and apoptosis as two distinctive cell survival and cell death mechanisms, respectively. Overall, the mechanisms that control CL asymmetry and translocations are still enigmatic, although there is clear evidence to indicate that mitochondrial damage is associated with presentation of CL on the OMM surface to signal and trigger intracellular mitochondrial clearance by mitophagy [84].

CL oxidation elicits intrinsic apoptosis

While mitophagy is a pro-survival rescue pathway, the continued accumulation of mitochondrial impairments and the insufficiency and/or failure of repair mechanisms may necessitate the elimination of the entire cell through the activation of the apoptotic program. This requires not only the presence of externalized CL, but also oxidation of its polyunsaturated acyl chain(s). Historically, the involvement of CLs in (BAX-mediated) apoptosis had been proposed and supported by the work of Kuwana et al. [86]. A later study

from the Orrenius laboratory, clearly demonstrated that there is no absolute requirement for CL for the execution of the apoptotic program in yeast cells (*S. cerevisae*) where BAX-dependent apoptosis was occurring in CL synthase deficient mutants (*Crd*) [87]. This apparent discrepancy may be reconciled if one considers that yeast cells grown under standard laboratory conditions simply do not contain oxidizable, polyunsaturated CL species [88–90]. Indeed, subsequent detailed studies revealed that CL peroxidation, catalyzed by cytochrome c, is required for the execution of intrinsic apoptosis in mammalian cells that have oxidizable CL species [91]. Detailed analysis of mechanisms and pathways involved in CL oxidation in apoptosis has been recently discussed in several reviews [37, 81, 92–95]. Briefly, externalization of CL in damaged mitochondria strongly enhances its availability for the intermembrane space hemoprotein, cytochrome c. This encounter results in the formation of cytochrome c/CL complex in which the normally hexa-coordinated hemoprotein adopts the penta-coordinate organization allowing for the emergence of the peroxidase catalytic competence [91]. As a consequence, the closest PUFA-phospholipid target, CL, gets peroxidized. Studies from several laboratories have confirmed and developed this paradigm. There is thus, a transformative structural shift for cytochrome c from being an electron carrier shuttling electrons between respiratory complexes III and IV while located on the outer surface of the IMM, to a CL-specific peroxidase function in apoptosis [34, 91, 96–99].

The peroxidase activity and CL oxidation precede and are required for mitochondria-initiated apoptosis whereby the sources of oxidizing equivalents are derived from dis-coordinated electron transport [33,36,82]. Structural details of cytochrome c/CL complexes and their significance for the peroxidase activation are still under detailed scrutiny whereby the dependence of the peroxidase activity on the level of protein unfolding are being explored using sophisticated state-of-the-art technologies including, solution NMR and solid state NMR [100, 101]. These developments in understanding CL oxidation and its role in apoptosis has also offered new opportunities for therapeutic interventions, and new types of regulators acting as suppressors of CL oxidation have been generated. For example, a series of mitochondria-targeted hemigramicidin nitroxides (eg., XJB131, XJB125) – acting as scavengers of electrons - have been designed, synthesized and formulated and found to have high therapeutic effectiveness in acute traumatic brain injury [102] and total body irradiation, among others. Another groups of regulators – imidazole substituted fatty acids displayed anti-apoptotic activity by preventing/suppressing cytochrome c/CL peroxidase activity via Fe-chelation [103].

One of the difficult and controversial issues originating from in vivo studies was the large diversification of CL oxidation products – dozens or even hundreds of different types of structurally distinctive oxidation products [104]. For some time, it remained unclear which of them are, indeed, directly related to the execution of apoptosis. This conundrum has been recently resolved by using a protocol of redox opto-lipidomics [77]. It has been demonstrated that only few out of a large number of detectable CL oxidation products represent real apoptotic signals. In fact, in cells exposed to several pro-apoptotic treatments such as actinomycin D, staurosporine, ionizing radiation or 10-nonylacridine orange, only C18:2 mono-oxygenated species of CLs were identified as predictive biomarkers of apoptosis [77].

Phagocytosis of extracellular mitochondria

When an effective biological solution evolves, it is frequently adapted and widely used. Autophagy of mitochondria and phagocytic removal of cells both depend on similar signaling systems although the processes are quite different. Interestingly, recent studies demonstrated that macrophages have evolved to retain mechanisms to target CLs on extracellular mitochondria for phagocytosis [105]. While the details and machinery of eliminating extracellular mitochondria remain a subject of interest, oxidation of CL has no role in extracellular mitochondrial phagocytosis. Similar to what has been shown for PS exposition on the surface of viable, non-apoptotic cells (see above), the integration of mitochondrial CL onto protein-free liposomes or onto healthy mitochondria that do not have surface CL also triggers engulfment through macrophage receptor CD36 [105]. The mechanisms of clearance in these model systems differs from the usual intracellular LC3-mediated mitophagy signaling of CL, wherein the CL externalized mitochondrial membrane does not leave the cell [84]. Thus, while both PS and CL can in principle signal clearance, there are spatial constraints that restrain signaling cross-talk between these lipids under normal physiology conditions, with CL being restricted to the mitochondria and PS being present in most other cellular membranes, most notably the plasma membrane, allowing CL to exclusively signal LC3 mediated mitophagy.

CL dependent intracellular clearance of damaged mitochondria by mitophagy, is a survival mechanism, and is therefore expected to occur without untoward activation of inflammatory cytokines. There are, however, several reports that under pathophysiological conditions, cells release CL-presenting mitochondria and mitochondrial membranes. Examples include, activation of neutrophils, stress, necrosis, and acute trauma [106–108]. Similarly, infections challenge the immune system by externalizing CL on the surface of bacterial membranes ([108] These CL-containing membranes are unique in that they lack the pro-phagocytic and anti-inflammatory signal PS [38, 51]. Moreover, they are also rich in immune-cell activating mtDNA or bacterial DNA, in addition to formyl peptides [109], which activate immune surveillance via an array of extracellular pattern recognition receptors, including Toll-like receptors (TLRs) [110], and intracellular inflammasomes platforms for pro-inflammatory cytokine production [111].

Hence, rapid clearance of these CL-expressing entities is essential to avoid an uncontrolled innate response, which can progress into local and systemic inflammatory maladies, culminating in chronic autoimmune disease. Macrophages address this eventuality by employing the same family of engulfment receptors discussed above for PS dependent phagocytosis. Among these, scavenger receptor CD36 stands out in its ability to target membranes, organelles and cells expressing eukaryotic or prokaryotic CL for clearance as evidenced using macrophages from CD36 null animals [105]. Interestingly, activation of many of the macrophage phagocyte receptors including CD36 is upregulated through activation of TLRs, and, moreover, there is also evidence to suggest cross-talk between TLRs and engulfment receptors [112, 113]. Thus, the host immune system has evolved to allow a balanced approach to appropriately deal with the unnecessary "self", the undesired "self", and the undesired "non-self" or foreign entities. In this scenario, both phagocytosis and immune surveillance systems work in parallel to remove the unnecessary and undesired,

so as to maintain cellular and tissue homeostasis, while at the same time preventing any deviations of the inflammatory responses.

In addition to signaling for removal of mitochondria and bacteria by phagocytosis, extracellular CLs have also been suggested to play a role in regulating inflammatory responses elicited by bacteria. In a recent study, it was determined that the ability of the gram-negative bacterial antigen, lipopolysaccharide (LPS) to activate inflammatory cytokine production via macrophage TLR4 receptors was significantly dampened in the presence of mitochondrial or bacterial CLs [105]. In contrast, substituting PS for CL showed no effects on cytokine production. This effect of CL was determined to operate by an extracellular mechanism as a consequence of competition between CL and LPS for the MD2 subunit of the TRL4/MD2 complex, in a manner similar to that reported for immature lipid A [114]. Thus, there are topographical differences between CL dependent cytokine suppression that acts by extracellular signaling via TLRs, and PS dependent cytokine suppression that occurs through intracellular mechanisms [115–117].

NF-κB, a transcription factor which acts downstream of cell surface receptors including macrophage TLRs drives inflammation through various pathways, including the priming of the NLRP3 inflammasome [118]. How the inflammasome is kept in check following its activation has remained unclear. Tschopp and co-workers showed that mitophagy/autophagy blockade resulted in the accumulation of damaged mitochondria, which in turn activated the NLRP3 inflammasome [118]. Recent studies have revealed a regulatory mechanism whereby NF-κB can restrain NLRP3 activation through mitophagy with specific recognition of the damaged organelles by p62 [119]. NF-κB exerted its anti-inflammatory activity by inducing delayed accumulation of the autophagy receptor p62 and macrophage-specific p62 ablation caused pronounced accumulation of damaged mitochondria and excessive IL-1β-dependent inflammation. Thus, mitophagy/autophagy might also prevent NLRP3 inflammasome activation from going awry.

It will be of interest to further examine the intracellular signaling events, including transcriptional responses, engendered by CL during removal of unnecessary organelles within the cell (mitophagy), or ingestion of extruded organelles (by other cells).

In contrast to the immunosuppressive function of extracellular CLs, there is evidence to indicate that intracellular CLs plays a direct role in activation of NLRP3 inflammasomes [120], which are tasked with the activation of inflammatory cytokines IL-1 and IL-18 as a prelude to pyroptosis in response to an intracellular pathogen. Thus, the signaling effects of CL are unique to its spatial location in the cell. Moreover, the consequences are unique and contrasting as well. For example, in the cytoplasm, CL plays a positive role in the maintenance of cell health by removal of damaged mitochondria during mitophagy. Cytoplasmic CL plays a suicidal role in cells infected with intracellular pathogens, through the activation of pyroptosis. The extracellular functions of CL are activated only under pathophysiological conditions resulting from infection (bacterial CLs), sterile cell damage (mitochondrial CLs) or infection induced host cell damage (bacterial CLs + mitochondrial CLs). Since macrophages cannot differentiate between bacterial and mitochondrial CLs [105], the signaling consequences are identical in each of these cases. Extracellular CL thus

plays a crucial role in pathophysiology, by promoting removal of mitochondrial membranes that lack PS as an elimination signal (as opposed to other intracellular membranes which have PS). Extracellular CL also plays another important role in restoration of tissue homeostasis following gram-negative bacterial infections by subduing further activation of TLR4 (Figure 2).

Concluding remarks

Reliability in biology frequently requires an excess of constituents including metabolites, enzymes, organelles, and cells. During times of change and stress, such as growth, injury, or infection, this excess can become unnecessary and detrimental. Elimination of the unnecessary applies to many facets of biology. Here we described how PS and CL, two distinct phospholipids, have evolved to signal transformative yet similar cellular processes that eliminate "the unnecessary". Discerning this information has involved generations of scientists who are curious about life's control mechanisms. Looking forward, in this research field, the progress being made in lipidomics and bioinformatics is likely to continue and expand while integrating knowledge from other research fields. It is thus a good time to understand "the unnecessary". As discussed here, lipid signaling also involves topographical molecular constraints to prevent promiscuous or inadvertent activation of phagocytosis and/or mitophagy in healthy cells. Indeed, available data suggest that one and the same signal, CL, may signal for widely disparate cellular and organismal outcomes depending upon its intra- or extracellular localization.

In pathological scenarios, e.g., burn injury or physical trauma or infection, the constraints placed by compartmentalization may break down and both PS and CL may inevitably co-signal through macrophage receptors. This is expected to occur during both sterile and non-sterile (pathogen-induced) tissue damage, which is characterized by the release of intracellular membranes and organelles, including both intact mitochondria and membrane fragments from damaged mitochondria [106–108]. The immunological consequence of simultaneous PS and CL signaling in this case would depend on several factors including, the proportion of each lipid, receptor expression pattern and activation status of the macrophages or other professional phagocytes being engaged [121–123], and the relative affinities for PS and CL for the expressed receptors.

Active research in the field of phospholipid signaling to eliminate the unnecessary has already revealed many essential mechanisms and pathways involved in these important processes in isolated model biochemical systems and cells. A more challenging task is the assessment of the role of these processes *in vivo*. It is likely that not only high analytical power of LC-MS protocols in the identification of different individual signals, but also more integral approaches that afford on-line recording of the signaling process may be useful. Among them, a promising technique may be registration of different types of chemiluminescence originating from the excited states of oxidatively modified lipids [124]. Assuming that (phospho)lipid hydroperoxides are unavoidable primary molecular products of lipid peroxidation and that their decomposition gives rise to multiple secondary products with carbonyl functions in the excited state, it is likely that chemiluminescence protocols – particularly with physical enhancers of chemiluminescence responses – may be used for

continuous monitoring of the peroxidation process in live cells. Given that highly portable chemiluminometers have become available, this approach may find promising applications in precision medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

CL	cardiolipin
IMM	inner mitochondria membrane
IMS	mitochondrial inter membrane space
LC	liquid chromatography
MAMS	mitochondria associated membranes
MLCL	monolyso-cardiolipin
MS	mass spectrometry
NPDKD	nucleoside diphosphate kinase
OMM	outer mitochondrial membrane
PS	phosphatidylserine
PSox	oxidized phosphatidylserine
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species

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Highlights

- Phospholipid (PL) signaling in elimination of organelles and cells is reviewed.

- Anionic PL membrane asymmetry generates distinct signals for elimination.

- Oxidation of cardiolipin is a required stage in mitochondrial apoptosis.

- Oxidation of externalized phosphatidylserine enhances phagocytosis efficiency.
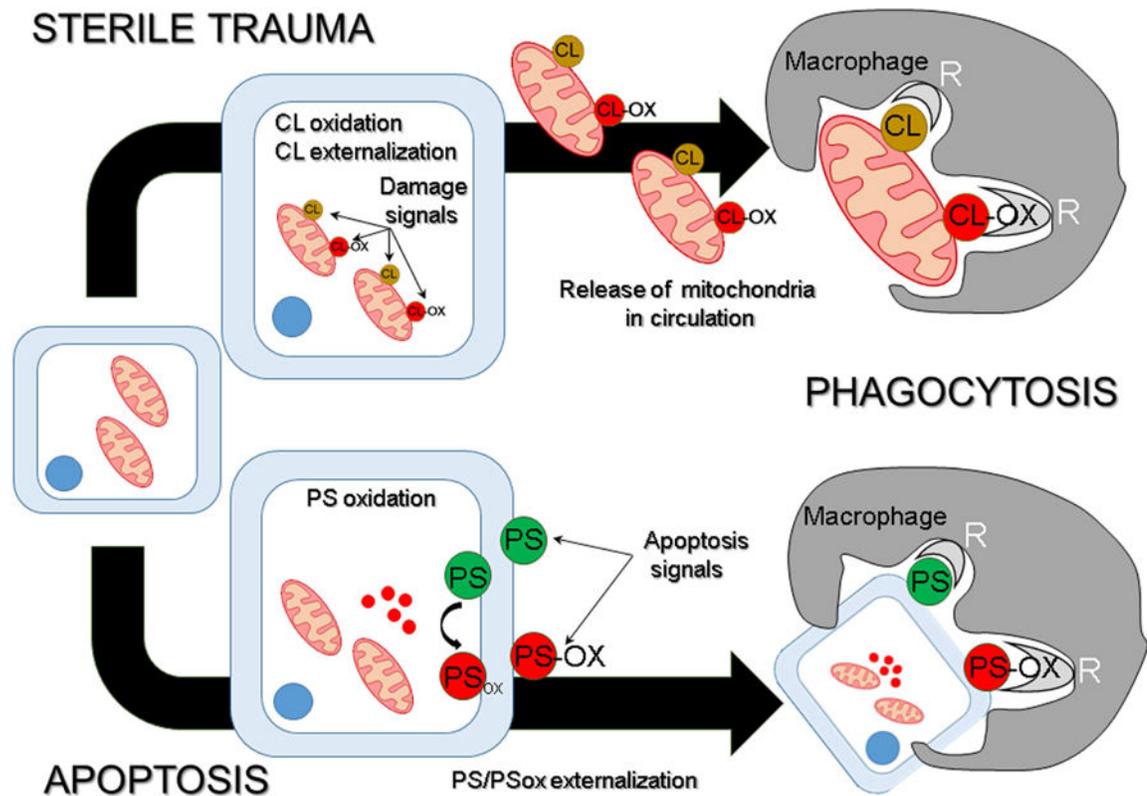
Figure 1. CL and PS as elimination signals

There are at least two different ways in which PS and CL participate in elimination signaling. CL and PS are localized in spatially distinct membranes – the mitochondria and the plasma membrane, respectively - that sets apart their availability as beacons for clearance. During sterile trauma, mitochondria containing CL and peroxidized CL (CLox) are released by damaged cells. When encountered by macrophages, CL and CLox trigger phagocytosis with similar efficacies, whereby these membranes are engulfed, for subsequent degradation in lysosomes. Induction of apoptosis results in externalization of PS on the cell surface. Oxidative stress induced apoptosis is further characterized by lipid peroxidation that results in the presentation of both PS and PSox on the dying cells. While both PS species are targeted by macrophages for engulfment, the efficiency of PSox-dependent clearance is substantially higher than PS-dependent clearance.
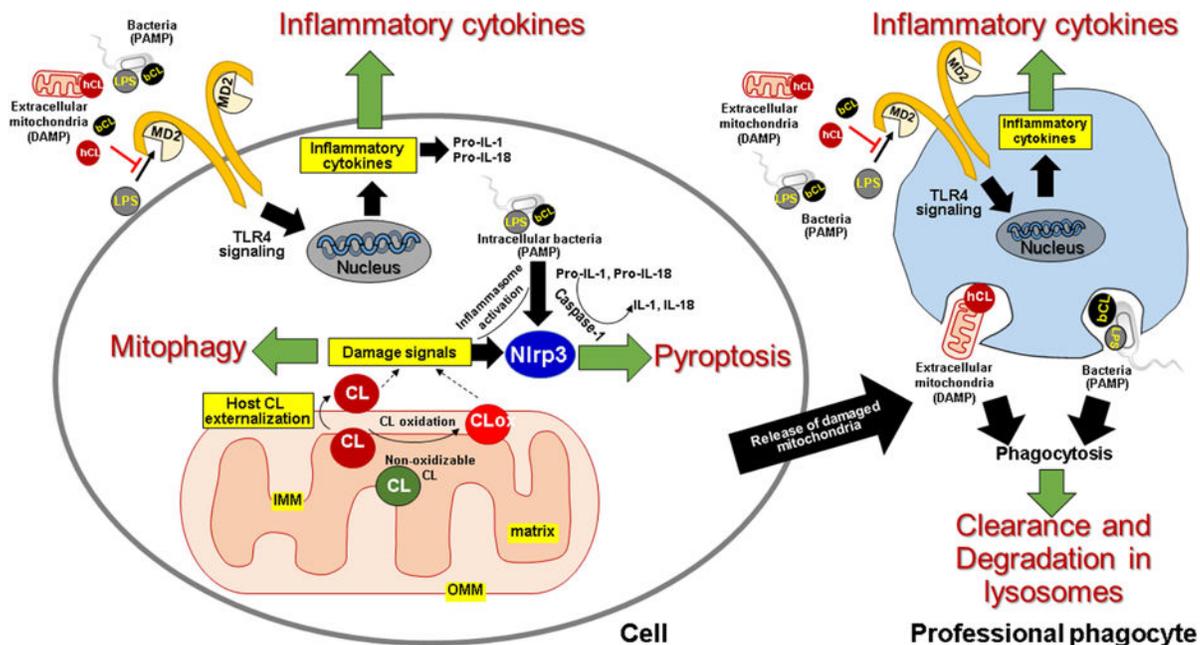
Figure 2. Role of cardiolipins in homeostasis

Intra- or extracellular signaling by CL is triggered by its externalization to the mitochondrial surface. *Intracellular CL:* In healthy cells, CL is exclusively localized in the inner mitochondrial membrane (IMM) specifically on the inner leaflet of the IMM that faces the matrix. Damage to the mitochondria results in loss of this asymmetry and movement of the lipid to the outer leaflet of the IMM, to the inner leaflet of the outer mitochondrial membrane (OMM), and finally to the outer leaflet of the OMM. Additionally, oxidative stress results in peroxidation of oxidizable CL (having polyunsaturaed acyl chains) by cytochrome c, to produce CLox, which may also be presented at the mitochondrial surface as a damage signal. Once at the mitochondrial surface, CL in the presence of cytoplasmic LC3, signals clearance of the damaged organelle by mitophagy, thereby restoring intracellular homeostasis. Under pathological conditions, such as presence of intracellular pathogens or presence of intracellular pathogen associated molecular patterns (PAMPs) like lipopolysaccharide (LPS), mitochondrial CL and/or CLox promotes activation of the NLRP3 dependent inflammasome pathway that directs cell death by pyroptosis. *Extracellular CL:* Mitochondria released following cell damage provide human CL (hCL) as a source of extracellular damage associated molecular patterns (DAMPs), which challenge host cells, including professional phagocytes. Similarly, infections challenge these cells with bacterial CL (bCL) and LPS. Both hCL and bCL signal engulfment of mitochondrial and bacterial membranes by professional phagocytes for subsequent degradation in lysosomes. hCL and bCL also interfere with binding of LPS to MD2, thereby attenuating Toll-like receptor (TLR)-4 dependent inflammatory cytokine production. The attenuation of cytokine response and phagocytic clearance both contribute to restoration of homeostasis.