

# Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums

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**Everyday we turnover billions of cells. The quick, efficient, and immunologically silent disposal of the dying cells requires a coordinated orchestration of multiple steps, through which phagocytes selectively recognize and engulf apoptotic cells. Recent studies have suggested an important role for soluble mediators released by apoptotic cells that attract phagocytes ("find-me" signals). New information has also emerged on multiple receptors that can recognize phosphatidylserine, the key "eat-me" signal exposed on the surface of apoptotic cells. This perspective discusses recent exciting progress, gaps in our understanding, and the conflicting issues that arise from the newly acquired knowledge.**

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Abbreviations used: LPC, lysophosphatidylcholine; Ptd-Ser, phosphatidylserine; S1P, sphingosine 1-phosphate; TAM, Tyro-3-Axl-Mer; UTP, uridine 5' triphosphate.

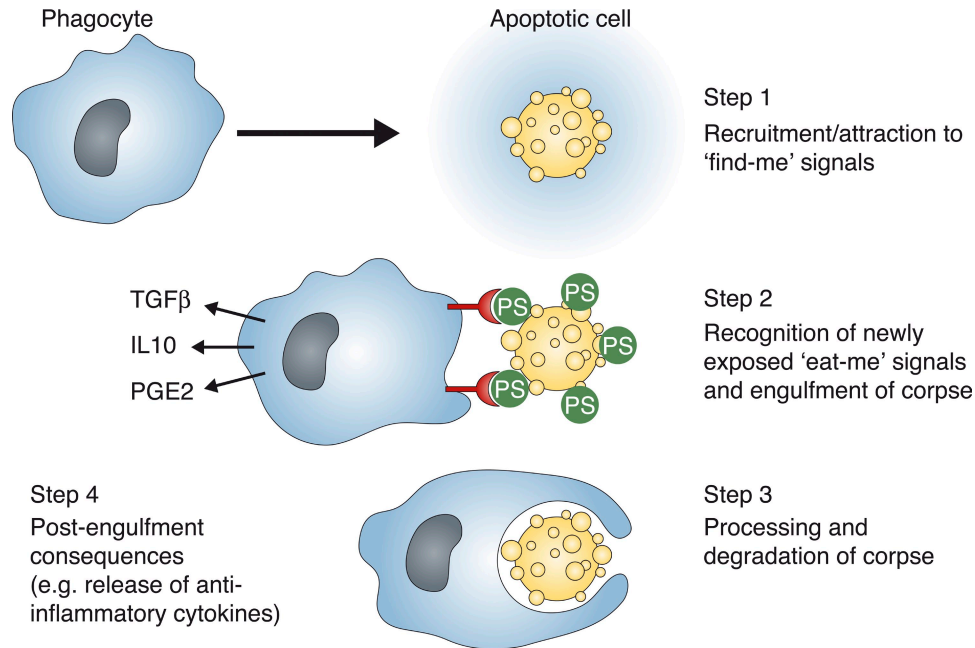
It is truly remarkable that our bodies turnover/recycle roughly one million cells every second (Henson and Hume, 2006; Ravichandran and Lorenz, 2007). These include excess cells that are being constantly generated in tissues as part of normal development, used/aged cells, and damaged cells that arise from disease or infections (Savill et al., 1993; Henson and Hume, 2006). The clearance of such dying cells is mediated either by phagocytes that are professional engulferers (such as macrophages and immature dendritic cells; Aderem and Underhill, 1999) or neighboring cells (such as fibroblasts and epithelial cells; Monks et al., 2008). The quick and efficient removal of unwanted cells is important for "making space" for living cells, and for maintaining the function of tissue, and in turn, a healthy organism (Savill et al., 2002; Henson, 2005; Henson and Hume, 2006). Uncleared dying cells can undergo secondary necrosis, and the release of intracellular contents from these cells has been linked to autoimmune diseases (Savill and Fadok, 2000; Nagata et al., 2010).

Under normal conditions, the clearance process is rapid such that even in tissues with high cellular turnover, very few apoptotic cells are seen; this led to an initial hypothesis that the basal capacity for cell clearance is very high (Gardai et al., 2006). However, there are many disease states where uncleared corpses/debris are seen, including systemic lupus erythematosus, atherosclerosis, Alzheimer's disease, and Parkinson's disease (Camins et al., 2008; Gorman, 2008; Calissano et al., 2009; Nagata et al., 2010). This suggests that the capacity for cell clearance may not be as

large as initially thought; rather, in the context of a tissue there may be a regulated balance between the numbers of apoptotic cells, the numbers of phagocytes, and their capacity for uptake. Therefore, defining the specific steps by which apoptotic cells are recognized and removed quickly is important for understanding diseases associated with defective clearance and for potentially manipulating the engulfment machinery for future therapeutic benefits.

The engulfment process can be broadly broken down into four major steps (Laubert et al., 2004; Fig. 1). The first step involves find-me signals released by apoptotic cells, which help attract phagocytes to the sites of death within a tissue (Gregory, 2009). The second step is the exposure of eat-me signals on the apoptotic cell surface, which promotes the specific recognition by the phagocyte and subsequent internalization of the corpse (Grimsley and Ravichandran, 2003). The third step is the processing of the ingested cargo by the phagocyte, where the engulfed corpse goes through a series of phagosome maturation steps, eventually leading to its degradation (Kinchen and Ravichandran, 2008, 2010). The fourth step, loosely denoted as post-engulfment consequences, involves the release by the phagocyte of antiinflammatory cytokines and other modifiers that are elicited during recognition and processing (Savill and Fadok, 2000; Savill

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**Figure 1. Different steps involved in efficient apoptotic cell clearance.** The find-me signals (such as low levels of nucleotides ATP and UTP, fractalkine, lysophosphatidylcholine, or sphingosine 1-phosphate) released by apoptotic cells help attract motile phagocytes to the proximity of the cell undergoing apoptosis. The phagocytes then use engulfment receptors on their surface to engage eat-me signals on apoptotic cells. For clarity, only the PtdSer on the apoptotic cells engaged by cognate receptors is depicted. Engagement of the engulfment receptors (linked to PtdSer recognition) has been shown to stimulate release of antiinflammatory cytokines such as TGF-β, IL-10, and prostaglandin E2 (PGE2). The intracellular signaling induced within the phagocyte by the ligand–receptor interactions leads to cytoskeletal rearrangements and internalization of the dying cell. The phagocyte processes the engulfed corpse through a series of steps, and proper digestion seems to be important for continued uptake of other dying cells by phagocytes.

et al., 2002; Henson, 2005). Collectively, these events mediate the selective and immunologically silent versus immunogenic removal of apoptotic cells in vivo (Green et al., 2009).

Over the past several years, several laboratories have helped identify the key molecules involved in these steps, as well as several molecular pathways that orchestrate the safe disposal of apoptotic cells (Wu and Horvitz, 1998a,b; Hamon et al., 2000, 2002; Reddien and Horvitz, 2000; Gumienny et al., 2001; Zhou et al., 2001a,b; deBakker et al., 2004; Kinchen et al., 2005; Yu et al., 2006). In this Perspective, I will focus on recent developments in two areas, the find-me and eat-me signals of apoptotic cells, which have caused significant excitement in the apoptosis and engulfment fields. As is often the case with interesting observations, these have also raised several fundamental issues that are puzzling or unexplained by the existing data. I will attempt to address some of the complexities and gaps in knowledge, and present some possible suggestions for moving forward. To keep this review focused, I primarily address events in the context of classical apoptosis that is largely nonimmunogenic; the reader is referred to other articles covering the modifications and responses of the immune system to necrotic and other immunogenic types of cell death (Green et al., 2009; Zitvogel et al., 2010).

#### Find-me signals: the beginnings of a fatal attraction

One of the striking anecdotal observations consistently observed over many years is that even in tissues known to have

a high turnover (e.g., thymus or bone marrow), in the basal state there are very few apoptotic cells seen. However, un-cleared apoptotic cells can become evident in these tissues when large numbers of cells are induced to undergo apoptosis, or in the context of defective engulfment (Henson and Hume, 2006; Elliott and Ravichandran, 2010). Recent studies have suggested that the apoptotic program is rather closely linked to the prompt removal of these cells (Gregory, 2009). Early evidence from elegant genetic studies in *Caenorhabditis elegans* suggested that apoptotic cells may be recognized by phagocytes and cleared well before the apoptotic cells are fully dead (Hoepfner et al., 2001; Reddien et al., 2001). This suggested that there might exist a mechanism by which the apoptotic cells advertise their own presence at the earliest stages of death. In addition, this led to the concept that find-me signals released from apoptotic cells can attract phagocytes to promote rapid clearance in vivo (Ravichandran, 2003).

Are there find-me signals in mammalian systems? Recently, several studies reported potential find-me signals released by apoptotic cells (Lauber et al., 2003; Gude et al., 2008; Truman et al., 2008; Elliott et al., 2009). These include lysophosphatidylcholine, fractalkine, nucleotides ATP and uridine 5' triphosphate (UTP), and sphingosine 1-phosphate (S1P). All of these are capable of attracting monocytes, although only fractalkine and nucleotides have been shown to be capable of functioning as find-me signals under in vivo conditions (Truman et al., 2008; Elliott et al., 2009). The identification of

several distinct find-me signals established the idea that apoptotic cells actually make an active effort to attract phagocytes. Several interesting questions ensue from these observations.

The basis behind the notion of find-me signals is that a chemotactic gradient would serve to attract the phagocytes. But what is the range of the gradient set up by these find-me molecules, and how is such a range determined? Most likely, the range would be determined by the tissue concentration of a given find-me molecule, and how quickly such a molecule is degraded. One could envision that if a find-me signal had a short half-life, and were restricted to the local tissue, it would act short range to attract resident phagocytes to the proximity of the dying cell. In contrast, if the find-me signals can get out of the tissues and into the circulation, they might attract phagocytes from circulation. As of now, we only know of short-range find-me signals. In the case of nucleotides, they can be readily degraded by extracellular nucleotidases (Elliott et al., 2009), and therefore their chemoattraction may be largely restricted to phagocytes resident within the tissue. In the context of clearing thymocytes undergoing apoptosis, disrupting the nucleotide find-me cue resulted in uncleared corpses in the thymus (Elliott et al., 2009). However, it is not known whether a given phagocyte responding to the call of an apoptotic thymocyte is a nearby first responder, or comes from the other end of the thymus. This would obviously depend on the distance of a gradient set up by the ATP/UTP (~100 nM) released by an apoptotic lymphocyte (Elliott et al., 2009), but this is really difficult to precisely establish in vivo. It is important to note that early apoptotic cells release very small amounts of ATP (<2% of intracellular ATP), which should be distinguished from necrotic cells that may release much of their ATP content. Marking particular macrophages and tracking their motion in real time toward a particular thymocyte may provide some answers. Lysophosphatidylcholine (LPCs; Lauber et al., 2003) and S1P (Gude et al., 2008) have not been tested in tissue models; therefore, it is difficult to assess their range. However, even if they were not rapidly degraded, LPC and S1P are present in high concentrations in circulation (much higher than what is reported to be released from dying cells), suggesting that they likely work locally and may not attract phagocytes from circulation. Although studies to date have considered the different find-me signals in isolation, if multiple find-me signals are released from the same apoptotic cell, they may act in concert, and may be more efficient in attracting phagocytes locally as well as from the circulation. This possibility needs to be explored.

Another interesting unanswered question is how the various find-me signals are released from apoptotic cells. It is clear from the existing work that these soluble mediators are, indeed, released when the apoptotic cells are intact, with no apparent leakage of cellular contents, and that the release is caspase dependent. This suggests that there may be channels or other mechanisms that mediate the release of these soluble mediators from inside the apoptotic cells. Given the varied nature of the find-me signals (lipid vs. protein vs. nucleotides) and their

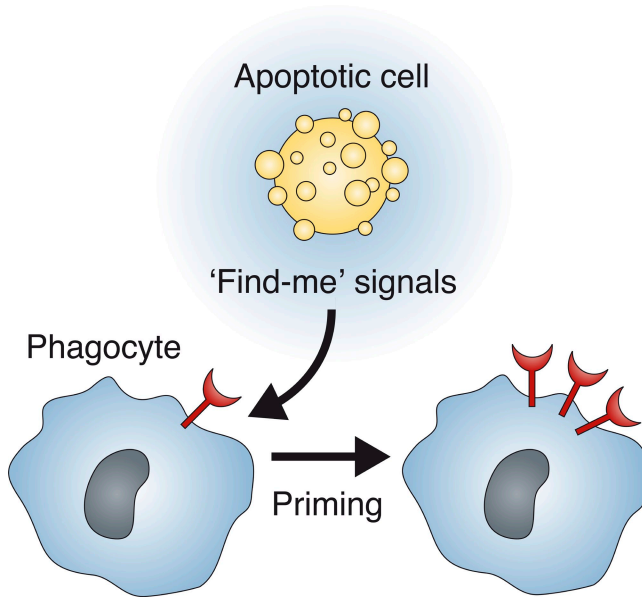
differing molecular sizes, it is likely there are distinct mechanisms for their release. Another important point is that apoptotic cells also appear to secrete stop signals, such as lactoferrin, which actively prevents neutrophil recruitment (Boumazou et al., 2009). It has long been known that clearance of apoptotic cells that occurs routinely in tissues often involves nonimmunogenic clearance via monocytes, without neutrophil recruitment and inflammation (Savill, 1997; Savill et al., 2002). Therefore, defining the specific channels or mechanisms of find-me signal release may be used for therapeutic purposes, for example to specifically attract monocytes for nonimmunogenic clearance and resolve inflammation.

### Find-me not the only function?

Another question is whether all apoptotic cells need to send out find-me signals. It is easy to envision why a dying thymocyte, which is likely not going to be cleared by a neighboring thymocyte (not phagocytic), would have to recruit thymus-resident phagocytes. However, an epithelial cell undergoing death, which might be eaten by a neighboring epithelial cell, may not need to send a find-me signal. Similarly, in *C. elegans*, neighboring cells often clear the apoptotic cells, and phagocytes need not travel to find the dying cells (Kinchen and Hengartner, 2005). This raises the question of why cells have find-me signals at all—or do find-me signals have other roles? It is noteworthy that lung epithelial cells also release ATP after apoptosis induction (Elliott et al., 2009). One intriguing possibility is that the find-me signals such as nucleotides or S1P could activate or prime phagocytes and improve their phagocytic capacity. In *Drosophila*, apoptosis induces up-regulation of the engulfment machinery in neighboring cells (MacDonald et al., 2006; Ziegenfuss et al., 2008). In mammals, the bridging molecule MFG-E8, which can decorate an apoptotic cell and facilitate engulfment, is expressed in activated, but not resting, macrophages (Hanayama et al., 2002). Thus, macrophages somehow must be recruited, up-regulate MFG-E8, garnish their apoptotic meal, and then engulf the target. One possibility is that the find-me signals released by apoptotic cells would serve not only to attract phagocytes, but also to up-regulate engulfment components in the recruited phagocytes (Fig. 2). If so, in the context of engulfment by a neighboring epithelial cell, perhaps the find-me signals act not as true find-me signals but rather help activate the engulfment machinery in their neighbor. Find-me signals may also influence immunogenic versus nonimmunogenic responses to apoptotic cells (Green et al., 2009; Zitvogel et al., 2010). Hopefully, future experiments will test this possibility.

### Eat-me signals: phosphatidylserine and its receptors

Although find-me signals can get phagocytes to the proximity of apoptotic cells within a tissue, the specific recognition of the dying cell among the neighboring live cells depends on eat-me signals exposed by the apoptotic cells (Lauber et al., 2004; Gardai et al., 2006). To date, multiple eat-me signals have been identified (Gardai et al., 2006). These include exposure of phosphatidylserine (PtdSer), changes in charge and



**Figure 2. Potential effect of find-me signals on phagocytes.** Find-me signals may prime phagocytes to up-regulate the engulfment machinery or cause an increase in expression of engulfment receptors (red) or other components that contribute to engulfment (such as cytoplasmic signaling proteins or soluble bridging molecules; not depicted).

glycosylation patterns on the cell surface, alteration of ICAM-1 epitopes on the cell surface, and exposure of calreticulin. Among these, the exposure of PtdSer on the outer leaflet of the plasma membrane is the most universally seen alteration on the surface of apoptotic cells (Fadok et al., 1992, 2000). In fact, PtdSer exposure is the best studied and the most accepted definition for calling a cell apoptotic (Fadok et al., 1998). However, several conundrums about PtdSer exposure and recognition are worthy of further consideration.

Over the past few years, there has been a paradigm shift in the thinking about how PtdSer on apoptotic cells gets recognized by phagocytes. Previously, it was thought that there would be a single PtdSer recognition receptor that would be universally used by phagocytes (Henson et al., 2001b). However, it now appears that multiple distinct receptors on phagocytes can engage PtdSer exposed on apoptotic cells (Bratton and Henson, 2008). These PtdSer recognition receptors come in two primary flavors – those that can bind directly to PtdSer, and those that indirectly bind PtdSer via soluble bridging molecules. Direct-binding PtdSer receptors include members of the TIM family (the prototype TIM-4, and also TIM-1 and TIM-3; Kobayashi et al., 2007; Miyanishi et al., 2007; Santiago et al., 2007; Ichimura et al., 2008; DeKruyff et al., 2010; Rodriguez-Manzanet et al., 2010; Wong et al., 2010), the seven transmembrane brain angiogenesis inhibitor 1 (BAI1; Park et al., 2007a), and the atypical EGF-motif containing membrane protein Stabilin-2 (Park et al., 2007b). In vitro studies coupled with in vivo studies in mouse models established a definitive role for the bridging molecule MFG-E8, which can bind PtdSer on apoptotic cells

with high affinity (Hanayama et al., 2002, 2004). A second region of MFG-E8 can simultaneously engage integrin  $\alpha_v\beta_3$  on phagocytes and thereby mediate PtdSer-dependent uptake of apoptotic cells. Two other bridging molecules, Gas6 and protein S, are involved in linking PtdSer exposed on apoptotic cells to Tyro-3–Axl–Mer family of receptors (denoted as TAM receptors) on phagocytes (Scott et al., 2001; Rothlin et al., 2007; Lemke and Rothlin, 2008). An important role for this family has been established through single and combined knockout of the TAM family members in mice; these receptors appear important for clearance of apoptotic cells in the eye, testes, thymus, and other tissues (Prasad et al., 2006; Lemke and Rothlin, 2008). In addition to the aforementioned receptors and bridging proteins, the membrane proteins CD36 and CD68, and the soluble thrombospondins (in turn binding to membrane receptors) have been suggested to be capable of binding PtdSer (Savill et al., 1991, 1992; Balasubramanian and Schroit, 1998; Imachi et al., 2000; Mevorach, 2000).

Why we need so many receptors and bridging molecules is an open question. The general consensus proposed in the field has been that not all receptors are expressed on all phagocytes, and therefore multiple modes of recognition are needed (Savill and Fadok, 2000; Henson et al., 2001a). Although this may be true for some phagocytes, many of these PtdSer recognition mechanisms were first established using macrophages. In fact, a typical peritoneal or bone marrow–derived macrophage (or macrophage cell lines such as J774) may express up to seven different known PtdSer recognition mechanisms. If we assume that these various PtdSer recognition modes are operational simultaneously, questions arise about the sheer number of PtdSer molecules that need to be exposed on a given apoptotic cell for binding by these various receptors, and the topology of the exposed PtdSer and membrane curvature needed for simultaneous recognition. From the phagocyte end, how can all of these receptors engage PtdSer on the same apoptotic cell? Because some of the PtdSer recognition receptors (such as TIM-4) appear abundant on the macrophage surface, one would expect these receptors to compete with each other for the same ligand. As soluble bridging molecules such as Gas6 and Protein S can also be quite abundant in serum/plasma (Anderson et al., 2003; Balogh et al., 2005), it is puzzling that any free PtdSer would even be accessible for binding by direct PtdSer–recognition receptors. The nature of the display of PtdSer on the surface of the apoptotic cell, how the PtdSer recognition receptors might engage PtdSer, and whether co-ligands on apoptotic cells work together with PtdSer to influence the specific recognition by phagocytes are discussed further in the following paragraphs.

#### Unanswered questions about the fundamental biology of PtdSer exposure

In considering the display of PtdSer, one needs to consider both the number of PtdSer molecules that are exposed on the surface of an apoptotic cell, as well as how these molecules are displayed for recognition by receptors on phagocytes. Are

there even enough PtdSer molecules on the surface to accommodate the various receptors? One study used Jurkat T cells to attempt to quantitate the number of endogenous PtdSer molecules that may be exposed during apoptosis (Borisenko et al., 2003). They estimated that the exposure of PtdSer on live cells is minimal ( $<0.9$  picomoles/million cells); in contrast, upon induction of apoptosis by anti-Fas or camptothecin, the PtdSer on the outer leaflet of the membrane goes up to  $>240$  picomoles/million cells. Another type of calculation based on the surface area of lymphocytes suggests that there may be  $\sim 5$ – $10$  million PtdSer molecules exposed on an apoptotic thymocyte (unpublished data). Although the precise numbers of individual PtdSer recognition receptors expressed on a single phagocyte are not available, typical membrane receptor numbers are in the range of  $10^3$  surface molecules/cell (Zagursky et al., 1995); if engulfment receptors are expressed at typical membrane receptor levels, it is likely that the number of PtdSer molecules exposed on an apoptotic cell might be enough to engage multiple PtdSer receptors. Because there is a  $>280$ -fold difference between apoptotic and live cells with respect to PtdSer exposure, and this number is achieved very early in the apoptotic process (within 1–2 h after apoptosis induction; Borisenko et al., 2003), it was suggested that this huge difference in PtdSer exposure between live and apoptotic cells may provide the specificity for phagocyte recognition of an apoptotic cell. Moreover, a threshold increase of PtdSer molecules (eightfold over basal state) needs to be exposed on a dying cell before it is subject to macrophage-mediated phagocytosis (Borisenko et al., 2003); this presumably allows neighboring live cells that may randomly expose small amount of PtdSer (for biological reasons independent of apoptosis) to be spared.

Although the absolute number of PtdSer molecules exposed may be sufficient to engage available receptors, two other issues must be considered. First, the aforementioned calculations assume that PtdSer is free floating on the membrane of the apoptotic cell and that every PtdSer molecule is accessible for recognition by a receptor. However, we know relatively little about how PtdSer is actually displayed on the surface of an apoptotic cell. Some studies suggested clustering of PtdSer molecules on the surface of a dying cell (Gardai et al., 2005), but the size of the clusters and the number of molecules per cluster, and how universal this is among different cell types undergoing apoptosis, are not known. Clustering of PtdSer molecules would become particularly relevant if multiple receptors bind to the PtdSer molecules within a single cluster. How can the various receptors topologically arrange themselves on the surface of the phagocyte to engage PtdSer displayed as a cluster on the apoptotic cell surface? Moreover, studies of annexin V binding to PtdSer estimated that each molecule of annexin V may cover up or cast a shadow over  $\sim 50$  phospholipid molecules on the surface (Cézanne et al., 1999). If the PtdSer were to exist in clusters, then the effective density of exposed PtdSer on the apoptotic cell membrane may drop quickly. In other words, within a PtdSer cluster, the binding of one receptor and its footprint could sterically alter

the ability of other receptors to engage PtdSer on the apoptotic cell.

Another point to consider is that at least a fraction of the exposed PtdSer is oxidized or modified in some other way (Tyurin et al., 2008). Some PtdSer recognition mechanisms (such as CD36 and MFG-E8) may prefer oxidized PtdSer (Borisenko et al., 2004; Fadeel et al., 2007; Tyurin et al., 2008). If so, and if the modified and native PtdSer molecules are simultaneously displayed, this may dictate affinity/avidity differences among these receptors for an apoptotic cell. Despite the fundamental importance of this scientific problem, relatively few groups are pursuing questions on the display of PtdSer on apoptotic cells. Moreover, the mechanism by which an apoptotic cell is induced to expose its PtdSer (loosely called PtdSer flipping) is also not well understood (Ravichandran and Lorenz, 2007). The few recent studies of genes regulating PtdSer flipping to the outer leaflet in model organisms have been inconsistent (Wang et al., 2007; Züllig et al., 2007; Venegas and Zhou, 2007; Darland-Ransom et al., 2008). New and directed efforts toward defining the biochemical and mechanistic aspects of PtdSer exposure on apoptotic cells, and the physical nature of the PtdSer displayed on the surface (perhaps through the recent advances in structural, electron micrographic, and imaging tools) are critically needed.

#### Quantitative and qualitative differences among PtdSer receptors

Another question is whether one PtdSer receptor is equal to any other PtdSer receptor. Not all of these receptors are expressed at the same density on the macrophage surface. For example, TIM-4 appears to be much more abundant than BAI1 on macrophages, whereas Sertoli cells of the testes express more BAI1 than TIM-4; however, a key counter argument to this density issue is that blocking of individual receptors via antibodies or small interfering RNA seems to suggest that most of these receptors play at least some role.

In addition, not all PtdSer recognition receptors may bind PtdSer with the same affinity or avidity. If this were the case, if one receptor with a higher affinity made the initial contact with PtdSer, a second receptor with an inherently lower affinity may be better able to engage PtdSer in this context. Such a cooperative or sequential binding would also avoid the problem of each of these receptors competing for the same ligand, should they all bind with the same affinity. Unfortunately, our knowledge of hierarchy among these receptors is minimal. In fact, no systematic studies directly comparing the affinities of different receptors exist. The observation that there may be a threshold level of PtdSer that needs to be exposed before phagocytosis of a target is initiated (Borisenko et al., 2003) also points to possible hierarchical binding should there be differences in affinity between the PtdSer recognition receptors. The affinity of these receptors for native versus modified PtdSer molecules also needs to be considered; if certain receptors were to prefer a modified moiety (Borisenko et al., 2004; Fadeel et al., 2007; Tyurin et al., 2008), this would allow sequential and cooperative

binding between the same PtdSer recognition receptors on the same phagocyte.

What about the differences in signaling pathways induced by the various PtdSer recognition receptors? The model of “tethering and tickling” proposed by Peter Henson several years ago (Henson and Hume, 2006) suggests that in the context of multiple different engulfment receptors engaging varied ligands, some may only serve an adhesion function, whereas the others may mediate signaling. This could also be true among different receptors that engage PtdSer. For example, the cytoplasmic and transmembrane regions of TIM-4 appear dispensable for promoting engulfment of apoptotic cells (Park et al., 2009), suggesting that other membrane proteins may conduct signaling subsequent to PtdSer engagement on apoptotic cells. Similarly, CD36 has a 4-aa cytoplasmic tail and likely uses other membrane proteins (such as the vitronectin receptor) for signaling (Savill et al., 1992; Albert et al., 1998). Moreover, MER is a tyrosine kinase (Lemke and Rothlin, 2008), whereas BAI1 is a seven-transmembrane protein that signals via the ELMO–Dock180–Rac complex (Park et al., 2009). Therefore, the possibility exists that distinct types of signals emanate, allowing a phagocyte to distinguish a live versus apoptotic cell.

### Is PtdSer sufficient?

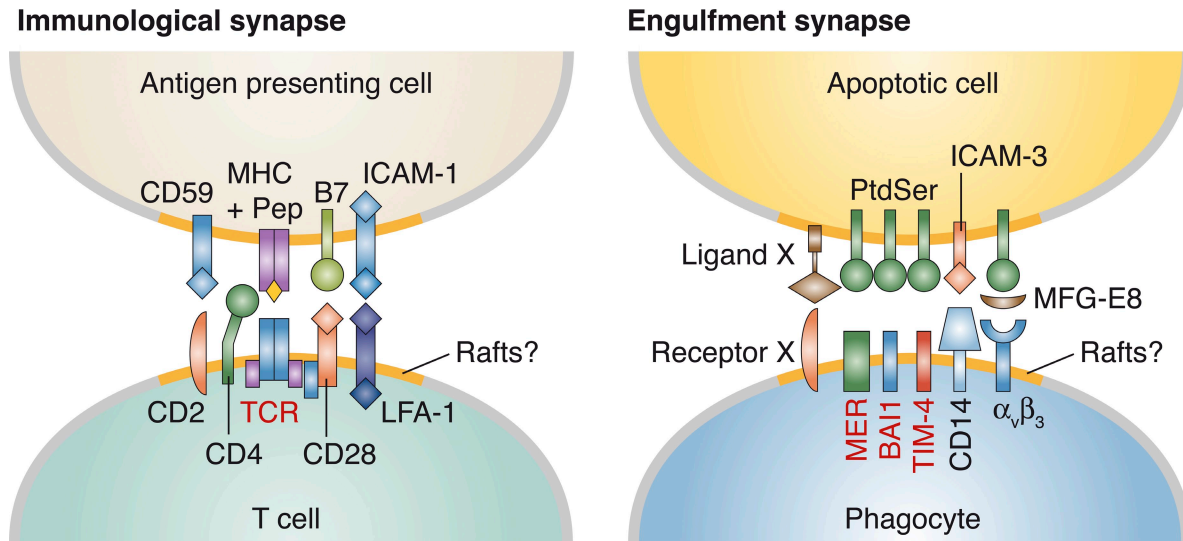
Based on the original work by Fadok et al. (1992), and subsequently confirmed by many groups, blocking PtdSer recognition via annexin V or other soluble agents that mask PtdSer potently inhibits engulfment of apoptotic cells (Fadok et al., 1998). In fact, no other ligand has been so consistently seen as relevant for engulfment in so many different contexts. This leads to the question of whether PtdSer is sufficient as an eat-me signal? One supportive argument is that PtdSer-containing liposomes were sufficient to induce responses mimicking those initiated by apoptotic cell recognition, such as antiinflammatory responses and cholesterol efflux from phagocytes (Fadok et al., 1998; Kiss et al., 2006). However, several counter arguments, albeit some anecdotal, also need to be considered. First, many cell types, such as macrophages and activated lymphocytes, routinely expose PtdSer at levels sufficient for detection, yet are spared from engulfment (Dillon et al., 2000; Hamon et al., 2000). Second, necrotic cells expose even greater levels of PtdSer (Borisenko et al., 2003), yet they elicit a different response from phagocytes than apoptotic cells (apoptotic cells are antiinflammatory, whereas necrotic cells are proinflammatory; Gallucci et al., 1999). Third, certain epithelial cells and non-professional phagocytes appear to engulf their apoptotic brethren much more efficiently than they engulf apoptotic thymocytes, although both target populations were comparably annexin V–positive (unpublished data). Previous studies have also suggested that in some cases, even when PtdSer is artificially incorporated into the outer leaflet of live cells, phagocytes do not engulf them (Borisenko et al., 2003). All of these observations suggest that ligands or binding parameters other than the exposure of PtdSer must be considered. The idea of additional ligands on apoptotic cells is not new, but their

characterization has lagged, perhaps because of the dominant role played by PtdSer in phagocytic recognition.

Could there be an additional ligand (denoted here as X), which works as a co-stimulatory ligand, such that phagocytes recognize cells displaying “PtdSer+X” as apoptotic cells (Fig. 3)? If one considers the analogy of a TCR interacting with a peptide+MHC complex (pMHC), it is well established that co-stimulatory molecules on the T cell surface (e.g., CD28) and their ligands on the APC surface (e.g., B7 family of molecules) critically regulate activation of naive T cells. In the absence of the co-stimulatory signals, even when the T cell and APC interact, the response of the T cell is stunted or aborted. Similarly, it is possible that X, coexpressed with PtdSer on the surface of apoptotic cells, may engage a counter receptor (the CD28 equivalent on the phagocyte), leading to a second signal. Such PtdSer+X recognition could be useful at several levels. First, it may provide additional specificity for PtdSer-dependent recognition and help phagocytes to avoid ingesting cells displaying PtdSer that was exposed independently of apoptosis. Second, engagement of PtdSer+X may help overcome don’t-eat-me signals simultaneously present on apoptotic cells. Third, X could help lower the threshold for PtdSer-dependent activation of phagocytes; e.g., an eightfold change in PtdSer levels is sufficient to promote macrophage phagocytosis of a target cell (Borisenko et al., 2003). Fourth, X, when engaged by its counter receptor on the phagocyte, may help modify the membrane curvature to allow proper apposition between the surfaces of the phagocytes and apoptotic cells. To date, there has only been one such second signal identified. Calreticulin was suggested by Gardai et al. (2005) as a possible second ligand that works together with PtdSer; although this study suggested a role for calreticulin in the system used, the calreticulin literature is rather varied depending on the nature of cell death and its immunogenicity (Gregory and Brown, 2005; Green et al., 2009; Zitvogel et al., 2010). Whether additional accessory molecules exist on the surface of early apoptotic cells (not secondarily necrotic), whether these are exposed basally or concurrent with PtdSer exposure, and what their receptors might be on the phagocyte remain to be determined. Recently, a phage display approach was used to identify additional PtdSer-binding proteins, although their relevance remains to be determined (Caberoy et al., 2009). Perhaps modification of such a method targeted toward identifying the second ligand could provide an enormous breakthrough in the field.

### Don't forget don't-eat-me signals

Although I primarily considered other molecules that may beneficially modify PtdSer recognition, we should not overlook “don’t-eat-me” signals, even when PtdSer is exposed. Presently, there is simply not enough data to know what the fold change in PtdSer exposure is on activated B or T lymphocytes or myoblasts that transiently expose PtdSer, and whether don’t-eat-me signals actively antagonize engulfment of these cells. With respect to don’t-eat-me signals, the best described to date are CD47 (Gardai et al., 2005, 2006) and



**Figure 3. A possible engulfment synapse during apoptotic cell clearance.** In a speculative model, an engulfment synapse may help phagocyte binding to and subsequent internalization of apoptotic target cells. There appears to be some clear similarities between T cell–APC recognition and phagocyte–apoptotic cell interactions (see text for more details). However, whereas the T cell–APC interaction is anchored by MHC+peptide interaction with the TCR, the PtdSer exposed on apoptotic cells perhaps may be more homogeneous (except for modifications such as oxidation, etc.), and is likely recognized by multiple PtdSer recognition receptors on the phagocyte. How the PtdSer is displayed on the apoptotic cells and how the different engulfment receptors are arranged on the phagocyte are not yet defined. One could speculate that a second signal analogous to B7 (ligand X) on the apoptotic cell may engage a co-stimulatory receptor analogous to CD28 (receptor X) on the phagocyte, thereby providing specificity, overcoming don't-eat-me signals, lowering the threshold for phagocyte priming, and/or altering membrane curvature.

CD31 (Brown et al., 2002). Because of the relevance of CD47 in red blood cells, the CD47 literature is clearly more advanced. Gardai et al. (2005) showed that CD47 could act as a potent don't-eat-me signal and inhibit engulfment of cells displaying PtdSer. They even suggested that in the absence of CD47, the nature of the PtdSer display might change. However, the extent of use of CD47 as a don't-eat-me signal by different cell types is unclear. At this point, only a handful of studies have systematically addressed don't-eat-me signals, and don't-eat-me mechanisms of action are not well understood. This may in part stem from technical issues. First, it is very difficult to use lack of engulfment as an assay, as in most typical phagocytosis assays the engulfment ranges from 5–50%. Second, given the current view that multiple ligands and receptors may be operational, it is difficult to systematically analyze such lack of engulfment in the eukaryotic context and define the role of individual biologically and functionally relevant molecules. However, model organisms, such as *C. elegans*, in which the death and uptake of single cells can be tracked in real time (Hoepfner et al., 2001), may prove useful for genetic studies of don't-eat-me signals, and in turn extend to mammalian systems.

**Concluding thoughts and future directions**

The engulfment field is relatively young; nevertheless, the appreciation that apoptotic cell clearance is a process relevant for many states of health and disease attracted many investigators from diverse fields, resulting in a significant increase in our knowledge during the past few years. But efforts to

address some key unanswered questions in the field may benefit from approaches that have proved highly useful in other systems dealing with recognition between two distinct cell types, such as T lymphocytes and APCs (Gascoigne et al., 2009; Fooksman et al., 2010; Reichardt et al., 2010; Fig. 3). The specific recognition of an apoptotic cell by a phagocyte is similar in several respects to T cell recognition of a pMHC complex on an APC. First, a T cell would have to recognize a specific antigen bearing APC among other APCs, analogous to a phagocyte trying to specifically recognize a dying cell among all the live cells within tissue or circulation. Second, like the T cell–APC interface, multiple receptor–ligand pairs are part of the phagocyte–apoptotic cell interface, although their roles are not well defined. Third, like T cells which can “sniff” many APCs without engaging in a strong interaction (Cahalan and Parker, 2005, 2008), macrophages engage live cells frequently, but quickly let them go because of the presence of don't-eat-me signals (Brown et al., 2002). Fourth, similar to a T cell–APC interaction where intracellular signals initiate T cell responses, the signals mediated during the phagocyte–apoptotic cell interaction also lead to the engulfment of the dying cell and secretion of antiinflammatory mediators, which are part of the immunologically silent clearance (Henson, 2005).

However, there are also several key differences, the most important being that the particular TCR–pMHC interaction critically anchors the T cell–APC conjugation. In the context of apoptotic cells, although PtdSer may be loosely considered as an equivalent of the pMHC, there is not an equivalent of

the TCR on the phagocytes. In contrast there seem to be multiple PtdSer recognition mechanisms. Furthermore, second signals analogous to those delivered by co-stimulatory molecules and accessory receptors in specific T cell–APC interactions have not yet been identified for the phagocyte–apoptotic cell interaction. One of the key reasons for our extensive knowledge in the T cell system is the existence of various monoclonal antibodies that were systematically generated toward various accessory and co-stimulatory molecules on T cells and APCs as part of the Cluster of Differentiation/Cell Differentiation Antigens/CD nomenclature initiative in the 1980s and early 1990s (Chorváth and Sedlák, 1998). Perhaps it may be time for the engulfment field to consider an analogous strategy. Although this may seem like rehashing of an old approach, this should not be dismissed, as our ability to clone and identify relevant molecules has improved tremendously because of current molecular and genetic tools. Another difference is that, contrary to a single cell type such as a T cell mediating pMHC recognition, the phagocyte can be essentially any cell in the body; nevertheless, a systematic effort with few defined phagocytic types could be hugely beneficial. In fact, one group used a version of this approach that led to the identification of TIM-4 as a PtdSer receptor (Miyaniishi et al., 2007). Also, such an effort could lead to identification of ligands of co-stimulatory molecules that work together with PtdSer.

Another area where the T cell–APC interaction is hugely advanced is in the understanding of the immunological synapse and the recruitment of molecules in and out of the synapse over the duration of T cell–APC contact (Gascoigne et al., 2009; Fooksman et al., 2010; Reichardt et al., 2010). Unfortunately, such detailed information simply does not exist for the phagocyte–apoptotic cell interface. An exciting possibility is that an engulfment synapse is formed by clustering of specific molecules on the surface of the phagocyte and the apoptotic cell. Given the recent advances in imaging, exemplified by elegant studies of the T cell–APC conjugate formation (Cahalan and Parker, 2008), a systematic effort toward defining how both direct and indirect PtdSer recognition receptors are topologically positioned on the phagocyte and how they move during recognition would help identify hierarchy and/or cooperative binding among the different receptors. Similarly, defining how PtdSer itself is displayed and moves during engagement by recognition receptors, as well as the relative localization of don't-eat-me signals, is critically needed. Moreover, simultaneous engagement of two receptors by ligands on apoptotic cells could alter the response; for example, MER can modify inflammatory responses via other receptors (Rothlin et al., 2007; Lemke and Rothlin, 2008).

Besides being fun basic science questions, these could have important implications for medicine. Failed clearance of apoptotic cells is a feature of diseases ranging from autoimmunity and atherosclerosis to neurological disorders (Elliott and Ravichandran). Apoptotic cell–based therapies are beginning to emerge (Wang et al., 2006, 2009; Green et al., 2009), but we really do not fully comprehend what constitutes

immunogenic versus nonimmunogenic cell death (Green and Kroemer, 2005; Green et al., 2009) and how this can be manipulated in a therapeutically beneficial way. Detailed understanding of how a phagocyte encounters and engulfs an apoptotic cell could be used to increase engulfment in situations deemed to be deficiencies of engulfment. Moreover, defining the subtleties of multiple receptors engaging the same or similar ligands may help us understand how the post-engulfment responses of phagocytes (such as antiinflammatory mediator signaling) may be modified. This type of information could be important for designing drugs that could trigger engulfment receptors to mimic physiological engulfment; the antiinflammatory mediators elicited by these drugs could be useful for resolving inflammation in many different conditions and treating autoimmunity.

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