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Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways

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Abstract

Prompt and efficient clearance of apoptotic cells is necessary to prevent secondary necrosis of dying cells, and to avoid immune responses to autoantigens. Recent studies have shed light on how apoptotic cells through soluble 'find-me signals' advertise their presence to phagocytes at the earliest stages of cell death. Phagocytes sense the find-me signal gradient, and in turn the presence of dying cells, and migrate to their vicinity. The apoptotic cells also expose specific eat-me signals on their surface that are recognized by phagocytes through specific engulfment receptors. This review covers the recent progress in the areas of find-me and eat-me signals, and how these relate to prompt and immunologically silent clearance of apoptotic cells.

We turnover billions of cells as part of normal homeostasis everyday (~1 million cells per second) (Henson, 2005; Nagata et al., 2010). The cells that are targeted for turnover can fall into several classes (Ravichandran and Lorenz, 2007). Excess cells are often generated as part of normal development or tissue maintenance, e.g. in the thymus during maturation of T cells, in the bone marrow during generation of neutrophils and other myeloid cells, in the testes during generation of germ cells, involution of mammary gland post lactation, or in specific niches of the brain as part of adult neurogenesis. Only a few of these newly generated cells survive or are considered fit enough to mature further, and the rest die and their corpses need to be cleared. Another situation requiring corpse removal is that many aged or used cells are removed on a daily basis in many tissues; some examples include red blood cells, and 'spent' rod cells in the eye. Also, damaged cells arise in many tissues due to either encounter with the environment (e.g. airway epithelial cells that come across noxious substances in polluted air), ultraviolet radiation (skin), or DNA damage as part of meiosis (germ cells). The dying cells are quickly recognized and removed by 'phagocytes, which can either be neighboring healthy cells or professional phagocytes recruited to sites of apoptotic cell death.

Over the past two decades, enormous strides have been made on various mechanisms of programmed cell death at the level of the dying cell (Gregory and Pound, 2010; Nagata et al., 2010). However, *in vivo*, the death process is closely linked to the removal of the corpses (Gregory and Pound, 2010). Failure to properly clear dying cells has been linked to non-resolving inflammation, many autoimmune conditions and developmental abnormalities

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(Hanayama et al., 2006; Munoz et al.; Nagata et al., 2010; Nathan and Ding, 2010). It could be argued that prompt, efficient and immunologically silent clearance of dying cells is perhaps the ultimate goal of the cell death program. It is noteworthy that many modalities of cell death have been described depending on morphological, biochemical and other features of the dying cells including caspase-dependent and independent apoptosis, regulated necrosis, and cell death associated with autophagy, and mitotic catastrophe (Galluzzi et al., 2011). However, depending on the nature of induction of death, the cell type and tissue context, a dying cell may show distinct, as well as overlapping or mixed features of cell death (Galluzzi et al., 2009). Since we have learned relatively more about removal of cells dying via caspase-dependent apoptosis, this review primarily focuses on our understanding clearance of these apoptotic cells.

Several distinguishable steps have been recognized in the engulfment and clearance of apoptotic cells. The phagocytes and the dying cells have to be in proximity, which is achieved through ‘find-me’ signals (Peter et al., 2009); the phagocytes then specifically engage the dying cells, achieved via eat-me signals on dying cells and receptors for the eat-me signals on phagocytes (Gardai et al., 2006); the phagocytes then physically engulf the dying cells through signaling induced by engulfment receptors and cytoskeletal reorganization of the phagocyte membrane (Ravichandran and Lorenz, 2007); and lastly, the corpse contents have to be processed within the phagocytes through phagolysosomal processing (Kinchen et al., 2008; Kinchen and Ravichandran, 2008; Park et al., 2011; Zhou and Yu, 2008). In the recent years, several new discoveries have been reported on all of the above aspects of engulfment. This review specifically covers recent discoveries that pertain to find-me and eat-me signals and how they relate to engulfment.

Find-me signals – the altruistic suicide notes

How do apoptotic cells advertise their presence?

It has long been observed by many investigators that even in tissues with known high turnover of cells (e.g. thymus, bone marrow, or testes) few apoptotic cells are seen in the steady state. However, when the apoptotic cell clearance process is disrupted, for example through genetic ablation of engulfment genes or pharmacological approaches that inhibit cell clearance, there is an accumulation of uncleared corpses that becomes readily apparent (Elliott et al., 2010; Henson, 2005; Lu et al., 2011; Nagata et al., 2010). This suggests that in the steady state the apoptotic cell clearance machinery is quick and efficient, such that the dying cells were sensed, recognized and cleared quickly. To use an analogy, we do not appreciate the garbage collector until there is a disruption in trash collection and we begin to notice the unpleasant odor. Similarly, as long as the engulfment machinery is intact and functional, uncleared apoptotic cells are not evident; however, disruption of cell clearance leads to accumulation of secondarily necrotic corpses in tissues often associated with autoimmunity toward self antigens derived from the uncleared corpses (Franz et al., 2006). This implies that the apoptotic cells must somehow ‘advertise’ their presence at the earliest stages of death to promote clearance. The very close link between cells beginning to die and their quick and efficient removal came first came from genetic studies in *C. elegans*. Using a mutant background where the caspase-mediated apoptosis was partially impaired, two groups (Hoeppner et al., 2001; Reddien et al., 2001) elegantly demonstrated that even under these partial death circumstances, if the engulfment machinery was intact, these ‘partially dead’ cells are efficiently recognized and removed; however, when the engulfment machinery is also impaired, then the partially dead cells abort the death program. This strongly suggests that the phagocytes are extremely efficient in sensing and detecting the dying cells at the earliest stages of apoptosis, and contribute to the ‘final stages’ of death.

In the above mentioned *C. elegans* studies, the phagocytes are often the healthy neighbors. In the context of mammalian cells, this is often not the case. For example, in the thymus, a dying thymocyte is unlikely to be engulfed by a neighboring healthy thymocyte (since immature thymocytes lack the cytoplasmic volume and cytoskeletal machinery to ingest another cell of the similar size); rather a resident macrophage or a dendritic cell within the thymus is more likely to mediate the clearance (Ravichandran, 2003). In such a case, the dying cell must be able to advertise its state of death and in turn recruit a phagocyte to its proximity. This led to the concept of soluble 'find-me' signals being released by apoptotic cells. Such soluble mediators would then set up a gradient within the tissue to attract the phagocytes to the proximity of the dying cells. The current notion is that the find-me signals, released by the dying cells at the earliest stages of apoptosis, would then be sensed by the phagocytes via receptors, and subsequent signaling within the phagocytes would induce the migration to the proximity of the dying cells (Peter et al., 2010).

Specific find-me signals, their release from apoptotic cells, and sensing by phagocytes

In the past few years, four possible find-me signals released from apoptotic cells have been reported. These include: the lipid lysophosphatidylcholine (LPC); sphingosine 1-phosphate (S1P); the fractalkine CX3CL1; and the nucleotides ATP and UTP (Figure 1). Remarkably, these are different types of molecules, yet they are all linked to monocyte or macrophage recruitment toward apoptotic cells. The discussion below focuses on what we currently know about the generation and release of these find-me signals, how phagocytes sense them, and whether or not they have been proven to be of relevance *in vivo*.

Lauber et al identified LPC as a find-me signal released by MCF-7 breast cancer cells based on the ability of their apoptotic cell supernatants to attract the THP-1 monocytic cell line (Lauber et al., 2003). Few other cell lines and different modes of apoptosis could lead to the release of LPC, but the MCF-7 cells were the best characterized. The authors also went on to show elegantly that the calcium-independent phospholipase A2 (iPLA2), upon cleavage by caspases, is likely responsible for the release of LPC from the dying cells (Lauber et al., 2003). This study brought the idea of find-me signals to the fore and that caspase-dependent release of soluble mediators could act as find-me signal to recruit monocytes from circulation in to tissues with apoptotic cells. However, a number of interesting questions were raised by this work. The first is the challenge of LPC setting up a concentration gradient. The concentration of LPC released by MCF-7 cells (at least as detected in the assay system) is small (Lauber et al., 2003). Since there is a high concentration of LPC in the serum or plasma (100μM), it is unclear how LPC can set up a gradient to attract phagocytes specifically to the proximity of apoptotic cells. While high concentration of LPC could be present locally near a dying cell and thereby still provide the gradient, higher LPC concentrations cause lysis of many cells. Perhaps LPC is not recognized in its native form, but rather is bound to other serum components (and therefore effectively unavailable), further modified in tissues, or LPC may function together with another soluble mediator(s). The second challenge with LPC as a find-me signal is that G2A (Peter et al., 2008), the receptor that is linked to LPC recognition by phagocytes, has been controversial and the validity of the original identification of G2A as the receptor LPC is questionable (Witte et al., 2005). Therefore, the specific receptor on phagocytes that mediates LPC dependent movement toward apoptotic cells remains to be determined. The third challenge is that the role of LPC as a find-me signal *in vivo* is not yet established. While the original studies clearly show that LPC can act as a find-me signal *in vitro* (Lauber et al., 2003), complementary *in vivo* studies of LPC dependent recruitment of phagocytes to apoptotic cells remain to be established.

The soluble molecule sphingosine 1-phosphate (S1P)has also been proposed as a find-me signal (Gude et al., 2008; Weigert et al., 2010). Gude et al suggested that induction of

apoptosis results in upregulation of S1P kinase 1 (SphK1) (Gude et al., 2008). The increased SphK1 was then linked to generation of S1P, and in turn S1P-dependent recruitment of macrophages to the apoptotic cell supernatants. However, a second study suggests that S1P kinase 2 (SphK2) itself is a target of caspase 1, and that the cleaved fragment of SphK2 could be 'released' from dying cells into the extracellular space where it would generate S1P (Weigert et al., 2010). Given the well-known role of S1P as a migratory signal, and the pharmacological interest in modifying S1P based signals, this observation is potentially very interesting. However, a number of key issues need to be resolved. First, all of the work to date suggesting S1P as a find-me signal has been done *in vitro*, and the relevance of S1P in recruiting phagocytes to apoptotic cells *in vivo* has not been determined. Staurosporine-induced cell death has been shown to induce caspase-1 and in turn cleavage of SphK2 (Weigert et al., 2010). However, caspase-1 is normally not induced during other forms of apoptosis and the mechanism for S1P generation remains to be better defined. With respect to sensing of S1P, it can be recognized by the receptors $S1P_1$ through $S1P_5$, yet which of these G-protein coupled receptors (GPCRs) are relevant for phagocytic recruitment to apoptotic cells is not known. Both sphingosine kinase 1 and 2 have been linked to S1P generation during apoptosis (Gude et al., 2008; Weigert et al., 2010), but presumably by distinct mechanisms: SphK1 protein level being increased during apoptosis versus cleavage of SphK2 by caspases. Since genetically targeted mice exist for both *Sphk1* and *Sphk2*, the specific enzyme question as well as the relevance of S1P as find-me signal should be addressable in an organismal context. Collectively, while S1P could serve as a find-me signal *in vitro*, its relevance *in vivo*, the receptors on phagocytes that help mediate recruitment to apoptotic cells, and the relative importance of S1P compared to other find-me signals need to be determined.

In addition to the two lipid mediators referred to above, a soluble fragment of fractalkine (CX3CL1) protein can also serve as a find-me signal for monocytes (Truman et al., 2008). Chris Gregory and colleagues demonstrated that a soluble chemokine fragment of fractalkine, which is normally on the plasma membrane and serves as an intercellular adhesion molecule, is released as a 60kDa fragment during apoptosis and can act as a chemoattractant (Truman et al., 2008). CX3CL1 release is caspase dependent, although it may not be direct (the cleavage being more likely done by extracellular ADAM protease family members). Truman et al also suggested that fractalkine (CX3CL1) is released as part of microparticles from early stages of apoptotic Burkitt Lymphoma cells (which are the malignant version of the germinal center B cells). CX3CR1, the receptor for CX3CL1, appeared important in sensing the chemokine and for inducing monocyte migration both *in vitro* and *in vivo*. Mice lacking CX3CR1 show a defect in the migration of macrophages to the germinal centers, where a high rate of apoptosis takes place. While the authors clearly acknowledge that the fractalkine might be more specific for Burkitt Lymphoma or germinal center B cells, this work through its *in vivo* studies using CX3CR1 establish fractalkine (CX3CL1) as a *bona fide* find-me signal.

The latest to join the find-me signal mediators are the nucleotides found in the supernatants of apoptotic cells. Elliott et al. demonstrated that the regulated release of triphosphate nucleotides ATP and UTP from early apoptotic cells can potently attract monocytes both *in vitro* and *in vivo* (Elliott et al., 2009). Nucleotide release was shown to occur in Jurkat cells, primary thymocytes as well as MCF-7 cells and lung epithelial cells after apoptosis induction via multiple modalities (crosslinking Fas, UV-treatment, etoposide etc.) and is dependent on caspase activity. Interestingly, less than 2% of the cellular ATP is released from early stage apoptotic cells when their plasma membrane is still intact. The released nucleotides in the apoptotic cell supernatants are 'chemotactic' for monocytes (i.e. inducing directional migration) rather than promoting 'chemokinesis' (i.e. random migration).

Importantly, the apoptotic cell supernatants injected in to a mouse dorsal airpouch could preferentially attract monocytes and this recruitment is abolished when the triphosphonucleotides from the supernatants were degraded (Elliott et al., 2009). Furthermore, using clearance of dying thymocytes (after Dexamethasone-induced apoptosis in the thymus) as a readout, enzymatically ablating the nucleotides, or blocking nucleotide recognition by phagocytes results in uncleared corpses in this *in vivo* model. The sensing of the released extracellular nucleotides was shown to involve the P2Y family of nucleotide receptors; more specifically, the P2Y2 member is relevant for sensing of nucleotides by monocytes both *in vitro* and in the context of P2Y2 genetically ablated mice. Collectively, these data identified release of the nucleotides ATP and UTP by early stage apoptotic cells and their sensing via P2Y2 on monocytes, and that this find-me signal circuit is relevant for apoptotic cell clearance *in vivo*.

As with other studies, a number of challenges remain in defining the role of nucleotides as a find-me signal. Extracellular nucleotides are often degraded by nucleotide triphosphatases (NTPases) (Knowles; Schetinger et al., 2007). Given the small amount of ATP released (about 100nM or ~2%), it is unclear how a nucleotide gradient would be established and what distances such a gradient may be able to 'travel' to attract phagocytes. The *in vivo* data in the airpouch model and the thymocyte clearance model suggest that a relevant gradient can be established by this amount of ATP and recognized by monocytes (Elliott et al., 2009), but the distance for this 'final call' remains unknown. Also, the NTPdases are expressed at different amounts in various tissues (Knowles; Schetinger et al., 2007), and therefore, may further regulate the distance for the attraction signal. It is noteworthy however, that diphosphate nucleotides (ADP or UDP), monophosphate (AMP) or adenosine show very poor chemotactic activity, suggesting that it is the triphosphates that were acting as find-me signals (Elliott et al., 2009). Another challenge is that while *P2ry2* ablated mice clearly show a defect in attracting monocytes (Elliott et al., 2009), there was still residual migration in these mice, suggesting other P2Y family members may also play a role. Furthermore, the signaling downstream of the P2Y receptors and the specific signaling that induces migration of monocytes remains to be determined. Nevertheless, nucleotides represent one of the better-understood find-me signals at this point.

In addition to the above four, the release of ribosomal protein S19 (which dimerizes during apoptosis) and binds to the complement receptor C5a r on monocytes has been suggested as a find-me signal (Yamamoto, 2007). However, it appears that S19 is likely released at very late stages of apoptosis. Also, a fragment of the tyrosyl tRNA synthetase (EMAPII) released from apoptotic cells has been shown to attract monocytes (Shalak et al., 2001). Surprisingly, EMAPII has inflammatory properties and also attracts (and activates) neutrophils. The neutrophil attraction and why this would be the case is unclear.

How are the find-me signals released?

One of the key features of find-me signals is that they are released when the apoptotic cells are still intact such that the released mediators can attract the phagocytes, which in turn would clear the apoptotic cells before they become secondarily necrotic. This implies that there has to be mechanisms in place that allow the direct or indirect generation and release of find-me signals that are coupled to induction of apoptosis. At present, we do not know how LPC is released from dying cells. The generation of S1P as reported by Weigert et al appears to involve caspase-1 dependent release of a fragment of Sphingosine kinase 2 (SphK2), which in turn would generate S1P. During apoptosis, a specific cleavage site in SphK2 results in a fragment of this enzyme retaining its enzymatic property. It was also suggested that this cleaved fragment of SphK2, presumably by binding to phosphatidylserine (PtdSer) on the inner leaflet of the plasma membrane, could be 'released' from dying cells when the PtdSer is flipped to the outer leaflet during apoptosis. SphK2

release into the extracellular space would then generate S1P. The timing of caspase dependent cleavage of SphK2 versus the exposure of phosphatidylserine (PtdSer), and the generality of this mechanism for other forms of apoptosis remain to be determined. For fractalkine release, Truman et al suggested that the 60kDa fragment of fractalkine was released as microparticles from early stages of apoptotic Burkitt Lymphoma cells. The microparticles were annexin V positive and suggested that they may be generated at the same time as PtdSer exposure.

The mechanism of nucleotide release during apoptosis has been better defined (Chekeni et al., 2010). Nucleotides are released through PANX1, a member of the pannexin family of channels on the plasma membrane. PANX1 is a four transmembrane protein (likely functioning as homo-hexamers) forming large pores that allow molecules up to 1 kDa to pass through the plasma membrane(D'Hondt et al., 2009). Silencing of PANX1 expression results in loss of nucleotide release from apoptotic cells, while overexpression of PANX1 strongly enhances the nucleotide release during apoptosis (Chekeni et al., 2010), and results in better recruitment of monocytes. Moreover, PANX1 currents are detectable upon induction of apoptosis, and this correlates with plasma membrane permeability. The uptake of dyes such as YO-PRO1 (and TO-PRO3), which have long been used to track plasma membrane permeability of early apoptotic cells, is dependent on PANX1 (Chekeni et al., 2010). Mechanistically, it was demonstrated that the C-terminal tail of PANX1 itself is a target of caspsase dependent cleavage (by caspases 3 and 7). Mutation of the cleavage site within PANX1 results in failure of nucleotide release, while truncation of PANX1 at the cleavage site leads to a constitutively active protein (Chekeni et al., 2010). In subsequent work, Vishva Dixit and colleagues demonstrated that cells from mice with a genetic ablation of *Panx1* are defective in nucleotide release during apoptosis as well as in recruiting macrophages (Qu et al., 2011). Thus, a model has emerged where apoptotic cells release the nucleotides ATP and UTP at the earliest stages of apoptosis via caspase dependent cleavage and opening or activation of PANX1 (Figure 2). These nucleotides are then be sensed by P2Y2 (and possibly other P2Y family members) and lead to migration of monocytes to the proximity of apoptotic cell clearance.

Other roles for find-me signals besides phagocyte attraction?

In addition to establishing a chemotactic gradient to aid in the location of the dying cells, find-me signals may also modulate the phagocytic activity of cells in the direct vicinity of the apoptotic cells. This becomes relevant when one considers situations where the dying cell is likely eaten by a neighbor (not a recruited phagocyte), yet releases find-me signals. For example, apoptotic airway epithelial cells release nucleotides (Elliott et al., 2009), yet neighboring epithelial cells can engulf other dying epithelial cells without any migration to the prey. This raises the question whether find-me signals have other roles besides recruiting phagocytes. An interesting possibility is that the find-me signals such as nucleotides or S1P could ‘activate’ or ‘prime’ phagocytes and improve their phagocytic capacity. In flies, apoptosis in the context of a tissue enhances the capacity of engulfment by the neighboring cells – for example, by inducing the upregulation of the engulfment machinery (MacDonald et al., 2006; Ziegenfuss et al., 2008). In mammals, the ‘bridging’ molecule MFG-E8 (which binds to PtdSer on apoptotic cells and facilitate engulfment by engaging the integrin $\alpha_v\beta_3$ on phagocytes, see below), is expressed by activated but not resting macrophages (Hanayama et al., 2002) and it has been demonstrated that the find-me signal fractalkine can induce production of the bridging molecule MFG-E8 by macrophages (Miksa et al., 2007). One possibility is that the find-me signals are sort of like a ‘smell of food’ that increases the ‘appetite’ of the phagocyte by upregulating the engulfment machinery components. If this were to be applicable to other cell types beyond macrophages, such as epithelial cells, perhaps the find-me signals do not quite act as the final call for a phagocyte, but rather help

boost the engulfment machinery in the neighbor and thereby actively promote its own clearance.

ATP has been typically thought of as a danger signal (Trautmann, 2009), and has been linked to recruitment of neutrophils during inflammatory conditions and for inducing anti-tumor immunity (Aymeric et al., 2010). Yet, apoptotic cell supernatants appear to preferentially recruit monocytes over neutrophils *in vivo* (Elliott et al., 2009), and the apoptotic cell clearance is usually anti-inflammatory and immunologically silent (Fadok et al., 1998b; Voll et al., 1997). There are perhaps several differences between the regulated release of nucleotides during apoptosis, and cytolysis (such as necrosis). Less than 2% of total cellular ATP is released via PANX1 by early apoptotic cells (Chekeni et al., 2010; Elliott et al., 2009), while damage-induced loss of membrane integrity may release a much higher quantity of nucleotides. Also, the notion of ATP as an inflammatory molecule derives from its ability to activate the ionotropic nucleotide receptor P2X7, which in turn results in activation of the inflammasome and release of pro-inflammatory cytokines (Bours et al., 2006; Di Virgilio, 2007). In fact, ATP derived from necrotic cells has been shown to result in sterile inflammation via inflammasome activation (Iyer et al., 2009). However, much higher ATP concentrations are necessary for P2X7 activation ($EC_{50} > 100\mu M$) than those necessary for activation of the receptors mediating monocyte chemotaxis (such as P2Y2; $EC_{50} < 1\mu M$) (Bours et al., 2006; Trautmann, 2009). Although early in vitro studies link ATP release via Panx1 to P2X7 activation, Dixit and colleagues have now clearly shown (using *Panx1* ablated mice) that Panx1 is dispensable for P2X7 and inflammasome activation (Qu et al., 2011). Interestingly, lower concentrations of ATP have been shown to have an anti-inflammatory effect by suppressing the secretion of inflammatory cytokines, while promoting the release of anti-inflammatory cytokines (Di Virgilio, 2007; Hasko et al., 2000; la Sala et al., 2001; Wilkin et al., 2002). Therefore, the concept of ATP as a universal danger signal might be too simplistic. How nucleotides and other find-me signals may influence immunogenic versus non-immunogenic responses to apoptotic cells remains to be better examined (Green et al., 2009; Zitvogel et al.).

‘Keep-out’ signals

The apoptotic cell clearance process is generally anti-inflammatory or non-phlogistic, with recruitment predominantly of monocytes and minimal neutrophils or inflammatory cells. Yet, none of the find-me signals discussed above are specific for monocytes alone. In fact, nucleotides have been long known to facilitate migration of neutrophils (directly or indirectly). Yet, when apoptotic cell supernatants are injected in the airpouch model, monocytes are predominantly recruited in a nucleotide-dependent manner, with minimal recruitment of neutrophils; under the same assay when bacterial lipopolysaccharide (LPS) is injected, neutrophils are predominantly recruited (Elliott et al., 2009). How is this possible? Gregory and colleagues have made an interesting observation that apoptotic cell supernatants also contain ‘keep-out’ signals for neutrophils (Bournazou et al., 2009). Specifically, they identified that lactoferrin is released from apoptotic cells (in a caspase-dependent process) and that lactoferrin can act as a potent stop signal for neutrophil migration toward many other chemokines. Lactoferrin does not inhibit monocyte migration under these conditions. Although the receptor(s) and the intracellular signals that mediate the neutrophil migration arrest are not known, this work suggested that apoptotic cells release both find-me signals and keep-out signals that preferentially recruit monocytes and/or facilitate the non-inflammatory clearance of the dying cells (Figure 3).

Eat-me signals - the beginnings of a good apoptotic meal

While the find-me signals help bring phagocytes to the proximity of the dying cell, the phagocyte still would have to specifically identify the dying cell among the sea of living

ones. This requires that the apoptotic cells express specific markers on their surface ('eat-me' signals) for recognition via specific receptors on the phagocytes. By analogy, while the find-me signals are similar to a fire alarm that brings the fire rescue squad to the street where a particular house is on fire, pinpointing the house in trouble would require either detecting the fire itself or the smoke (i.e. eat-me signals). At present many eat-me signals have been recognized as being exposed on the surface of apoptotic cells. These include exposure of PtdSer, changes in charge and glycosylation patterns on the cell surface, alteration in intercellular adhesion molecule-1 (ICAM-1) epitopes on the cell surface, and exposure of the endoplasmic reticulum resident protein, calreticulin (Lauber et al., 2004; Nagata et al., 2010).

Among these, the exposure of PtdSer on the outer leaflet of the plasma membrane has received the most attention (Fadok et al., 2000; Fadok et al., 1992). In fact, PtdSer exposure is *the* most universally seen alteration on the surface of apoptotic cells, observed in many different cell types after multiple modalities of apoptosis induction. PtdSer exposure is perhaps the generally accepted definition for calling a cell 'apoptotic' (Fadok et al., 1998a). However, as discussed below, PtdSer exposure alone may not be sufficient and PtdSer may work in conjunction with other eat-me markers to facilitate specific recognition of an apoptotic cell by a phagocyte. In the section below, I have detailed some of the recent information on PtdSer exposure and its recognition by engulfment receptors. For a more thorough discussion on all of the other eat-me signals, the reader is referred to some of the other more detailed reviews (Gardai et al., 2006; Lauber et al., 2004; Nagata et al., 2010; Savill et al., 2002).

PtdSer exposure - common principles and some complications

PtdSer is normally found on the inner leaflet of the plasma membrane in healthy cells with very little exposure on the outer leaflet of most healthy cells (Fadok et al., 1998a; Fadok et al., 1992). However, when the ells are induced to undergo apoptosis, there is a dramatic change in the amounts of PtdSer exposed on the outer leaflet (Fadok et al., 1998a; Fadok et al., 1992). One of the fascinating things about PtdSer exposure is that it occurs very early during the apoptosis program (i.e. on apoptotic cells that retain their membrane integrity) and is readily detectable using many commercially available preparations of annexin V, as well as antibodies that recognize PtdSer head group. Valerian Kagan colleagues quantitated the number of endogenous PtdSer molecules exposed on Jurkat T cells during apoptosis (Borisenko et al., 2003). While live cells have minimal PtdSer (<0.9picomoles/million cells) Jurkat cells undergoing apoptosis (either by anti-Fas or camptothecin), displayed >240 picomoles/million cells of PtdSer on the surface. This translates to roughly >280 fold increase between apoptotic and live cells with respect to PtdSer exposure, and this is achieved very early in the apoptotic process (1-2 hours after apoptosis induction) (Borisenko et al., 2003). This change in PtdSer concentrations on the outer leaflet is proposed to provide the 'specificity' for phagocyte recognition of an apoptotic cell. Remarkably, masking phosphatidylserine on apoptotic cells either through soluble annexin V of soluble fragments of engulfment receptors or bridging molecules that recognize PtdSer (see below) blocks phagocytosis of apoptotic cells. Since many cell types expose PtdSer during apoptosis and blocking PtdSer attenuates engulfment, it has clearly been established that phosphatidylserine represents an essential eat-me signal for clearance of apoptotic cells.

However, the question that has repeatedly arisen over the recent years is whether phosphatidylserine exposure is sufficient for phagocytic uptake (Ravichandran, 2010). In early studies, since liposomes containing PtdSer are taken up preferentially (over phosphatidylcholine) and could elicit certain responses of apoptotic cells from macrophages, phosphatidylserine exposure along was considered sufficient. However, some other studies have also suggested that even when PtdSer is artificially incorporated into the outer leaflet

of live cells, phagocytes fail to engulf them (Borisenko et al., 2003). Interestingly, many cell types such as macrophages and activated lymphocytes routinely expose PtdSer sufficient for detection (via annexin V), yet are spared from engulfment (Dillon et al., 2000; Hamon et al., 2000). Most recently, Nagata and colleagues identified a new calcium dependent phospholipid scramblase (TMEM16F) that could promote the exposure of PtdSer on live BaF3 cells (in the presence of calcium addition) (Suzuki et al., 2010); however, despite the PtdSer exposure, these cells are not taken up by macrophages. This suggests phosphatidylserine alone is not sufficient. The simplest interpretation of the data is that PtdSer on apoptotic cells is recognized by phagocytes in conjunction with one or more of the other eat-me signals exposed on apoptotic cells. What these other eat-me signals are remain to be precisely established. To date, there has only been one such second signal identified. Calreticulin was suggested by Gardai and Henson as a possible second ligand that works together with PtdSer (Gardai et al., 2005), but this remains to be better defined. However, calreticulin can also be recognized directly, and the literature reporting the exposure of this molecule is variable depending on the nature of cell death and its immunogenicity (Green et al., 2009; Gregory and Brown, 2005; Zitvogel et al.).

There are also other possibilities for why forced expression of PtdSer on the outer leaflet may not be sufficient for engulfment. It appears that a threshold increase of PtdSer molecules (8 fold over basal state) needs to be exposed before an apoptotic cell becomes a target for macrophage-mediated phagocytosis (Borisenko et al., 2003). We do not have enough quantitative information on the number of PtdSer molecules exposed by activated lymphocytes or in BaF3 cells overexpressing TMEM16F (Suzuki et al., 2010). Perhaps the lower exposure of PtdSer on live cells allows neighboring live cells that may randomly expose small amount of PtdSer (for biological reasons independent of apoptosis) to be spared. Another important consideration is that live cells express ‘don't eat-me signals’ that may need to be turned off during apoptosis to allow recognition by phagocytes. For example, CD47 and CD31 have both been independently suggested to inhibit engulfment of healthy cells (Brown et al., 2002; Gardai et al., 2005). The presence or absence of CD47 has also been reported to even alter the display of PtdSer on the cell surface. Another point to consider is that during apoptosis, at least a fraction of the exposed PtdSer may be oxidized or modified in other ways (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 this were the case, then live cells that expose phosphatidylserine (independent of apoptosis) or support the synthetic conditions discussed above may not be comparable to exposure of phosphatidylserine by apoptotic cells (Appelt et al., 2005). Therefore, the threshold of phosphatidylserine exposure, other eat-me markers on the dying cells, as well as the don’t eat-me signals could all regulate how phosphatidylserine is recognized by phagocytes. It is important to note that these are not mutually exclusive possibilities and could all contribute to the specific phosphatidylserine-dependent recognition of apoptotic cells by phagocytes.

Engulfment receptors

Studies from a number of laboratories over many years have identified a multitude of engulfment receptors on phagocytes that can recognize the various eat-me signals exposed on apoptotic cells. These include Scavenger receptors, lectins, complement receptors, oxidized low density lipoprotein (ox-LDL) recognizing receptors, CD14, CD36, CD68, LDL-receptor related protein, tyrosine kinases of the Tyro3-Axl-Mer family, as well as several other recent PtdSer recognition receptors (see below). Why we need so many receptors is an open question. The consensus in the field has been that not all receptors are expressed on a given phagocyte, and therefore multiple modes of recognition are necessary (Henson et al., 2001a; Savill and Fadok, 2000). Similarly, depending on the nature of the

induction of apoptosis, there may be differences in exposure of eat-me signals that in turn may dictate the usage of particular combination of engulfment receptors by a given phagocyte. A number of years ago, Peter Henson suggested a model of 'tethering and tickling' that in the context of multiple different engulfment receptors binding to varied ligands on apoptotic cells, some may only serve an adhesion function, while the others may mediate signaling (similar to the situation that is well established when T cells engage an antigen presenting cell).

Over the past decade, there has been a significant change in the thinking about how phagocytes recognize PtdSer on apoptotic cells. It was initially thought that there would be a 'single' PtdSer recognition receptor universally used by all phagocytes (Henson et al., 2001b). Now it appears that there are multiple distinct receptors on phagocytes capable of recognizing PtdSer (Bratton and Henson, 2008). The PtdSer recognition receptors appear to come in two kinds – those that are membrane proteins that can directly bind and recognize PtdSer, and those that indirectly recognize PtdSer through binding of soluble bridging molecules (Figure 4). The PtdSer receptors capable of directly recognizing PtdSer include members of the T cell immunoglobulin and mucin (TIM)family (TIM-4, as well as TIM-1 and TIM-3) (DeKruyff et al. 2011; Ichimura et al., 2008; Kobayashi et al., 2007; Miyanishi et al., 2007; Rodriguez-Manzanet et al.; Santiago et al., 2007; Wong et al. 2010), brain angiogenesis inhibitor 1 (BAI1) that is a seven transmembrane protein belonging to the adhesion type II GPCR family (Park et al., 2007a), and the atypical epidermal growth factor (EGF)-motif containing membrane protein Stabilin-2 (Park et al., 2007b).

With respect to indirect recognition of PtdSer on apoptotic cells, the soluble protein MFG-E8 has garnered the most attention recently (Akakura et al., 2004; Asano et al., 2004; Hanayama and Nagata, 2005; Hanayama et al., 2002; Hanayama et al., 2004; Thorp and Tabas, 2009). One region of MFG-E8 can bind PtdSer on apoptotic cells with high affinity (Hanayama et al., 2002; Hanayama et al., 2004), while a second region of MFG-E8 can simultaneously engage integrin $\alpha_v\beta_3$ on phagocytes; thus, through this bridging function, MFG-E8 can mediate PtdSer-dependent uptake of apoptotic cells. *In vitro* studies coupled with *in vivo* studies in mouse models have now established a definitive role for MFG-E8 as an important bridging molecule. Interestingly, MFG-E8 is not expressed on resting macrophages, but upregulated in activated macrophages. As discussed above, perhaps find-me signals may 'promote' expression of MFG-E8 on macrophages. Two other bridging molecules, Gas6 and Protein S, can also recognize PtdSer exposed on apoptotic cells; they are in turn recognized by Tyro-3-Axl-Mer family of receptors (denoted as TAM receptors) on phagocytes (Lemke and Rothlin, 2008; Rothlin et al., 2007; Scott et al., 2001). An important role for this family has been established through single and combined genetic ablation of the TAM family members in mice; these receptors appear important for clearance of apoptotic cells in the eye, testes, thymus and other tissues (Lemke and Rothlin, 2008; Prasad et al., 2006). In addition to the above 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 (Balasubramanian and Schroit, 1998; Imachi et al., 2000; Mevorach, 2000; Savill et al., 1991; Savill et al., 1992).

How these various receptors signal intracellularly to mediate the physical rearrangement of the phagocyte cytoskeleton to facilitate corpse uptake is not fully defined. It appears that there is considerable variation even among receptors recognizing a single ligand PtdSer. The cytoplasmic and transmembrane regions of TIM-4 appear not necessary for TIM-4-mediated apoptotic cell uptake (Park et al., 2009), suggesting that TIM-4 may only serve as 'tethering' function and use other membrane receptors for signaling. Similarly, CD36 has a short cytoplasmic tail (four amino acids) and likely uses other membrane proteins (such as the

vitronectin receptor) for signaling (Albert et al., 1998; Savill et al., 1992). BAI1 is a 7-transmembrane protein that signals via the evolutionarily conserved ELMO1-Dock180-Rac complex (Park et al., 2009) to promote cytoskeletal rearrangement. Therefore, the possibility exists that distinct types of signals emanate within the phagocytes that may help signal amplification as well as allow a phagocyte to distinguish between live and apoptotic cells.

Future challenges

In summary, over the past few years, significant progress has been in made in defining find-me and eat-me signals of apoptotic cells and how they regulate cell clearance *in vivo*. The new knowledge has also raised a number of new questions or challenges, some of which are discussed below.

While the concept that the early steps of apoptosis and phagocytosis are intimately tied (i.e. two sides of the same coin) originated from the *C. elegans* studies to date no find-me signals have been identified in either *C. elegans* or Drosophila. Since neighboring cells usually mediate engulfment of dying cells in *C. elegans*, perhaps the motility of phagocytes is not necessary. However, the hemocytes and glia in Drosophila are motile and therefore find-me signals could be relevant. The advantage of the model organisms is that the number of genes within a functional pathway are often less redundant, thereby making genetic manipulations of a find-me signal and determining effects at an organism level likely more amenable. In contrast, we already know of 4 or more find-me signals in the mammalian system and redundancy among them is inevitable. Also, the possibility that find-me signals (such as nucleotides, S1P, or fractalkine) could also activate the engulfment machinery is quite attractive, as this may be more relevant for situations where the apoptotic cells may need to boost the engulfment capacity of a neighboring healthy cell. Testing this possibility initially in model organisms and further in the mammalian system (e.g. lung or gut epithelial cells) could prove interesting. Also, while the concept that nucleotides can be released via pannexin channels during apoptosis is well established, it is unclear how apoptotic cells release other find-me signals or the keep-out signals (such as lactoferrin, a much larger molecule). Since both find-me and eat-me signals are found in the same apoptotic cell supernatants, distinct yet cooperative mechanisms must exist and these remain to be defined. Also, we have relatively little knowledge of find-me signals that may be released in types of cell death other than apoptosis and how other types of cell death (e.g. necrosis, pyroptosis, cell death associated with autophagy etc.) attract phagocytes.

With respect to eat-me signals, although phosphatidylserine as a key recognition molecule on apoptotic cells is established beyond doubt, we still have relatively low resolution on the topology of phosphatidylserine exposed on the apoptotic cells, and how it is engaged by specific engulfment receptors. It is also puzzling that we have so many engulfment receptors that can recognize phosphatidylserine, yet whether they simultaneously, sequentially, or cooperatively bind the exposed PtdSer on apoptotic cells is unclear. If these receptors do bind simultaneously to PtdSer on apoptotic cells, then how do they avoid competition among each other and how are steric issues of recognition resolved? We also do not have much information on whether PtdSer recognition receptors function as monomers, dimers or higher order oligomers during PtdSer recognition. Intriguingly, some of the engulfment receptors also appear to have a soluble version (comprising the region of PtdSer binding), and whether these soluble fragments are decoys or promoters of engulfment remains to be determined.

While the eat-me signals on ‘early’ apoptotic cells have been the focus here, clearly apoptotic cells that escape detection by the engulfment machinery could undergo secondary necrosis. The precise molecular features of late apoptotic cells and necrotic cells, and how

the phagocytes may engage them are just beginning to emerge (Beer et al., 2008; Franz et al., 2007; Peter et al., 2010). For example, certain carbohydrate moieties on the late apoptotic cells appear to progressively change and they also tend to expose molecules generally considered intracellular (such as endoplasmic reticulum components) (Beer et al., 2008; Franz et al., 2007). Similarly, annexin I, exposed on secondarily necrotic cells appears to block inflammatory responses of phagocytes (Blume et al., 2009). How early apoptotic cells progress to secondarily necrotic cells, how the pattern of eat-me signals is modified on their surface, and which phagocyte receptors would mediate their recognition remain to be determined. Another area to be explored further is the type of eat-me signals exposed on cells undergoing different types of cell death and how phagocytes might recognize such eat-me signals.

Lastly, the signaling via the engulfment receptors need to be more systematically analyzed and compared, as this would have direct relevance for defining the anti-inflammatory cytokine production downstream of apoptotic cell recognition. The latter is obviously important for both understanding autoimmune diseases that arise as a consequence of failed corpse clearance, as well as a therapeutic approach to dampening unwanted inflammation in many disease states.

References

Akakura S, Singh S, Spataro M, Akakura R, Kim JI, Albert ML, Birge RB. The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. Exp Cell Res. 2004; 292:403–416. [PubMed: 14697347]

Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, Bhardwaj N. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J Exp Med. 1998; 188:1359–1368. [PubMed: 9763615]

Appelt U, Sheriff A, Gaipl US, Kalden JR, Voll RE, Herrmann M. Viable, apoptotic and necrotic monocytes expose phosphatidylserine: cooperative binding of the ligand Annexin V to dying but not viable cells and implications for PS-dependent clearance. Cell Death Differ. 2005; 12:194–196. [PubMed: 15540112]

Asano K, Miwa M, Miwa K, Hanayama R, Nagase H, Nagata S, Tanaka M. Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. J Exp Med. 2004; 200:459–467. [PubMed: 15302904]

Aymeric L, Apetoh L, Ghiringhelli F, Tesniere A, Martins I, Kroemer G, Smyth MJ, Zitvogel L. Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. Cancer Res. 2010; 70:855–858. [PubMed: 20086177]

Balasubramanian K, Schroit AJ. Characterization of phosphatidylserine-dependent beta2-glycoprotein I macrophage interactions. Implications for apoptotic cell clearance by phagocytes. J Biol Chem. 1998; 273:29272–29277. [PubMed: 9786940]

Beer A, Andre S, Kaltner H, Lensch M, Franz S, Sarter K, Schulze C, Gaipl US, Kern P, Herrmann M, Gabius HJ. Human galectins as sensors for apoptosis/necrosis-associated surface changes of granulocytes and lymphocytes. Cytometry A. 2008; 73:139–147. [PubMed: 18186087]

Blume KE, Soeroes S, Waibel M, Keppeler H, Wesselborg S, Herrmann M, Schulze-Osthoff K, Lauber K. Cell surface externalization of annexin A1 as a failsafe mechanism preventing inflammatory responses during secondary necrosis. J Immunol. 2009; 183:8138–8147. [PubMed: 20007579]

Borisenko GG, Iverson SL, Ahlberg S, Kagan VE, Fadeel B. Milk fat globule epidermal growth factor 8 (MFG-E8) binds to oxidized phosphatidylserine: implications for macrophage clearance of apoptotic cells. Cell Death Differ. 2004; 11:943–945. [PubMed: 15031725]

Borisenko GG, Matsura T, Liu SX, Tyurin VA, Jianfei J, Serinkan FB, Kagan VE. Macrophage recognition of externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells--existence of a threshold. Arch Biochem Biophys. 2003; 413:41–52. [PubMed: 12706340]

Bournazou I, Pound JD, Duffin R, Bournazos S, Melville LA, Brown SB, Rossi AG, Gregory CD. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. J Clin Invest. 2009; 119:20–32. [PubMed: 19033648]

Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC. Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther. 2006; 112:358–404. [PubMed: 16784779]

Bratton DL, Henson PM. Apoptotic cell recognition: will the real phosphatidylserine receptor(s) please stand up? Curr Biol. 2008; 18:R76–79. [PubMed: 18211846]

Brown S, Heinisch I, Ross E, Shaw K, Buckley CD, Savill J. Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment. Nature. 2002; 418:200–203. [PubMed: 12110892]

Chekeni FB, Elliott MR, Sandilos JK, Walk SF, Kinchen JM, Lazarowski ER, Armstrong AJ, Penuela S, Laird DW, Salvesen GS, et al. Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis. Nature. 2010; 467:863–867. [PubMed: 20944749]

D'Hondt C, Ponsaerts R, De Smedt H, Bultynck G, Himpens B. Pannexins, distant relatives of the connexin family with specific cellular functions? Bioessays. 2009; 31:953–974. [PubMed: 19644918]

DeKruyff RH, Bu X, Ballesteros A, Santiago C, Chim YL, Lee HH, Karisola P, Pichavant M, Kaplan GG, Umetsu DT, et al. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. J Immunol. 2011; 184:1918–1930. [PubMed: 20083673]

Di Virgilio F. Liaisons dangereuses: P2X(7) and the inflammasome. Trends Pharmacol Sci. 2007; 28:465–472. [PubMed: 17692395]

Dillon SR, Mancini M, Rosen A, Schlissel MS. Annexin V binds to viable B cells and colocalizes with a marker of lipid rafts upon B cell receptor activation. J Immunol. 2000; 164:1322–1332. [PubMed: 10640746]

Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature. 2009; 461:282–286. [PubMed: 19741708]

Elliott MR, Zheng S, Park D, Woodson RI, Reardon M, Juncadella IJ, Kinchen JM, Zhang J, Lysiak J, Ravichandran KS. Unexpected requirement for ELMO1 in apoptotic germ cell clearance in vivo. Nature. 2010; 467:333–337. [PubMed: 20844538]

Fadeel B, Quinn P, Xue D, Kagan V. Fat(al) attraction: oxidized lipids act as “eat-me” signals. HFSP J. 2007; 1:225–229. [PubMed: 19404423]

Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ. 1998a; 5:551–562. [PubMed: 10200509]

Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest. 1998b; 101:890–898. [PubMed: 9466984]

Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J Biol Chem. 2001; 276:1071–1077. [PubMed: 10986279]

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol. 1992; 148:2207–2216. [PubMed: 1545126]

Franz S, Gaipl US, Munoz LE, Sheriff A, Beer A, Kalden JR, Herrmann M. Apoptosis and autoimmunity: when apoptotic cells break their silence. Curr Rheumatol Rep. 2006; 8:245–247. [PubMed: 16839503]

Franz S, Herrmann K, Furnrohr BG, Sheriff A, Frey B, Gaipl US, Voll RE, Kalden JR, Jack HM, Herrmann M. After shrinkage apoptotic cells expose internal membrane-derived epitopes on their plasma membranes. Cell Death Differ. 2007; 14:733–742. [PubMed: 17170754]

Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, Bazan NG, Blagosklonny MV, Blomgren K, Borner C, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ. 2009; 16:1093–1107. [PubMed: 19373242]

Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2011. Cell Death Differ. 2011 Jul 15. doi: 10.1038.

Gardai SJ, Bratton DL, Ogden CA, Henson PM. Recognition ligands on apoptotic cells: a perspective. J Leukoc Biol. 2006; 79:896–903. [PubMed: 16641135]

Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenborg PA, Michalak M, Henson PM. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell. 2005; 123:321–334. [PubMed: 16239148]

Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nat Rev Immunol. 2009; 9:353–363. [PubMed: 19365408]

Gregory CD, Brown SB. Apoptosis: eating sensibly. Nat Cell Biol. 2005; 7:1161–1163. [PubMed: 16319968]

Gregory CD, Pound JD. Cell death in the neighbourhood: direct microenvironmental effects of apoptosis in normal and neoplastic tissues. J Pathol. 2010; 223:177–194. [PubMed: 21125674]

Gude DR, Alvarez SE, Paugh SW, Mitra P, Yu J, Griffiths R, Barbour SE, Milstien S, Spiegel S. Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a “come-and-get-me” signal. FASEB J. 2008; 22:2629–2638. [PubMed: 18362204]

Hamon Y, Broccardo C, Chambenoit O, Luciani MF, Toti F, Chaslin S, Freyssinet JM, Devaux PF, McNeish J, Marguet D, Chimini G. ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. Nat Cell Biol. 2000; 2:399–406. [PubMed: 10878804]

Hanayama R, Miyasaka K, Nakaya M, Nagata S. MFG-E8-dependent clearance of apoptotic cells, and autoimmunity caused by its failure. Curr Dir Autoimmun. 2006; 9:162–172. [PubMed: 16394660]

Hanayama R, Nagata S. Impaired involution of mammary glands in the absence of milk fat globule EGF factor 8. Proc Natl Acad Sci U S A. 2005; 102:16886–16891. [PubMed: 16275924]

Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. Nature. 2002; 417:182–187. [PubMed: 12000961]

Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, Nagata S. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. Science. 2004; 304:1147–1150. [PubMed: 15155946]

Hasko G, Kuhel DG, Salzman AL, Szabo C. ATP suppression of interleukin-12 and tumour necrosis factor-alpha release from macrophages. Br J Pharmacol. 2000; 129:909–914. [PubMed: 10696089]

Henson PM. Dampening inflammation. Nat Immunol. 2005; 6:1179–1181. [PubMed: 16369556]

Henson PM, Bratton DL, Fadok VA. Apoptotic cell removal. Curr Biol. 2001a; 11:R795–805. [PubMed: 11591341]

Henson PM, Bratton DL, Fadok VA. The phosphatidylserine receptor: a crucial molecular switch? Nat Rev Mol Cell Biol. 2001b; 2:627–633. [PubMed: 11483996]

Hoeppner DJ, Hengartner MO, Schnabel R. Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans. Nature. 2001; 412:202–206. [PubMed: 11449279]

Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. J Clin Invest. 2008; 118:1657–1668. [PubMed: 18414680]

Imachi H, Murao K, Hiramine C, Sayo Y, Sato M, Hosokawa H, Ishida T, Kodama T, Quehenberger O, Steinberg D, Takahara J. Human scavenger receptor B1 is involved in recognition of apoptotic thymocytes by thymic nurse cells. Lab Invest. 2000; 80:263–270. [PubMed: 10701695]

Iyer SS, Pulskens WP, Sadler JJ, Butter LM, Teske GJ, Ulland TK, Eisenbarth SC, Florquin S, Flavell RA, Leemans JC, Sutterwala FS. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. Proc Natl Acad Sci U S A. 2009; 106:20388–20393. [PubMed: 19918053]

Kinchen JM, Doukoumetzidis K, Almendinger J, Stergiou L, Tosello-Trampont A, Sifri CD, Hengartner MO, Ravichandran KS. A pathway for phagosome maturation during engulfment of apoptotic cells. Nat Cell Biol. 2008; 10:556–566. [PubMed: 18425118]

Kinchen JM, Ravichandran KS. Phagosome maturation: going through the acid test. Nat Rev Mol Cell Biol. 2008; 9:781–795. [PubMed: 18813294]

Knowles AF. The GDA1_CD39 superfamily: NTPDases with diverse functions. Purinergic Signal. 7:21–45. [PubMed: 21484095]

Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, Butte MJ, Nagumo H, Chernova I, Zhu B, et al. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. Immunity. 2007; 27:927–940. [PubMed: 18082433]

la Sala A, Ferrari D, Corinti S, Cavani A, Di Virgilio F, Girolomoni G. Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. J Immunol. 2001; 166:1611–1617. [PubMed: 11160202]

Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the corpses. Mol Cell. 2004; 14:277–287. [PubMed: 15125832]

Lauber K, Bohn E, Krober SM, Xiao YJ, Blumenthal SG, Lindemann RK, Marini P, Wiedig C, Zobywalski A, Baksh S, et al. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell. 2003; 113:717–730. [PubMed: 12809603]

Lemke G, Rothlin CV. Immunobiology of the TAM receptors. Nat Rev Immunol. 2008; 8:327–336. [PubMed: 18421305]

Lu Z, Elliott MR, Chen Y, Walsh JT, Klibanov AL, Ravichandran KS, Kipnis J. Phagocytic activity of neuronal progenitors regulates adult neurogenesis. Nat Cell Biol. 2011 Aug 2. ePub.

MacDonald JM, Beach MG, Porpiglia E, Sheehan AE, Watts RJ, Freeman MR. The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons. Neuron. 2006; 50:869–881. [PubMed: 16772169]

Mevorach D. Opsonization of apoptotic cells. Implications for uptake and autoimmunity. Ann N Y Acad Sci. 2000; 926:226–235. [PubMed: 11193038]

Miksa M, Amin D, Wu R, Ravikumar TS, Wang P. Fractalkine-induced MFG-E8 leads to enhanced apoptotic cell clearance by macrophages. Mol Med. 2007; 13:553–560. [PubMed: 17673941]

Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. Nature. 2007; 450:435–439. [PubMed: 17960135]

Munoz LE, Lauber K, Schiller M, Manfredi AA, Herrmann M. The role of defective clearance of apoptotic cells in systemic autoimmunity. Nat Rev Rheumatol. 6:280–289. [PubMed: 20431553]

Nagata S, Hanayama R, Kawane K. Autoimmunity and the clearance of dead cells. Cell. 2010; 140:619–630. [PubMed: 20211132]

Nathan C, Ding A. Nonresolving inflammation. Cell. 2010; 140:871–882. [PubMed: 20303877]

Park D, Han CZ, Elliott MR, Kinchen JM, Trampont PC, Das S, Collins S, Lysiak JJ, Hoehn KL, Ravichandran KS. Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein. Nature. 2011 Aug 23. ePub.

Park D, Hochreiter-Hufford A, Ravichandran KS. The phosphatidylserine receptor TIM-4 does not mediate direct signaling. Curr Biol. 2009; 19:346–351. [PubMed: 19217291]

Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, Klibanov AL, Mandell JW, Ravichandran KS. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature. 2007a; 450:430–434. [PubMed: 17960134]

Park SY, Jung MY, Kim HJ, Lee SJ, Kim SY, Lee BH, Kwon TH, Park RW, Kim IS. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. Cell Death Differ. 2007b

Peter C, Waibel M, Radu CG, Yang LV, Witte ON, Schulze-Osthoff K, Wesselborg S, Lauber K. Migration to apoptotic “find-me” signals is mediated via the phagocyte receptor G2A. J Biol Chem. 2008; 283:5296–5305. [PubMed: 18089568]

Peter C, Wesselborg S, Herrmann M, Lauber K. Dangerous attraction: phagocyte recruitment and danger signals of apoptotic and necrotic cells. Apoptosis. 2010; 15:1007–1028. [PubMed: 20157780]

Peter C, Wesselborg S, Lauber K. Molecular suicide notes: last call from apoptosing cells. J Mol Cell Biol. 2009; 2:78–80. [PubMed: 20008330]

Prasad D, Rothlin CV, Burrola P, Burstyn-Cohen T, Lu Q, Garcia de Frutos P, Lemke G. TAM receptor function in the retinal pigment epithelium. Mol Cell Neurosci. 2006; 33:96–108. [PubMed: 16901715]

Qu Y, Misaghi S, Newton K, Gilmour LL, Louie S, Cupp JE, Dubyak GR, Hackos D, Dixit VM. Pannexin-1 is required for ATP release during apoptosis but not for inflammasome activation. J Immunol. 2011; 186:6553–6561. [PubMed: 21508259]

Ravichandran KS. “Recruitment signals” from apoptotic cells: invitation to a quiet meal. Cell. 2003; 113:817–820. [PubMed: 12837239]

Ravichandran KS. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. J Exp Med. 2010; 207:1807–1817. [PubMed: 20805564]

Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. Nat Rev Immunol. 2007; 7:964–974. [PubMed: 18037898]

Reddien PW, Cameron S, Horvitz HR. Phagocytosis promotes programmed cell death in C. elegans. Nature. 2001; 412:198–202. [PubMed: 11449278]

Rodriguez-Manzanet R, Sanjuan MA, Wu HY, Quintana FJ, Xiao S, Anderson AC, Weiner HL, Green DR, Kuchroo VK. T and B cell hyperactivity and autoimmunity associated with niche-specific defects in apoptotic body clearance in TIM-4-deficient mice. Proc Natl Acad Sci U S A. 2010; 107:8706–8711. [PubMed: 20368430]

Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. Cell. 2007; 131:1124–1136. [PubMed: 18083102]

Santiago C, Ballesteros A, Martinez-Munoz L, Mellado M, Kaplan GG, Freeman GJ, Casasnovas JM. Structures of T cell immunoglobulin mucin protein 4 show a metal-Ion-dependent ligand binding site where phosphatidylserine binds. Immunity. 2007; 27:941–951. [PubMed: 18083575]

Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol. 2002; 2:965–975. [PubMed: 12461569]

Savill J, Fadok V. Corpse clearance defines the meaning of cell death. Nature. 2000; 407:784–788. [PubMed: 11048729]

Savill J, Hogg N, Haslett C. Macrophage vitronectin receptor, CD36, and thrombospondin cooperate in recognition of neutrophils undergoing programmed cell death. Chest. 1991; 99:6S–7S.

Savill J, Hogg N, Ren Y, Haslett C. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. J Clin Invest. 1992; 90:1513–1522. [PubMed: 1383273]

Schetinger MR, Morsch VM, Bonan CD, Wyse AT. NTPDase and 5′-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors. 2007; 31:77–98. [PubMed: 18806312]

Scott RS, McMahon EJ, Pop SM, Reap EA, Caricchio R, Cohen PL, Earp HS, Matsushima GK. Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature. 2001; 411:207–211. [PubMed: 11346799]

Shalak V, Kaminska M, Mitnacht-Kraus R, Vandenabeele P, Clauss M, Mirande M. The EMAPII cytokine is released from the mammalian multisynthetase complex after cleavage of its p43/proEMAPII component. J Biol Chem. 2001; 276:23769–23776. [PubMed: 11306575]

Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. Nature. 2010; 468:834–838. [PubMed: 21107324]

Thorp E, Tabas I. Mechanisms and consequences of efferocytosis in advanced atherosclerosis. J Leukoc Biol. 2009

Trautmann A. Extracellular ATP in the immune system: more than just a “danger signal”. Sci Signal. 2009; 2:6.

Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, Melville L, Melrose LA, Ogden CA, Nibbs R, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood. 2008; 112:5026–5036. [PubMed: 18799722]

Tyurin VA, Tyurina YY, Kochanek PM, Hamilton R, DeKosky ST, Greenberger JS, Bayir H, Kagan VE. Oxidative lipidomics of programmed cell death. Methods Enzymol. 2008; 442:375–393. [PubMed: 18662580]

Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature. 1997; 390:350–351. [PubMed: 9389474]

Weigert A, Cremer S, Schmidt MV, von Knethen A, Angioni C, Geisslinger G, Brune B. Cleavage of sphingosine kinase 2 by caspase-1 provokes its release from apoptotic cells. Blood. 2010; 115:3531–3540. [PubMed: 20197547]

Wilkin F, Stordeur P, Goldman M, Boeynaems JM, Robaye B. Extracellular adenine nucleotides modulate cytokine production by human monocyte-derived dendritic cells: dual effect on IL-12 and stimulation of IL-10. Eur J Immunol. 2002; 32:2409–2417. [PubMed: 12207325]

Witte ON, Kabarowski JH, Xu Y, Le LQ, Zhu K. Retraction. Science. 2005; 307:206. [PubMed: 15653487]

Wong K, Valdez PA, Tan C, Yeh S, Hongo JA, Ouyang W. Phosphatidylserine receptor Tim-4 is essential for the maintenance of the homeostatic state of resident peritoneal macrophages. Proc Natl Acad Sci U S A. 2010; 107:8712–8717. [PubMed: 20421466]

Yamamoto T. Roles of the ribosomal protein S19 dimer and the C5a receptor in pathophysiological functions of phagocytic leukocytes. Pathol Int. 2007; 57:1–11. [PubMed: 17199736]

Zhou Z, Yu X. Phagosome maturation during the removal of apoptotic cells: receptors lead the way. Trends Cell Biol. 2008; 18:474–485. [PubMed: 18774293]

Ziegenfuss JS, Biswas R, Avery MA, Hong K, Sheehan AE, Yeung YG, Stanley ER, Freeman MR. Draper-dependent glial phagocytic activity is mediated by Src and Syk family kinase signalling. Nature. 2008; 453:935–939. [PubMed: 18432193]

Zitvogel L, Kepp O, Kroemer G. Decoding cell death signals in inflammation and immunity. Cell. 2010; 140:798–804. [PubMed: 20303871]

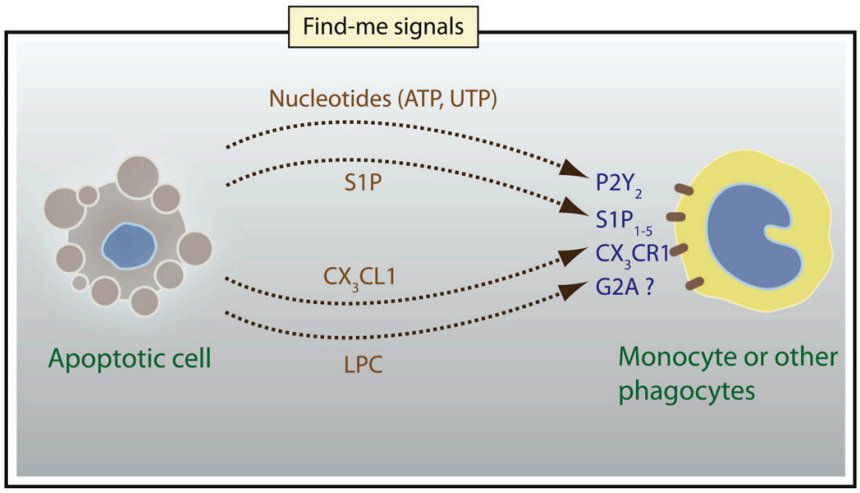

Figure 1. Find-me signals in phagocyte attraction

The known find-me signals released by apoptotic cells are depicted. Of these, the nucleotides and P2Y2, and the CX3CL1 and CX3CR1 modules have been shown to be operational *in vivo*, but the roles of S1P and LPC remain to be established in an *in vivo* model. The receptor LPC, although suggested to be G2A, is unclear and remains to be proven.

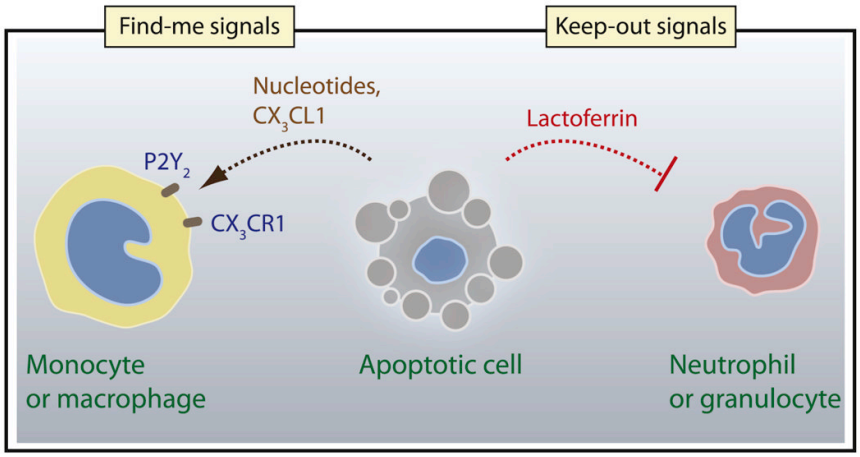

Figure 2. A balance between find-me signals and keep-out signals

Apoptotic cells release both find-me and keep-out signals that help preferentially recruit monocytes or non-inflammatory cells over neutrophils. Lactoferrin is identified as one of the keep-out signals. It remains to be determined whether lactoferrin may be 'disabled' in necrotic cells that promote inflammation.

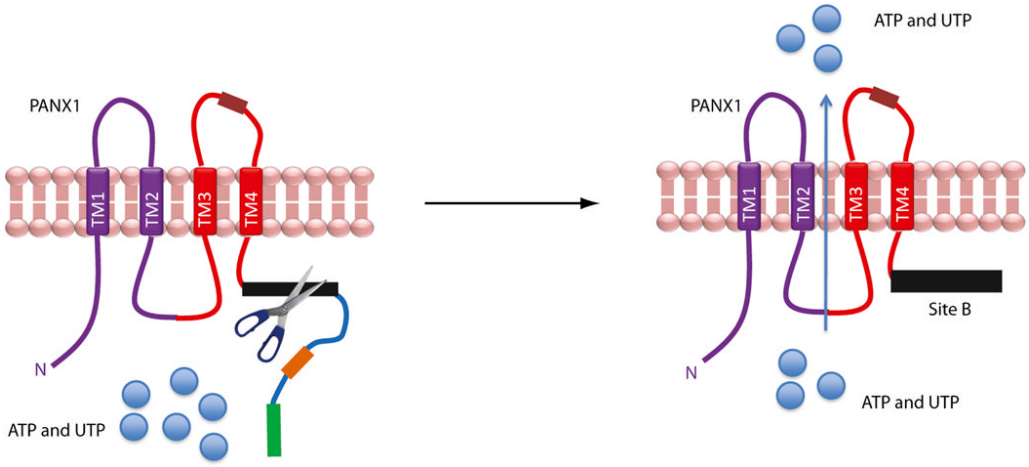

Figure 3. PANX1 dependent release of nucleotides from apoptotic cells

A specific proteolytic site within the cytoplasmic tail of PANX1 has been identified as a target of executioner caspases 3 and 7 during apoptosis (scissors). This leads to opening of the channel and the release of nucleotides ATP and UTP from the apoptotic cells. Please note that PANX1 is thought to function as a hexamer and that the multi-subunit complex forms a channel through which nucleotides are released across the plasma membrane.

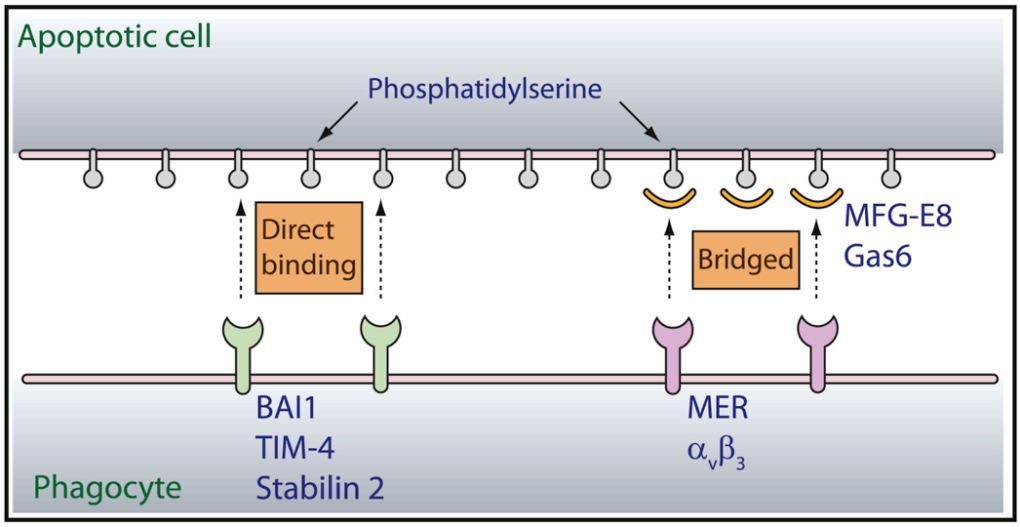

Figure 4. Direct and indirect recognition of phosphatidylserine on apoptotic cells

Two modes of recognition of PtdSer exposed on apoptotic cells involve the direct recognition by phagocytic receptors such as BAI1, TIM-4 and Stabilin 2, and the indirect recognition where bridging molecules such as MFG-E8 and Gas6 bind PtdSer that are in turn recognized by membrane proteins such as MER and $\alpha_v\beta_3$. The two modes of recognition are not mutually exclusive and likely occur simultaneously. Also, for the sake of clarity only the PtdSer recognition is shown here, but there are number of other ligands on apoptotic cells and their cognate receptors on phagocytes have also been identified that may also participate in the specific recognition process.