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Phagocytosis of apoptotic cells in homeostasis

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Our bodies collectively turnover about 200–300 billion cells every day. Such turnover is an integral part of embryonic and postnatal development, as well as routine tissue homeostasis. This process involves induction of programmed cell death in specific cells within the tissues, and the specific recognition and removal of dying cells by a clearance crew composed of the professional, non-professional, and specialized phagocytes. In the past few years, remarkable progress has been made in uncovering many features of apoptotic cell clearance. Some of these new observations challenge the way we view dying cells themselves, and how healthy cells interact with and respond to dying cells. Here, we focus on the homeostatic removal of apoptotic cells in tissues.

Among the different forms of cell death, caspase-dependent apoptosis is thought to account for the majority of homeostatic cellular turnover[1]. Apoptosis is characterized by cell rounding and shrinking, chromatin condensation, and the formation of plasma membrane blebs or apoptotic bodies[2]. Apoptotic cell death helps to eliminate cells that are old or no longer needed, without causing damage to the surrounding tissues or initiating an immune response. As part of routine homeostasis, different tissues turnover varying numbers of apoptotic cells, with some tissues undergoing an impressive rate of renewal: hematopoiesis produces billions of cells daily, many with short lifespans (such as neutrophils); epithelial cells of the gastrointestinal tract, which cover an area equivalent in size to a tennis court, are turned over every 4–5 days; in the thymus and the bone marrow, millions of thymocytes and immature B cells, respectively, are eliminated during maturation; in the brain, adult neurogenesis produces thousands of new neurons daily, but only a few survive; and in the testes, spermatogenesis produces millions of germ cells, of which many undergo apoptosis. In addition, there are increased homeostatic turnovers under certain conditions, such as during involution of the mammary gland post-lactation and weaning[3]. In some situations, pieces of cells (rather than whole cells) are phagocytosed, for example during neuronal pruning. Finally, there are situations where the number of apoptotic cells increases beyond the normal rate within a given tissue, such as during an infection or acute tissue injury.

In these contexts, disposal of apoptotic cells needs to be performed quickly and without eliciting inflammation in the local tissue milieu[2, 4]. Under homeostatic conditions, the tissue

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resident phagocytes mediate the corpse removal. In cases of increased cell death, due to infections (epithelial cell apoptosis during lung infections) or sustained ‘sterile’ inflammation (atherosclerotic plaques), corpse clearance is mediated both by resident phagocytes and those recruited from the circulation. Failures in clearing apoptotic cells at early stages of death and their progression to a secondary necrotic state can induce tissue inflammation due to the release of cellular contents or exposure of otherwise sequestered intracellular moieties[2]. The critical decision of whether or not to initiate an immune response to the dying cell is made by the cell clearance machinery, in response to molecules released by and/or exposed on the dying cells. The phagocyte ultimately responds by actively suppressing or eliciting inflammation[2, 4].

Phagocyte types and the tissue contexts

Homeostatic corpse removal within a tissue is determined by the composition of the local ‘clearance crew’. Phagocytes that ingest apoptotic cells have been previously divided into ‘professional’ and ‘non-professional’ phagocytes. Based on existing evidence we suggest a third category, ‘specialized’ phagocytes’ (Fig. 1).

Professional phagocytes include macrophages and immature dendritic cells. Macrophages have long been known as professional engulfers of apoptotic cells due to their high capacity for engulfment *in vitro* and *in vivo*[5, 6]. Although early studies used macrophages of different sources (native, thioglycollate-elicited, bone marrow derived etc.), our understanding of macrophage types has substantially grown in recent years[7, 8]. Elegant series of studies now suggest that embryonic yolk-sac derived stem cells colonize most tissues and contribute to the resident macrophage pool[9, 10]. This self-renewing population differentiates into specific types of tissue resident macrophages, such as peritoneal macrophages, Kupffer cells in the liver, alveolar macrophages in the lung, and microglia in the brain. These resident macrophage-like cells clear dying cells and debris: Kupffer cells clear aged red blood cells[11], while microglia clear dying neurons and prune mature neurons[12]. Besides resident phagocytes, circulating monocytes can also be recruited during infection or injury. Recruited phagocytes can cooperate (or compete) with the resident phagocytes, and thereby influence the immune response[13].

Non-professional phagocytes, such as epithelial cells and fibroblasts, have recently gained appreciation for their ability to clear apoptotic cells under routine homeostatic conditions (Fig. 1). Although termed ‘non-professional’ due to their lower efficiency of phagocytosis compared to professional phagocytes, non-professional phagocytes play a major role in tissues where macrophages are scarce or when macrophage access to apoptotic cells is not readily achieved – such as in the alveoli of the lung or the intestinal epithelium. The importance of non-professional phagocytes in corpse clearance has been revealed in several contexts. In macrophage-deficient animals, apoptotic cells generated during development continue to be cleared, albeit with lower efficiency[14]. Similarly, airway epithelial cells engulf dying apoptotic airway epithelial cells, requiring the small GTPase Rac1 (which functions downstream of several engulfment receptors)[15]. Epithelial cell-specific deletion of Rac1 results in increased susceptibility to allergen-induced airway inflammation and decreased production of anti-inflammatory mediators[15]. Similarly, intestinal epithelial cells

can also engulf their neighbors *in vivo*, contributing to the regulation of inflammatory sequelae (Lee *et al.*, unpublished observations). Moreover, during involution of the mammary tissue post-lactation, the epithelial cells of the mammary gland (rather than macrophages) function as primary engulfers[3]. Because epithelial cells vastly outnumber professional phagocytes and are likely the first to contact a dying adjacent epithelial cell, engulfment by neighboring cells might help maintain the tissue barrier while providing the benefits of anti-inflammatory cytokine production[15].

Specialized phagocytes are hybrid, multi-functional phagocytes that are increasingly recognized for their importance in specific tissue contexts. The best examples are Sertoli cells of the testes and retinal pigment epithelial cells (RPE) of the eye (Fig. 1). Sertoli cells, which are non-hematopoietic and post-mitotic, line the epithelium of seminiferous tubules and make up the blood-testes barrier. Sertoli cells clear millions of apoptotic germ cells that arise during spermatogenesis. Their hybrid function is exemplified by the fact that a single Sertoli cell is often in contact with 30–40 germ cells in various stages of differentiation. In addition to serving as nurse cells for the developing spermatocytes, the Sertoli cells phagocytose those germ cells that display improper meiosis or other developmental abnormalities, and disruption of either apoptosis or engulfment can affect spermatogenesis[16, 17]. Another example of specialized phagocytes are the RPE cells. RPE are long-lived cells that play a critical role in the homeostatic photoreceptor outer segment removal that occurs daily in a circadian fashion (with RPE uptake triggered by light onset)[18]. Each RPE is estimated to engulf thousands of outer segment discs over its lifetime. Failures in RPE-mediated removal of outer segments can severely affect the integrity of retinal layers and contribute to a predisposition for adverse conditions, such as retinitis pigmentosa[18].

Accessing and identifying apoptotic cells

Based on studies by a number of groups, engulfment of apoptotic cells includes distinguishable steps (Fig. 2). First, the dying cell releases ‘find-me’ signals to attract and/or activate the phagocytes. The phagocytes then distinguish the apoptotic cell from healthy living cells via specific engulfment receptors, which recognize ‘eat-me’ signals on the dying cell. Next, the phagocyte undergoes extensive cytoskeletal rearrangement to internalize corpses that are often the same size (e.g. an epithelial cell eating its neighbor). The final step is the processing of the ingested cargo and elicitation of specific phagocyte responses, primarily the secretion of anti-inflammatory mediators that help dampen the local immune response.

The release of find me signals is a critical first step in many tissues, as it recruits a potentially distant phagocyte to the dying cell. In some tissues this is particularly important, as in the developing thymus; a dying thymocyte is unlikely to be eaten by its neighbor, as lymphocytes generally lack the capacity to engulf apoptotic cells. Therefore, motile resident phagocytes have to be recruited to the proximity of apoptotic thymocytes. This is achieved through the release of find-me signals from the dying cell, including: nucleotides (ATP, UTP), the chemokine fractalkine (CX3CL1), and the lipids lysophosphatidylcholine and sphingosine-1-phosphate[19–23]. Among these, only nucleotides and a nucleotide receptor on

the phagocyte (P2Y2) are linked to the clearance of apoptotic thymocytes *in vivo*[19]. It is possible that find-me signals may serve other functions, such as during the removal of dying epithelial cells by a viable neighbor, when recruitment is not required. Since apoptotic epithelial cells also release find-me signals[19], perhaps these signals influence/enhance the engulfment capacity of the neighbor(s). For example, CX3CL1 stimulates phagocyte expression of the milk fat globule-EGF factor 8 (MFG-E8), which bridges apoptotic cells to the phagocytes to facilitate engulfment[24].

Next is the recognition of specific eat-me signals on apoptotic cells by engulfment receptors on the phagocytes. To date, the best-studied eat-me signal on apoptotic cells is the exposure of the lipid phosphatidylserine (PtdSer), which is evolutionarily conserved from *Caenorhabditis elegans* to humans[25, 26]. In living cells, PtdSer is actively restricted to the inner leaflet of the plasma membrane[27], and recent elegant studies from the Nagata group have identified apoptosis-mediated as well as calcium-induced modes of PtdSer exposure[28–30]. In addition to PtdSer, other moieties that are variably exposed on apoptotic cells include a modified form of intracellular adhesion molecule-3 (ICAM-3), oxidized low-density lipoprotein, calreticulin, annexin I, cell surface-bound thrombospondin and complement C1q, as well as changes in the surface protein charge and glycosylation status[31]. Conversely, viable cells avoid their removal by displaying ‘don’t-eat-me’ signals CD47 and CD31 or by binding to the CD300a receptor on the phagocyte, and suppressing phagocyte functions[32–34].

Engulfment receptors linked to homeostatic clearance

Multiple apoptotic cell recognition and engulfment receptors have been identified in inflammatory and/or homeostatic contexts. These receptors come in different flavors, such as scavenger receptor family members, immunoglobulin domain containing proteins, 7-transmembrane proteins, tyrosine kinases, etc.[31]. Why we have many different types of engulfment receptors and how they provide specificity is still unclear. In some respects, the diversity of engulfment receptors is similar to that of accessory proteins linked to T cell interaction with antigen presenting cells (APC). While the exposed PtdSer could be viewed as loosely analogous in function to the MHC molecule on an APC, the distinction between the phagocyte-apoptotic cell interaction and the T cell-APC interface lies in the lack of an equivalent to the T cell receptor (TCR) on phagocytes. Rather, the role of the TCR seems to be distributed among the different engulfment receptors. What we have learned so far from studies in animals with specific deletions of individual PtdSer receptors is that, while there is redundancy in function, at least in some cases there are specific needs for particular engulfment receptors. Since not all engulfment receptors are expressed on all phagocyte types, the differences in expression between professional and non-professional phagocytes might influence the homeostatic turnover of dying cells. In fact, a diverse set of phenotypes have been reported in mice with alterations in various molecules linked to PtdSer recognition (Table 1).

We discuss below three specific receptors that engage phosphatidylserine: TIM-4, BAI1, and MerTK (Fig. 3). TIM-4 can bind PtdSer directly, but does not have a signaling capacity on its own (i.e., a tethering receptor), while BAI1 is a 7-transmembrane protein that can directly

engage PtdSer and also relay intracellular signaling to mediate engulfment. MerTK, on the other hand, is a membrane tyrosine kinase that cannot engage PtdSer directly, but uses bridging molecules that bind PtdSer on apoptotic cells. We chose these receptors as they highlight some of the complexities in PtdSer recognition, and are linked to homeostatic cell turnover.

BAI1, with the homologs BAI2 and BAI3, belongs to the adhesion subfamily of G-protein-coupled receptors[35]. Originally identified as an inhibitor of angiogenesis, BAI1 plays a functional role in diverse biological processes, including phagocytosis of apoptotic cells, myoblast fusion, synaptogenesis, and tumor growth[36–40]. Via its thrombospondin repeats, BAI1 can directly bind PtdSer [39]. Upon PtdSer recognition, BAI1 interacts with a cytoplasmic signaling module composed of ELMO1 and Dock180, which function as a guanine exchange factor for Rac1, thereby inducing actin cytoskeletal rearrangements and facilitating the apoptotic cell uptake[39].

Although BAI1 deficient mice are grossly normal, several key homeostatic defects are seen. Adult mice with global deletion of BAI1 have smaller skeletal muscle fibers and display delayed healing after muscle injury; since myoblast fusion also appears to involve PtdSer exposure, these results likely reflect an interesting additional function of BAI1[37]. In peritoneal macrophages, binding of apoptotic cells to BAI1 triggers signaling that promotes cholesterol efflux [41], and contributes to the maintenance of lipid homeostasis (see further below). It has also been independently reported that mice lacking BAI1 have deficits in spatial learning and memory. This could be due to BAI1 function in regulating postsynaptic density[40]. BAI1 expression is particularly high in the brain, testes, and certain hematopoietic compartments[39]. Although BAI1 mRNA levels in macrophages are lower than those of TIM-4 or MerTK mRNA (unpublished observations), macrophages from BAI1 and TIM-4 deficient mice have comparable deficiencies in the uptake of apoptotic cells[41]. However, direct comparisons of BAI1 mRNA and protein levels have not been reported to date. BAI1 expression may also be regulated post-transcriptionally, or BAI1 might influence engulfment via mechanisms not requiring high expression.

TIM-4 belongs to a family of cell surface glycoproteins that were originally identified as regulators of T cell function[42]. The discovery of TIM-4 as a PtdSer recognition receptor was closely followed by the recognition of other members of the TIM family (such as TIM-1 and TIM-3) as PtdSer receptors[43, 44]. However, unlike BAI1, TIM4 does not activate direct downstream signaling, but rather acts as a tethering receptor[45]. Although integrins can function cooperatively with TIM-4 for signaling *in vitro*[46], the co-signaling receptor(s) for TIM-4 under endogenous expression conditions is unclear. An elegant study in zebrafish showed that BAI1 and TIM-4 may act at distinct stages of engulfment with possible cooperation between the receptors, where BAI1 contributes to phagosome formation, while TIM-4 contributes to phagosome stabilization[47].

In mice, TIM-4 expression is high on tissue resident macrophages, dendritic cells, and particularly peritoneal macrophages[44]. Macrophages lacking TIM-4 show reduced apoptotic cell engulfment[48, 49]. Global TIM-4 deficient mice also variably develop signs of autoimmunity[48–50], whereas mice with TIM-4 overexpression display reduced secondary

immune responses[51]. These data suggest that homeostatic clearance of apoptotic cells can be influenced by TIM-4, with potential links to immune tolerance. Conditional deletion of Tim-4 in specific cell types is needed for better characterization of its function in immune responses.

Mer tyrosine kinase (MerTK) is a member of the TAM receptor family, which includes Tyro, Axl and Mer receptor tyrosine kinases[52]. TAM receptors possess immunoglobulin-like domains and fibronectin repeats in the extracellular region and a cytoplasmic tyrosine kinase domain. TAM receptors engage PtdSer on apoptotic cells indirectly, via the soluble ligands Protein S and Gas-6[52]. There are differential requirements for Protein S and Gas-6 in mediating TAM receptor ligation and downstream signaling[53, 54]. Although MerTK has been reported as a specific marker of macrophages[55], it should be noted that many epithelial cells express high levels of MerTK.

TAM receptors are linked to homeostatic clearance of apoptotic cells in several contexts. Single or combined deletion of TAM family members leads to an accumulation of apoptotic germ cells in the testes, with complete lack of mature sperm in mice lacking all three TAM receptors[56]. Also, mice lacking MerTK show progressive blindness (by 8–12 weeks) due to the deficiency in the circadian RPE-dependent removal of rod outer segments in the retina, thereby revealing the specific and critical requirement for MerTK in the function of retinal epithelial cells[57]. Moreover, while losing all three TAM receptors does not affect embryonic development, adult mice show decreased clearance of apoptotic cells and develop severe systemic autoimmunity[56]. This latter phenotype has been linked to TAM receptors function as powerful inhibitors of the immune response[58].

Processing the apoptotic cargo

A fascinating but understudied area of apoptotic cell clearance is how phagocytes process the ingested cargo. When a phagocyte engulfs an apoptotic cell, it may double its protein, lipid, and carbohydrate content, yet professional phagocytes manage to rapidly engulf multiple corpses. In tissues that turnover a large number of cells, such as the thymus, the number of macrophages is much lower than that of thymocytes undergoing death. Therefore, a single phagocyte must eat more than one corpse, likely in succession. Several studies suggest that the process of engulfment itself influences the capacity of the phagocyte to engulf additional corpses, linked to increased expression of engulfment receptors via nuclear receptors (LXR, PPARδ, PPARγ and RXR)[59–61] (Fig. 2). Continued clearance of apoptotic cells by the phagocyte is also positively regulated by increased expression of UCP2, a mitochondrial uncoupler of oxidative phosphorylation from ATP synthesis[62]. Whether LXR, PPARδ, PPARγ, RXR and UCP2 expression and induction differ between the professional and non-professional phagocytes under both homeostatic and inflammatory conditions remains to be established.

Among the ingested components degraded in the phagocytic lysosomes, degradation of DNA is of particular importance, as ‘escaped’ DNA can induce breaks in self-tolerance and lead to the rise of autoimmunity[4]. A key situation where this happens in homeostasis is during erythropoiesis. During the definitive stage of erythropoiesis, DNA from erythroblasts

is extruded in structures called pyrenocytes (nuclei surrounded by membrane decorated with phosphatidylserine[63]). Pyrenocytes are engulfed by neighboring macrophages in a MerTK-dependent fashion[64], allowing erythropoiesis to proceed[65]. The enzyme that degrades DNA in the lysosomes is DNase II[65]. DNase II is highly expressed in the macrophage, and macrophages lacking DNase II cannot digest the DNA from engulfed apoptotic cells and cannot support erythropoiesis[65]. In fact, failed DNA digestion leads to the activation of the cyclic cGAS-STING nucleic acid sensing pathway, with production of type I interferon (IFN) and lethal anemia[66]. Although these mice are rescued from anemia by the added deletion of the IFN-type I receptor, they develop arthritis from excessive tumor necrosis factor (TNF) production, suggesting that undigested DNA from apoptotic cells can induce inflammatory disease[67].

Certain components of the ingested apoptotic cell, such as cholesterol, can also be disposed of in other ways. In macrophages, the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 help efflux intracellular cholesterol to the lipid-rich high-density lipoprotein (HDL), which is then taken up by the liver and excreted in the bile[68]. Impairments in cholesterol efflux are linked to dyslipidemia and atherosclerosis[69]. When macrophages engage apoptotic cells, they rapidly increase their ABCA1 expression and cholesterol efflux in a PtdSer dependent manner[70]. Surprisingly, this early induction of ABCA1 does not require the canonical LXR-mediated pathway (although LXR can be relevant after prolonged exposure of apoptotic cells[41]). Instead, the BAI1-ELMO1-Dock180-Rac1 signaling module mediates ABCA1 upregulation and cholesterol efflux[41]. Furthermore, in atherosclerosis-prone mice on a high-fat diet, deletion of BAI1 results in lower serum concentrations of HDL, a risk factor for cardiovascular disease, whereas BAI1 overexpression results in higher ratios of serum HDL to cholesterol and LDL[41], suggesting that BAI1 regulates normal lipidemia.

Cell clearance and anti-inflammatory responses

Corpse clearance commences at the earliest stages of apoptosis, prior to the loss of the plasma membrane integrity, to avoid the release of cellular contents. During homeostatic conditions, this occurs rather efficiently, and there is hardly any inflammatory cell recruitment even in tissues with high cellular turnover. However, once the plasma membrane integrity is lost due to the secondary necrosis of late stage apoptotic cells, the released cellular contents can engage receptors for damage-associated molecular patterns (DAMPs) and contribute to immune responses to self-antigens[71]. The mechanisms of clearing late apoptotic and necrotic cells include opsonization with lectins, properdin, pentraxins, thrombospondin and heparan sulfate proteoglycans. Interestingly, many of the opsonins that facilitate clearance of these cells also facilitate pathogen clearance[72]. Perhaps the concurrent recognition of the late stage dying cell and the infectious pathogen contributes to faster recovery from infectious injury and resolution of inflammation. Treatment with recombinant human MFG-E8 reduced disease in two mouse models of colitis[73], suggesting that enhancing clearance of all PtdSer exposing cells can be of benefit in inflammation. Although delayed or impaired clearance of dying cells (Table 1) can aggravate inflammatory disease, administration of early stage apoptotic cells helps reduce disease severity in inflammation models, likely via elicitation of anti-inflammatory mediators[74]. This suggests that the benefit

versus inflammatory potential of apoptotic cells is in a delicate balance, and likely critical in designing apoptotic cell-based therapies for inflammatory diseases.

Should homeostasis be breached by tissue inflammation with infiltrating cells, the dying cells can include bystander cells and short-lived immune cells (such as neutrophils) that need to be removed during resolution of inflammation. Besides apoptosis, other forms of cell death may also be involved, including primary and secondary necrosis, pyroptosis and necroptosis[75]. Neutrophils recruited to the sites of bacterial infection can also die via neutrophil extracellular trap formation (NETosis)[76], with release of nuclear chromatin and histones to facilitate trapping and killing of bacteria. Due to the release of cellular contents, NETosis is generally thought to incite inflammation, though certain types of NETs can contribute to its resolution[77]. Necroptosis is a non-apoptotic cell death triggered by TNF (a cytokine abundantly present at the sites of inflammation), or by other stimuli when apoptosis is blocked[75]. The clearance of necroptotic cells is not yet fully defined. In fact, fascinating but unexplored topics are the relative contribution of different forms of cell death to maintaining homeostasis in any given tissue, how the cells that die by different mechanisms within the same tissue are removed (by the same phagocytes?), and how decisions are made about the phagocyte responses.

Rethinking apoptosis and PtdSer exposure

Apoptosis is closely linked to regenerative processes, as a dying cell can stimulate proliferation in the surrounding viable cells through ‘apoptosis-induced compensatory proliferation’[78]. This is observed even in the simple metazoan *Hydra*, where a caspase-dependent apoptotic response caused by injury induces proliferation of the surrounding cells[79] (Fig. 4a). Similarly, apoptosis is a requirement for regenerative processes in *Xenopus*[80], planaria (flatworm)[81], newt[82], and even the mammalian liver[83].

Interestingly, caspases that are activated during cell death can also regulate subsequent induction of inflammation[84]. When apoptotic caspases are missing, viral infection causes Bax and Bak-dependent mitochondrial membrane permeabilization, leading to release of mitochondrial DNA, activation of the cGAS-STING pathway via cytosolic DNA recognition, and type I IFN induction (Fig. 4b)[84]. This suggests that the caspase-dependent death that occurs during most homeostatic conditions could have evolved to dampen local inflammation that may have been adapted by viruses that induce cell lysis.

PtdSer can also be transiently exposed on viable cells. Since such transient PtdSer exposure does not lead to engulfment, PtdSer exposure alone may not be sufficient for stimulating phagocytosis. It is likely that ‘eat-me’ signals in addition to PtdSer, perhaps in combination with the lack of ‘don’t-eat-me’ markers, might be required to ‘confirm’ the impending cell death to the phagocyte[85]. In T cells, exposure of PtdSer is triggered by TCR stimulation or the ATP receptor P_2X_7[86]. PtdSer on activated T cells contributes to the downregulation of immune responses by engaging Protein S and triggering TAM receptor mediated anti-inflammatory signaling in antigen presenting cells[87]. Therefore, PtdSer exposure in this context acts as a rheostat of the immune response, instead of an ‘eat-me’ signal. Transient PtdSer exposure is also observed upon activation of neutrophils and mast cells[88, 89]. The

distinction between apoptotic versus non-apoptotic PtdSer exposure is that the latter is reversible and generally lasts only minutes or even seconds. Exposure of PtdSer was noted during myoblast fusion into skeletal muscle myotubes *in vitro*[90] and subsequently, fusion-inducing cues have been shown to cause death of some myoblasts, and the caspase-dependent PtdSer-exposure is required for the fusion to occur[37]. Furthermore, the PtdSer receptor BAI1 and its homolog BAI3 act as promoters of myoblast fusion, as mice deficient in BAI1 and BAI3 develop smaller myofibers and show delayed healing after muscle injury[37, 91] (Fig. 4c).

PtdSer exposure is also exploited by several microorganisms due to the anti-inflammatory nature of PtdSer-dependent apoptotic cell clearance (Fig. 4d). This was first reported in *Leishmania*, which exposes PtdSer on the cell surface during the amastigote stage of the life cycle[92]. PtdSer promotes internalization of amastigotes by the macrophage while also inhibiting the immune response via induction of transforming growth factor-β (TGF-β). Similar mechanisms of evasion have been reported in *Toxoplasma gondii*[93] and *Trypanosoma cruzi*[94]. Enveloped viruses also use PtdSer for cellular entry in a process termed ‘apoptotic mimicry’[95]. The list of viruses that utilize this mechanism is growing rapidly, including HIV[96], Vaccinia[95], Ebola[97, 98], Dengue[99], and Pichinde viruses[100]. Remarkably, even non-enveloped viruses, conventionally thought to require cell lysis for viral transmission, have been suggested to use PtdSer decorated vesicles for packaging of multiple virions for transfer into the new host cell[101]. Similarly, many PtdSer receptors are linked to viral entry[100]. Finally, certain PtdSer receptors can also bind bacteria and fungi, including BAI1[102], TREM-2[103], Stabilin-2[104], CD36 and SCARF-1[105]. Thus, rethinking the role of PtdSer receptors both in the context of apoptotic cell clearance as well as non-apoptotic homeostatic functions and pathogen encounters is warranted.

Impending challenges

In terms of how apoptotic cell clearance regulates homeostasis in tissues, a number of interesting questions remain to be addressed. The first challenge is understanding the role of specific receptors. It is unclear whether there is preference in utilizing particular engulfment receptors or clearance mechanisms to achieve the distinction between homeostatic from inflammatory apoptotic cell turnover. The second challenge is defining the anti-inflammatory responses. The difference between phagocytosis of apoptotic cells versus other targets (such as bacteria or other pathogens) is that routine apoptotic cell uptake is generally not immunogenic; furthermore, it elicits the production of mediators that actively suppress inflammation in the local tissue milieu. However, our understanding of the phagocyte molecular events leading to specific downstream consequences is just beginning to be defined[41, 106]. Engagement of apoptotic cells is well known to induce TGF-β, which is linked to the differentiation of immunosuppressive regulatory T cells (T_{reg} cells). Whether routine apoptotic cell clearance plays a role in generating T_{reg} cells specific for self-antigens not expressed in the thymus remains to be explored. The third major challenge is in understanding the ‘labor distribution’ between professional and non-professional phagocytes. An intriguing question is whether there is crosstalk between professional and non-professional phagocytes under homeostatic conditions, and whether this might influence the phagocytic capacity of either. Furthermore, in many inflammatory conditions, there are

different phagocytes present (resident macrophages, non-professional phagocytes, and recruited phagocytes). It is not known whether professional phagocytes redirect non-professional phagocytes, (such as epithelial cells), to shift their efforts toward proliferation or matrix production for tissue recovery. Moreover, non-professional phagocytes can also produce anti-inflammatory cytokines[15], but there may be differences in the spectrum of factors produced and their contribution to the maintenance of the local anti-inflammatory state. Such knowledge could be useful for therapeutic targeting and accelerating tissue recovery after injury. The fourth challenge is deciphering the 'metabolomics' of apoptotic cargo processing. We know relatively little about how the target-derived metabolites are processed and used by the phagocyte, or in the phagocyte neighborhood. Release of some of these metabolites may also provide a means for communication between cells in a tissue. In this context, the tumor cell secretion of lactate regulates macrophage phenotypes in a tumor environment[107]; perhaps similar strategies exist whereby a non-professional phagocyte engulfing an apoptotic cell secretes metabolites that regulate the activation status of macrophages in the local environment. A comprehensive determination of the metabolomics of engulfment could be of relevance to human diseases such as obesity and diabetes. Thus, better defining homeostatic clearance of apoptotic cells could have important implications in our understanding of basic physiology, immune tolerance, and responses to infection.

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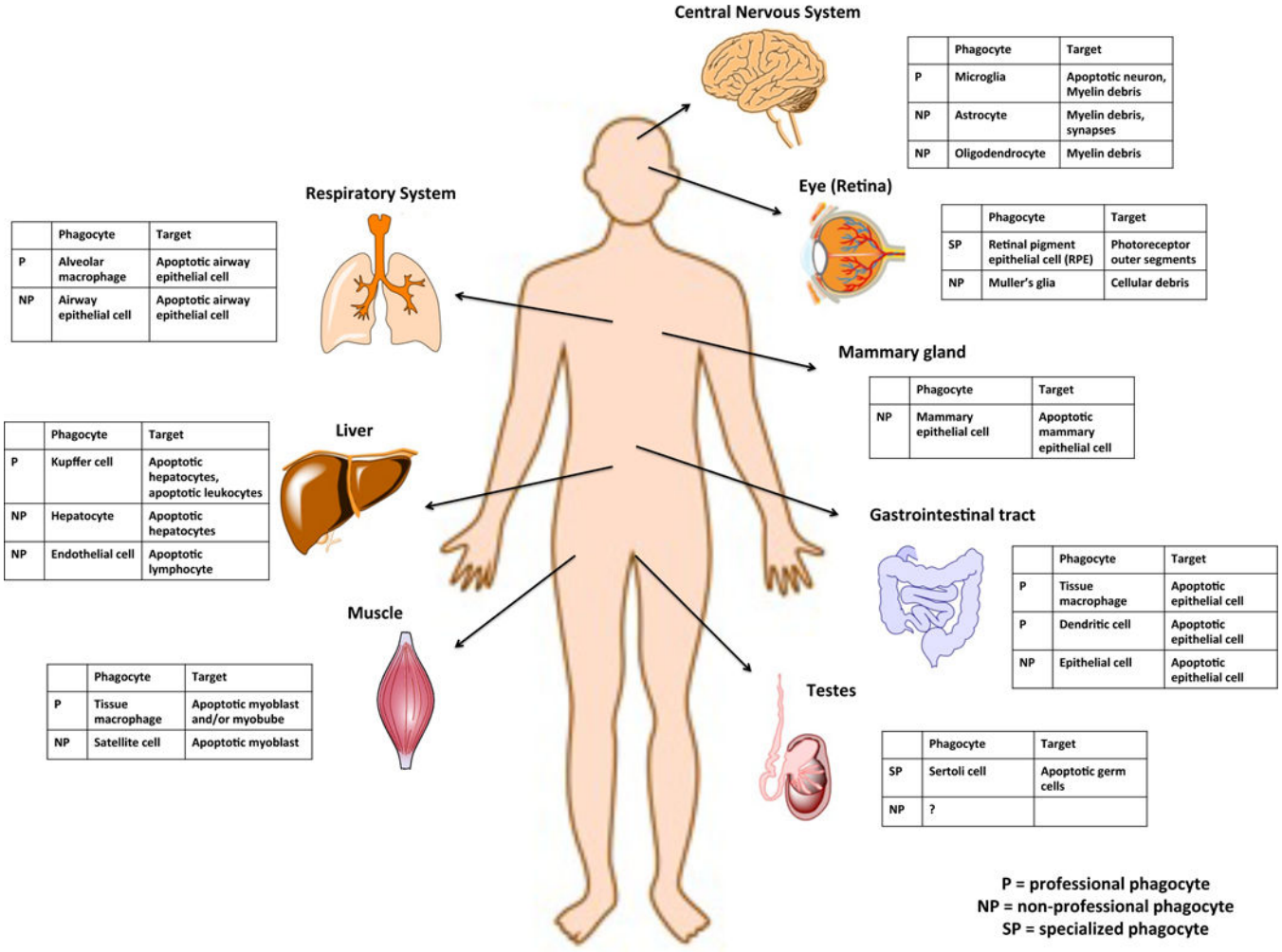

Fig. 1.

Homeostatic clearance of apoptotic cells via different phagocytes. In many tissues of the body, clearance of apoptotic cells is performed by the professional phagocytes (P), which include the tissue resident macrophages and immature dendritic cells. Many non-hematopoietic cells also have phagocytic functions in *ex vivo* or *in vitro* systems. These non-professional phagocytes (NP) include epithelial cells, hepatocytes and endothelial cells of the liver[11], astrocytes, oligodendrocytes and neuronal progenitor cells of the central nervous system[108–111], or the Muller's glia of the eye[112]. Satellite cells of the skeletal muscle have also been reported to engulf apoptotic myoblasts[37]. Specialized phagocytes (SP) are multifunctional cells that engulf apoptotic cells and include retinal pigment epithelial cell (RPE)[113], and Sertoli cells in the testes[16].

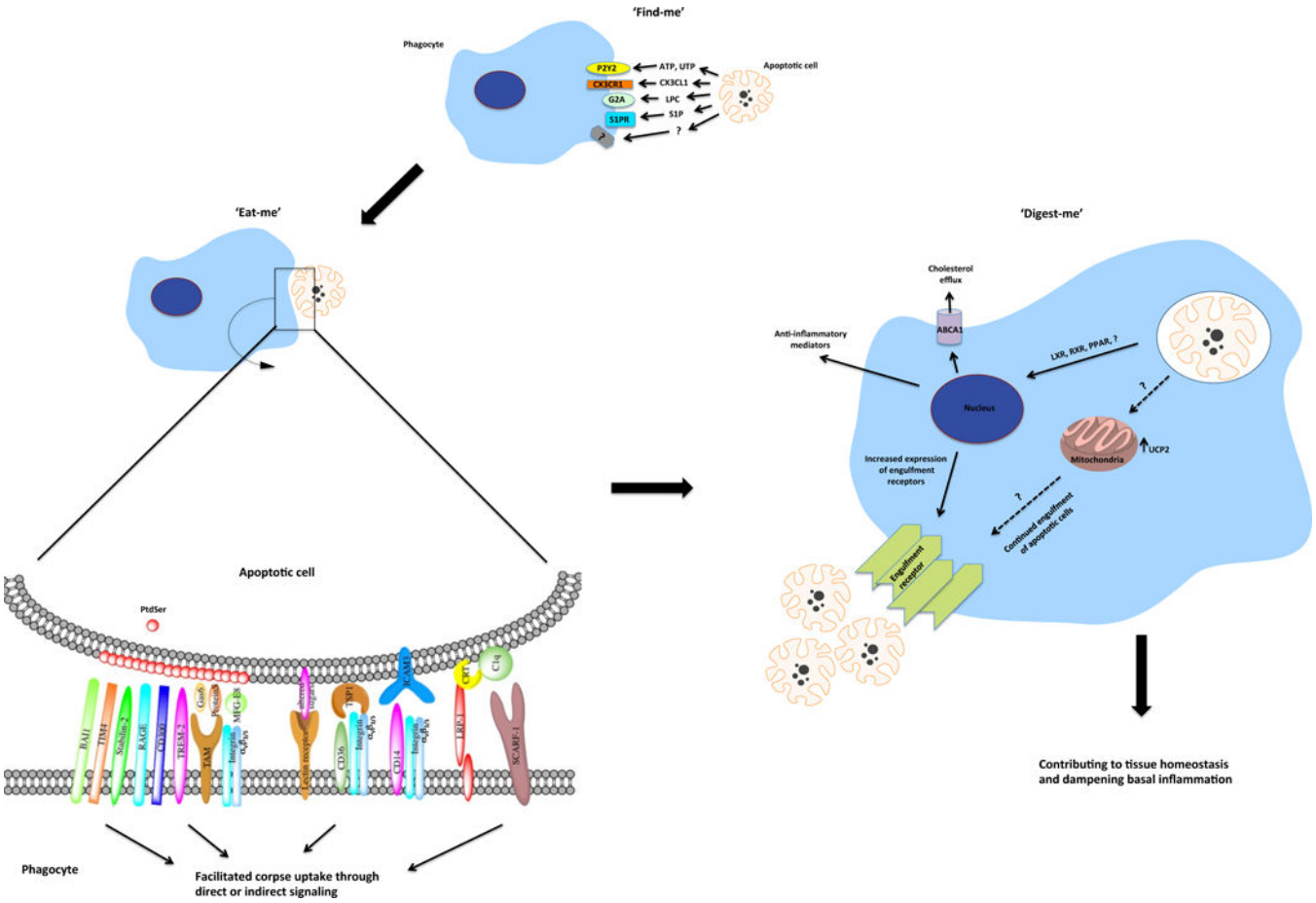

Fig. 2.

Steps during phagocytosis of apoptotic cells. When a cell initiates the apoptotic program, it releases soluble ‘find-me’ signals that attract phagocytes. The apoptotic cell is distinguished from the nearby living cell via the exposure of ‘eat-me’ signals, the most prominent of which is the phosphatidylserine (PtdSer). Eat-me signals are recognized by different engulfment receptors on the phagocytes, resulting in signaling events that facilitate the apoptotic corpse uptake. Engulfment also elicits the transcriptional up-regulation of the cholesterol efflux transporter ABCA1, and increased expression of engulfment receptors. Within the mitochondria, the levels of the uncoupling protein UCP2 are increased, enabling the continued uptake of apoptotic corpses. Anti-inflammatory mediators are expressed and secreted, contributing to tissue homeostasis and inhibition of local inflammation. PtdSer, phosphatidylserine; P2Y2, purinergic P2 receptor Y2; CX3CR1, CX3C chemokine receptor-1; G2A, G protein-coupled receptor G2A; S1PR, sphingosine-1-phosphate receptor; ATP, adenosine triphosphate; UTP, uridine triphosphate; CX3CL1, chemokine (C-X3-C motif) ligand-1, also known as Fractalkine; LPC, lysophosphatydilcholine; S1P, sphingosine-1-phosphate; LXR, liver X receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator activated receptor; BAI1, brain angiogenesis inhibitor-1; TIM4, T cell immunoglobulin and mucin domain containing molecule-4; RAGE, receptor for advanced glycation end products; TREM-2, triggering receptor expressed on myeloid cells-2; TAM, Tyro Axl Mer family receptor; LRP1, low density lipoprotein receptor related

protein-1; SCARF-1 is also known as SREC-1, scavenger receptor expressed by endothelial cell-1; Gas6, growth arrest specific-6; MFG-E8, milk fat globule EGF factor-8; TSP1, thrombospondin-1; ICAM3, intracelullar adhesion molecule-3; CRT, calreticulin.

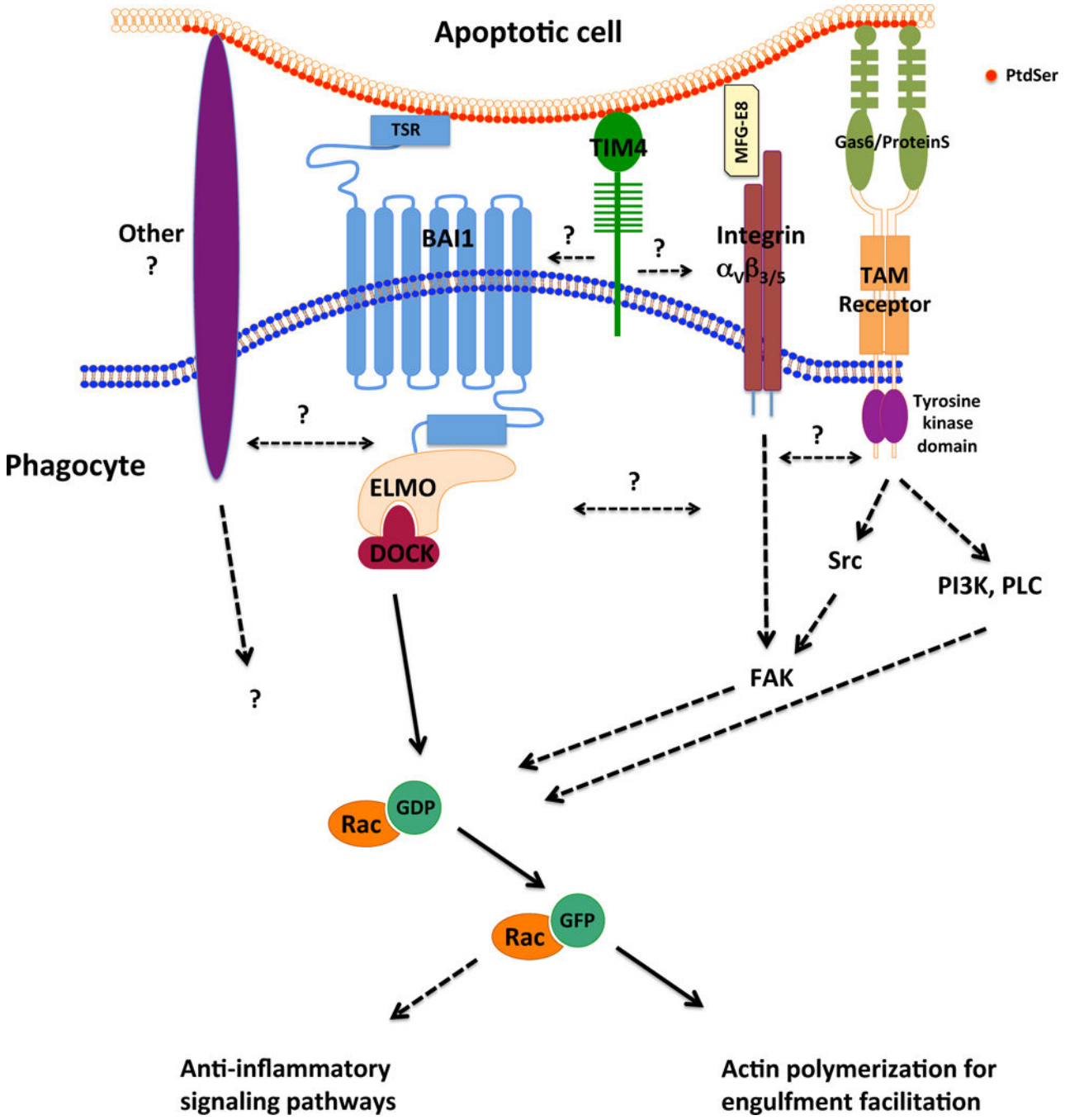

Fig. 3.

Signaling pathways elicited by three PtdSer recognition receptors. Binding of the apoptotic cell to the phagocyte triggers signaling pathways. BAI1 is a 7-transmembrane receptor that directly binds the PtdSer on the surface of an apoptotic cell, resulting in the recruitment of the Engulfment and cell motility (ELMO)/Downstream of Crk (DOCK) complex, which functions as a guanine exchange factor for the small GTPase Rac[39]. Rac activation promotes actin cytoskeleton remodeling required for the engulfment of the apoptotic corpse. Integrins $\alpha_V\beta_3$ or $\alpha_V\beta_5$ and the Tyro Axl Mer (TAM) family receptors bind apoptotic cells indirectly,

via PtdSer-bound bridging molecules MFG-E8, Gas6 or ProteinS, resulting in the activation of the focal adhesion kinase (FAK) and contributing to the activation of Rac[114]. TAM receptors are tyrosine kinases that also activate cell signaling pathways involving the kinases Src and phosphatidylinositol-3-kinase (PI3K) and phospholipase C (PLC)[114, 115]. TIM4 functions as a tethering receptor bringing the apoptotic cell in contact with signaling engulfment receptors, and signal through co-receptors. The extent of the connection between the signals elicited by different engulfment receptors awaits further characterization.

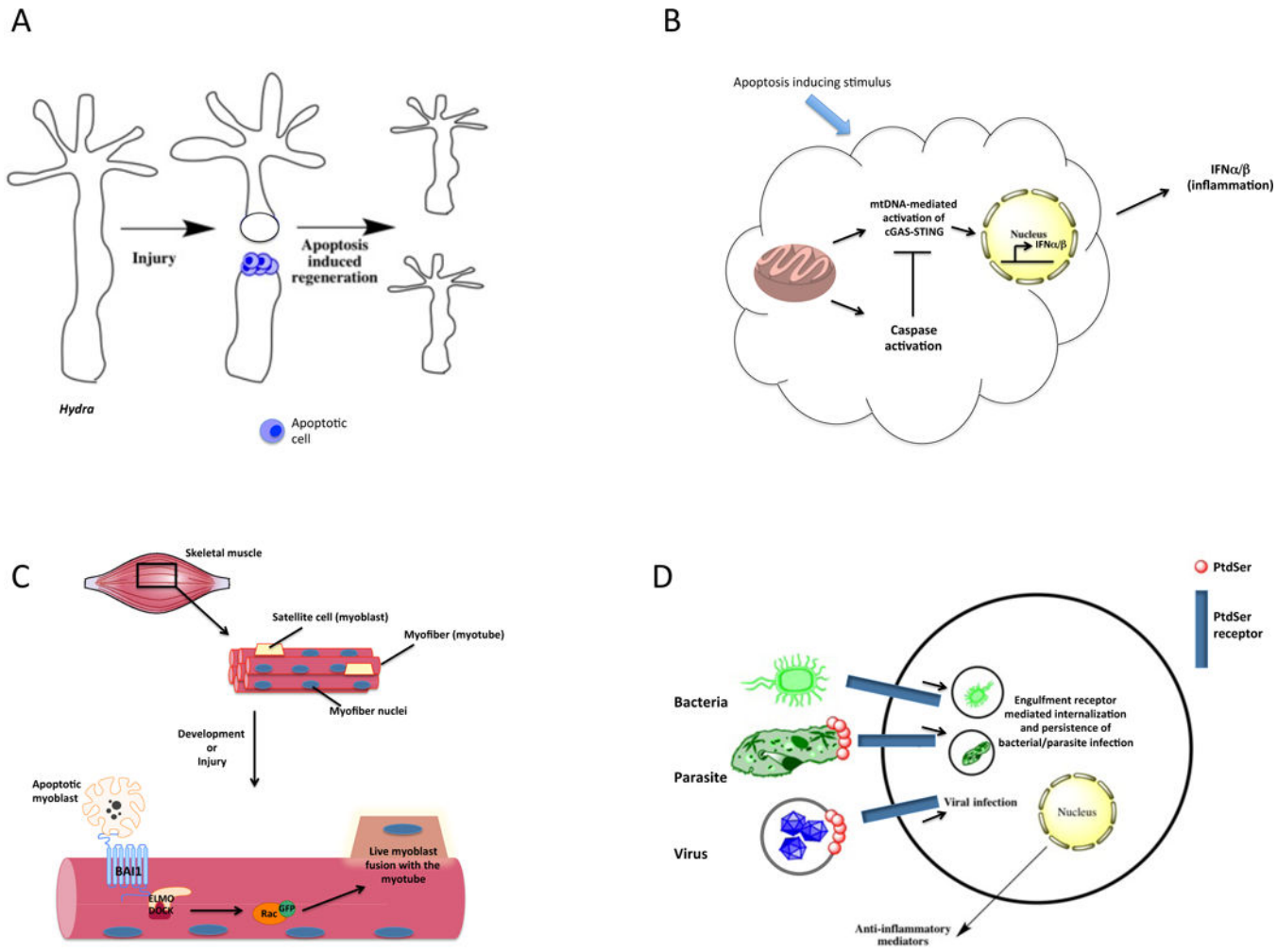

Fig. 4.

Additional and non-obvious functions of apoptotic cells. (**a**) Regeneration: In the metazoan *Hydra*, tissue injury can lead to apoptosis of the cells, which stimulates regenerative processes in the nearby viable tissues via a process called ‘apoptosis-induced compensatory proliferation’. Apoptosis is required for the re-growth of the new *Hydra* head. (**b**) Caspase-dependent inhibition of interferon production: In the context of a viral infection, apoptosis leads to the activation of caspases that is linked to inhibiting interferon-α/β (IFN-α/β) production induced by the mitochondrial DNA (mtDNA)-mediated activation of the cGAS-STING pathway. (**c**) Myoblast fusion: During muscle development and regeneration after muscle injury, apoptosis of myoblasts triggers BAI1 signaling through the ELMO-DOCK complex, leading to Rac activation. This pathway contributes to the fusion of healthy myoblasts with the nascent myotube and promotes muscle development and regeneration. (**d**) Pathogen exploitation of engulfment receptors: Bacteria, parasites and even viruses have evolved to utilize the apoptotic cell engulfment receptors for the cellular entry and induction of anti-inflammatory signaling in the host cell (phagocyte), aiding in the establishment and persistence of infection.

Table 1

Phenotypes in mice lacking receptors linked to PtdSer recognition.

Mouse strain	Homeostasis phenotype	Induced phenotype	Comment	References
Direct PtdSer receptors				
Adgrb1$^{-/-}$ (BAI1)	• Smaller muscle fibers • Defects in spatial learning and memory	• Decreased muscle regeneration, • Western diet-induced dyslipidemia on *Ldlr*$^{-/-}$ background		37, 40, 41
Timd4$^{-/-}$ (TIM4)	• Increased cellularity in the peritoneum • see Comment	• Decreased susceptibility to hepatic ischemia/ reperfusion injury	Reports of autoimmune disease in this strain vary.	48–50, 116.
TIM4-Tg	•	• Reduced secondary immune response		51
Stab1$^{-/-}$ (Stabilin-1)	•	• Slower growth and dissemination of transplanted B16 tumors (see Comment)	Same phenotype observed in mice with the conditional deletion of Stabilin-1 in macrophages or hematopoietic and endothelial compartment.	117
Stab2$^{-/-}$ (Stabilin-2)	•	• Reduced dissemination of transplanted B16 tumors		118
Stab1$^{-/-}$ *Stab2*$^{-/-}$ $^{-/-}$ (double deficient)	• Glomerular fibrosis with proteinuria (not intrinsic to kidney) • Premature mortality	•		119
Ager$^{-/-}$ (RAGE)	• Age-related lung fibrosis	• Increased inflammation and decreased efferocytosis in LPS-induced lung inflammation • Increased asbestos-induced lung fibrosis • Decreased bleomycin-induced lung fibrosis • Increased survival in CLP model of septic shock		120–124
Cd300lf$^{-/-}$ (CD300f)	•	• Increased autoimmunity on FcγRIIb deficient background		125

Mouse strain	Homeostasis phenotype	Induced phenotype	Comment	References
Trem2$^{-/-}$	• Age-related deficiency in CNS microglia • see Comment	• Decreased recovery from cuprizone-induced demyelination	Opposing phenotypes observed in two mouse models of Alzheimer's disease.	126–129
Indirect PtdSer receptors				
Mer-TK	• Loss of vision and defects in clearance of photoreceptor outer segments • Decreased clearance of apoptotic cells in testes and decreased fertility • Autoimmunity	• Increased AOM/DSS-induced colon cancer • Increased disease in endotoxin challenge • Increased contact hypersensitivity response • Increased EAE • Improved motor function after focal cerebral ischemia		56, 57, 130–137
Itgav$^{fl/fl}$ Tek-Cre (Integrin subunit α_v)	• Colitis • Autoimmunity	• Protected from EAE		138, 139
Itgav$^{fl/fl}$ *Lyz2-Cre*	• Colitis	• Delayed disease development in EAE		138, 139
Itgav$^{fl/fl}$*Nes*-Cre	• Axonal degeneration, seizures, motor dysfunction • Premature death	•		140
Itgav$^{fl/fl}$ Gfap-Cre	• Eyelid tumors (see comment)	•	Specific strain of GFAP-Cre was used.	141
Itgb5$^{-/-}$ (Integrin subunit β_5)	• Age-related loss of vision	•		142
Bridging molecules				
Mfge8$^{-/-}$ (MFG-E8)	• Age-related dermatitis • See comment	• Increased DSS-induced colitis • Decreased AOM/DSS-induced colon cancer • Increased disease and decreased efferocytosis in LPS-induced lung inflammation • Accelerated disease in a mouse model of diabetes • Decreased neuronal loss after LPS-induced inflammation • Improved motor function after focal cerebral ischemia • *Mfge8*$^{-/-}$ bone marrow reconstitution of *Ldlr*$^{-/-}$ mice increases Western	Reports of autoimmune disease in this strain vary.	50, 143–149

Mouse strain	Homeostasis phenotype	Induced phenotype	Comment	References
		diet induced atherosclerosis		
Gas6$^{-/-}$	•	• Increased AOM/DSS-induced colon cancer • Increased demyelination and decreased recovery from cuprizone-induced demyelination • Increased susceptibility to hepatic ischemia/reperfusion injury • Decreased disease in induced glomerulonephritis • Protected against thrombosis • Delayed GVHD		150–156
Protein S deficiency in T cells	•	• Increased immune response • Increased T cell-induced colitis in *Rag*$^{-/-}$ mice		87
Other receptors				
Cd36$^{-/-}$	•	• Resistant to high fat diet-induced obesity • Reduced efferocytosis in bleomycin-induced lung injury and in skin wound healing		157–160
Lrp1$^{fl/fl}$*Lyz2*-Cre	•	• Increased atherosclerosis on high fat diet on *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$*Ldlr*$^{-/-}$ backgrounds		161–163
Lrp1$^{fl/fl}$*Itgax*-Cre	•	• Increased susceptibility to HSV-1		164
Scarf1$^{-/-}$	• Autoimmunity	•		165
C1qa$^{-/-}$	• Autoimmunity, • Defective synaptic refinement • Epilepsy	• Defective wound healing • Epilepsy • Increased atherosclerosis on high fat diet on *Ldlr*$^{-/-}$ background		166–170