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Cell Removal: Efferocytosis

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Abstract

In metazoans, removal of cells in situ is involved in larval maturation, metamorphosis, and embryonic development. In adults, such cell removal plays a role in the homeostatic maintenance of cell numbers and tissue integrity as well as in the response to cell injury and damage. This removal involves uptake of the whole or fragmented target cells into phagocytes. Depending on the organism, these latter may be near-neighbor tissue cells and/or professional phagocytes such as, in vertebrates, members of the myeloid family of cells, especially macrophages. The uptake processes appear to involve specialized and highly conserved recognition ligands and receptors, intracellular signaling in the phagocytes, and mechanisms for ingestion. The recognition of cells destined for this form of removal is critical and, significantly, is distinguished for the most part from the recognition of foreign materials and organisms by the innate and adaptive immune systems. In keeping with the key role of cell removal in maintaining tissue homeostasis, constant cell removal is normally silent, i.e., does not initiate a local tissue reaction. This article discusses these complex and wide-ranging processes in general terms as well as the implications when these processes are disrupted in inflammation, immunity, and disease.

Contents

INTRODUCTION

The concept of tissue cell loss and replacement is inherent in the cellular nature of life and the substantial structural changes during development in multicellular animals—a point well recognized by the classical embryologists of the nineteenth century. It had long been apparent that cells can die in situ, and potential mechanisms for cell removal were later conceptualized in Metchnikoff's phagocyte theories (see Tauber 2003).

However, not until the 1970s was a more precise process identified, by Wyllie and colleagues (Kerr et al. 1972): a state of natural, programmed cell death (PCD) that they termed apoptosis. These investigators and others at approximately the same time suggested that apoptosis led to local removal by phagocytosis. Subsequently, multiple forms of PCD have been, and are increasingly being, identified and are associated with an increasing spectrum of mechanisms by which dying cells are recognized and removed. Two general observations help define these processes. Unlike recognition and removal of foreign organisms or particles, the clearance of apoptotic cells is mostly quiet, i.e., not associated with a concurrent inflammatory or immune response, and is thus conceptually very much part of the normal maintenance of tissue homeostasis. In addition, it became increasingly apparent that the uptake process (forms of phagocytosis) differed mechanistically from that involved in cellular ingestion of bacteria, fungi, and a wide variety of inert particles, both in the initial mechanisms of recognition and in the actual ingestion. This led us to

coin the term efferocytosis (deCathelineau & Henson 2003) to depict the uptake and removal of apoptotic cells and, indeed, of cells undergoing the increasing variety of nonapoptotic forms of PCD. A recent, simple guide to efferocytosis by Kumar & Birge (2016) is a useful introduction to these processes.

An important element of this general metazoan property of dying cell removal is its overall efficiency. This means that examination of normal tissues, even when significant cell removal is ongoing, can often reveal only minimal evidence of actual dying cells. An example is the mammalian (murine) thymus, where lymphocyte PCD is ongoing and can be massively enhanced by administration of corticosteroids, but with almost no detection of dying cells in the tissue unless the animals are genetically defective in their ability to carry out efferocytosis (Scott et al. 2001). An implication is that detection of apoptotic cells in tissues should be considered not only as an indication of enhanced cell death but also, or alternatively, as altered cell clearance.

For this article, which could cover extensive research on the subjects of cell removal in animals, plants, and fungi (and even single-cell organisms), I take a personal view, outlining some key concepts surrounding these processes in animals, some speculations on their mechanisms and relevance, and what I believe to be significant possibilities and challenges for future study and exploitation. This article does not represent a history of the studies of apoptotic cell removal. Accordingly, the accompanying Literature Cited is deliberately selective and no more than illustrative.

SELECTIVITY OF CELL RECOGNITION AND REMOVAL

The critical initial question for understanding cell removal is that of recognition. The innate and adaptive immune systems have evolved many strategies to avoid recognition of normal cells in the body to focus on foreign and altered structures so as to kill and/or remove them as a way to preserve tissue integrity and function, as well as to establish a protective memory response for the future. And yet huge numbers of ($>10^{11}$) cells are removed in the normal adult mammal on a daily basis and during mammalian embryogenesis without engendering a noticeable local or systemic reaction. How then are these cells recognized as being destined for removal, and how are these recognition processes different from those involved in classical immune responses to foreignness; i.e., how do such processes provide the potential to exploit the latter without compromising the normal processes of homeostasis? Furthermore, are the changes on dying cells that provide the recognition unique to different cell types and/or for different modes of cell death, i.e., homeostatic versus nonhomeostatic (e.g., toxic, infectious) cell damage? In contrast, or additionally, might viable cells express inhibitory signals to potential phagocytes to actively prevent recognition?

To no surprise, these recognition mechanisms seem to be highly redundant; substantially complex; and certainly variable, with different forms of cell death. An initial dualistic concept contrasting apoptosis with necrosis suggested homeostatic noninflammatory and actively anti-inflammatory responses and clearance mechanisms for the former and more proinflammatory and immunogenic consequences for the latter. This construct has had some value but has since proven too simplistic and indeed potentially misleading (see below). Many different modes of cell death have been identified and classified where possible by mechanism (Galluzzi et al. 2012), and the list continues to expand. Whereas Kerr et al.'s (1972) original description of apoptosis was morphological in nature (see also Green & Fitzgerald 2016), standard apoptosis may now be considered as PCD driven through intracellular pathways of caspase activation, but with multiple initiation and pathway differences. Importantly, in many of these PCD processes, the cell retains its general structure and integrity, at least long enough for its efferocytic removal if the appropriate phagocyte is available, thereby avoiding cell disruption and exposure of the tissue to potentially toxic and immunogenic intracellular materials.

Overall cell integrity is maintained even in the common situation in which apoptosis includes subsequent blebbing, because the blebs (cell fragments) maintain their membrane integrity and, as they are smaller, may be easier for phagocytes to ingest. Thus, new surface markers on a dying, but still relatively intact, cell are implicated for recognition. Indeed, a host of these markers, and the receptors that recognize them, have been suggested (outlined in multiple reviews, e.g., Birge et al. 2016, Elliott & Ravichandran 2016, Gardai et al. 2006, Green et al. 2016, Krysko et al. 2008, Kumar & Birge 2016). The most common and well characterized of these surface changes is exposure of phosphatidylserine (PS) (Fadok et al. 1993, Segawa & Nagata 2015), an anionic phospholipid normally confined to the inner leaflet of the plasma membrane and maintained at that location by ATP-dependent aminophospholipid translocases that flip any exposed PS back to the inner leaflet. During apoptosis and other forms of PCD, activation of so-called phospholipid scramblases move the PS across the membrane and, along with loss of translocase activity (likely often because of inadequate ATP generation in the dying cell), collectively results in PS exposure on the cell surface. Researchers have described a wide variety of PS receptors on potential phagocytes, as well as PS-recognizing bridge molecules (opsonins) that link the PS to other phagocyte receptors such as αv integrins and scavenger receptors. The complexity indicates either a high degree of redundancy or much more diversity of recognition in the cases of different cells, cell types, and modes of cell injury and death than is currently appreciated.

Given all these potential signals for removal, one might reasonably wonder what prevents the accidental recognition of normal living cells. In an analogy with the intriguing concept that cells in multicellular organisms need constant stimuli to keep them alive, i.e., to prevent them from undergoing PCD (Raff et al. 1994), one possibility is that living cells exhibit constitutive inhibitory signals to prevent their removal: so-called "don't eat me" signals. Upon the loss of such signals, for example, during the initiation of cell death, normal surface structures might be recognized. As a potential example of the broad scope of these recognition processes and their involvement in normal cell behavior and turnover, cell activation (for example, in neutrophils) can lead to transient exposure of PS on the outer leaflet without the initiation of cell death pathways (Frasch et al. 2008). This development can be enough to mediate removal of the activated cells, likely as part of the normal resolution of acute inflammation. A general candidate in mammals for "don't eat me" inhibitory stimulation is CD47 on the target cell; CD47 delivers an antiefferocytic signal to the phagocyte via SIRPα (also termed SHPS-1) (Gardai et al. 2005). Loss and/or redistribution of the CD47 during cell death or activation removes the inhibition and allows for uptake and removal. This system is currently being exploited for enhancing cell removal in, for example, cancer and atherosclerosis (Kojima et al. 2016, Weiskopf et al. 2016). The broader extent of such inhibitory processes is currently unknown, but one could reasonably expect many more (e.g., Brown et al. 2002).

An additional set of questions addresses the need for the phagocyte to find the dying cell in the first place. This is not an issue for the removal of cells undergoing PCD while in contact with potentially phagocytic (efferocytic) near-neighbor cells, as seen during development of nematode larvae or during, in some cases, epithelial remodeling in mammals (see below). However, finding the target cell is relevant for removal by migratory cells such as macrophages and would seem to require some attractant processes initiated by the dying cell. In fact, a number of chemotactic molecules ("find me" signals) have been identified, and these include nucleotides and phospholipids that are released by cells undergoing apoptosis and that appear to serve this function (see Elliott & Ravichandran 2016).

As an example of the extent of normally operating cell removal, huge numbers of leukocytes and erythrocytes are removed from the circulation on a daily basis by phagocytes in the liver, spleen, and bone marrow. Although this removal may not always be specifically due to apoptosis or other forms of PCD, loss of phospholipid asymmetry and exposure of PS are likely involved, as

are potential loss of the normal "don't eat me" signals and the engulfment mechanisms of efferocytosis. However, another set of related questions addresses the mechanisms for removal of frankly necrotic cells, i.e., cell fragments or cells that are dying from postapoptotic necrosis, programmed necrosis, or chemical and physical injury. As noted below, necrotic cell recognition is more often associated with proinflammatory responses from the incipient phagocyte and presumably involves a set of recognition processes either different from or overriding those associated with immunologically silent and anti-inflammatory apoptotic cell removal. Clearance of cell debris, however, is essential for tissue repair and inflammation resolution, i.e., occurs regularly during resolution of inflammation and injury. How many of these discriminatory effects are generally driven by the programming state of the phagocyte versus how many are driven by the spectrum of ligands (and their phagocyte receptors) on cells/particles is unclear and certainly highly variable.

Importantly, although most efferocytic recognition signals are notably different from those associated with phagocytosis in the standard innate immune system– or adaptive immune system–mediated removal of foreign materials or cells, the distinction is by no means complete. As examples, C1q and other collectins can participate in both immunological and efferocytic cell removal, and cells undergoing PCD can also fix the complement component C3 (although the initiation mechanisms are not always clear), with consequent C3b-mediated endocytosis. Nuclear materials, including DNA, can be detected on the surfaces of some cells dying by PCD processes and can also be recognized, as can antibodies binding to normally inaccessible intracellular but now exposed cell constituents, even before postapoptotic cell dissolution.

The generality of the recognition and signaling processes involved in efferocytosis can be evidenced not only by the commonality of intracellular signaling leading to the cell clearance noted below, but also by the finding that PS exposure appears to be a critical recognition signal across multiple animal systems, including nematodes and insects. Not surprisingly, efferocytosis-like processes have also been exploited by parasites, for example, *Leishmania* (Wanderley et al. 2013), as a mechanism for gaining access to intracellular compartments in the phagocyte. Another point is that enveloped viruses, almost by definition because they cannot rectify the phospholipid asymmetry of their acquired membranes, can interact with the PS-recognizing systems on cells, with a potential contribution to uptake of the virus and/or downstream signaling that may alter subsequent cellular responses (e.g., Watanabe et al. 2002).

EFFEROCYTIC CELLS AND MECHANISMS OF UPTAKE

The preeminent phagocytic cells in many animal phyla are macrophages, which are thought to carry out most efferocytic cell removal. Accordingly, macrophages have received the most study. Monocytes and dendritic cells (DCs) in mammals are also effective, although there is some DC specialization, depending on the key transcription factors involved in DC development (Desch et al. 2011). However, cell removal is essential for development and homeostasis in all multicellular organisms, and so-called nonprofessional phagocytes are clearly able to carry out this function. Thus, even in mammals, many cell types, including epithelial cells, endothelial cells, and fibroblasts, have been implicated in these processes. An example is the normal process of postlactation involution of the mammary gland, in which some mammary epithelial cells develop phagocytic (efferocytic) potential and ingest the remaining epithelial cells as the latter become apoptotic (Monks et al. 2008). Much of the early characterization of apoptotic cell uptake mechanisms and the associated endocytic signal pathways came from extensive genetic studies in *Caenorhabditis elegans*, in which, during larval development, 131 specific cells undergo apoptosis and are engulfed by near-neighbor cells. These studies, spearheaded by Horvitz and colleagues and many other investigators (see Hengartner 2000, Lettre & Hengartner 2006, Tosello-Trampont et al. 2007,

Yuan & Horvitz 2004), helped define not only the molecules and signaling involved in apoptosis but also the unique signal pathways that operate in the uptake processes common to both nonprofessional efferocytic cells and the highly efficient macrophages. A number of reviews outline these pathways in relation to the different receptors mentioned above (e.g., Birge et al. 2016, Elliott & Ravichandran 2016, Kumar & Birge 2016). Early studies supported a striking commonality in these pathways downstream of engagement of a wide variety of candidate receptors, with critical roles played by the Rho family of low-molecular-weight GTPases. These studies suggested intriguing opposing effects of Rac1 (having a proefferocytic effect) and RhoA (having an inhibitory effect) (see below and deCathelineau & Henson 2003, Elliott & Ravichandran 2016), leading to potential implications for endogenous regulatory and/or deliberately manipulable ways of altering the efficacy of cell clearance. More recently, additional processes and signaling pathways have been shown to mediate uptake processes, including a pathway associated with autophagy (in the phagocyte) termed LAP (LC3-associated phagocytosis) (see Green et al. 2016, Martinez et al. 2015).

Again, redundancy is the apparent observation but begs once again the key question: Are these different pathways, processes, and mechanisms for cell removal truly redundant, or do they apply selectively to different cells, forms, and stages of cell death or even to cell damage, such as before the point beyond which the cell cannot recover? In this context, the relationship of the increasingly investigated processes of cellular senescence with those of cell removal is of potential interest. How do senescent cells avoid efferocytic recognition?

Less well understood are the actual physical mechanisms of apoptotic cell uptake into the efferocyte into what may be termed the efferosome [the LAPasome for the LAP process (Green et al. 2016)], let alone the subsequent digestion and disposition of the engulfed contents (see below). We have suggested that the uptake represents essentially a two-step process: tethering of the target dying/dead cell to the efferocyte surface and then stimulation of the actual uptake process [a tether-and-tickle process (Gardai et al. 2006, Hoffmann et al. 2001; see also Toda et al. 2012)]. A functional differentiation between recognition receptor classes for these two steps has been suggested, although some receptors can effect both steps. The ability of recognition molecules such as TIM4 and CD14, for example, to initiate tethering but not ingestion (Park et al. 2009, Savill 1998) raises interesting functional questions about normal control and regulation of the entire efferocytic process; as noted throughout this article, this area is ripe for investigation and manipulation.

Multiple mechanisms of cellular endocytosis have been described, and such mechanisms are often classified on the basis of target particle size as phagocytosis (for sizes >1–2 μm), pinocytosis (for sizes <1 μm), or macropinocytosis (for intermediate sizes) (Conner & Schmid 2003). Different uptake mechanisms, signaling, receptor and membrane processes, and endocytosed material disposition are suggested for different forms of each of these mechanisms of endocytosis. Where does efferocytosis fit in? Some of our earlier studies (Gardai et al. 2006, Hoffmann et al. 2001, Ogden et al. 2001) suggested a form of stimulated macropinocytosis, with a loose plasma membrane enclosure of the apoptotic cell resulting in a spacious phagosome (efferosome) and thus involving concurrent uptake of surrounding fluid. However, a more formal phagocytic cup—with closely applied membranes [the zipper mechanism (Swanson & Baer 1995)] and a tight endosome, i.e., one without fluid uptake—has also been proposed (Krysko et al. 2008). Intriguingly, once again, the different stages of apoptosis and consequently different ligands and receptors for apoptotic cell uptake may result in different ingestion processes. Mechanisms for final closure of the efferosome are only now receiving much attention (for example, see Elliott & Ravichandran 2016, Nakaya et al. 2008). A number of these recent investigations of efferocytic mechanisms indicate the intracellularly local and highly temporal balance between the effects of Rho and those of Rac and emphasize the need to study intracellular distribution and the effects of such effector molecules

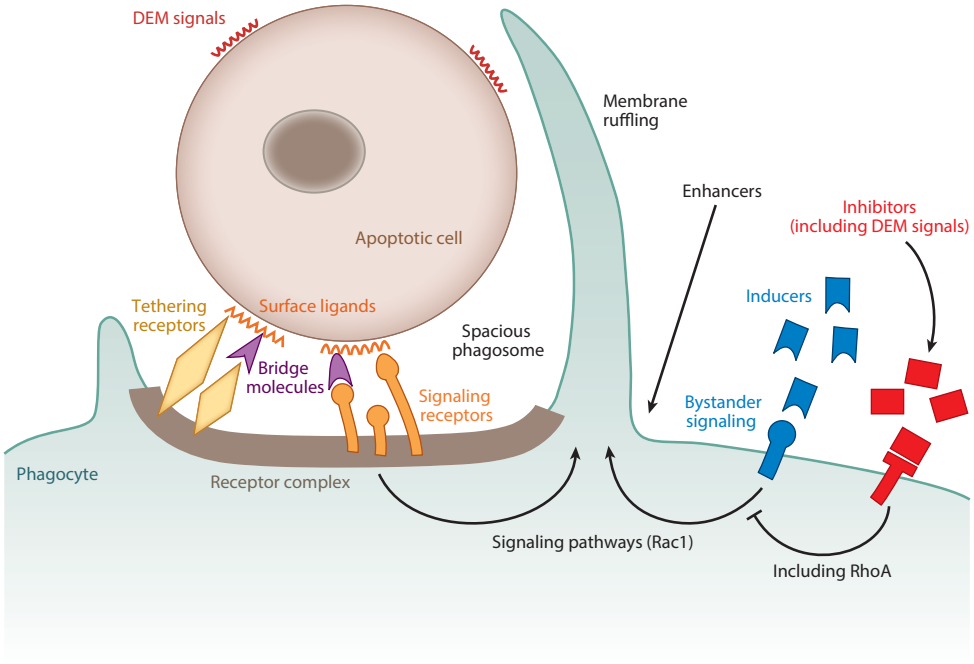

Figure 1

Efferocytosis. Shown are some receptors and modulating processes involved in the uptake of cells undergoing apoptosis and other forms of programmed cell death (PCD), along with related cell fragments, apoptotic bodies, etc. The initial recognition of the dying cell by receptors induces both tethering to the efferocyte and signaling for uptake. This process includes a number of bridge molecules (opsonins) that recognize surface ligands (including phosphatidylserine) on the target. Such ligands then bind to receptors on the ingesting cell. Ingestion is promoted by Rac1 and is generally antagonized by RhoA. Normal cells may resist recognition and removal by expressing inhibitory "don't eat me" (DEM) stimuli on their surface. Such stimuli are removed or disarranged during PCD. Soluble stimuli (inducers) from the environment can initiate uptake of tethered cells by bystander signaling, and similarly, inhibitory stimuli can suppress efferocytosis. Finally, another group of stimuli (enhancers) may generally prime or enhance the overall efferocytic process. Adapted with permission from Janssen et al. (2016), copyright © 2016, American Society for Microbiology.

and processes rather than relying on the older whole-cell approaches. A number of investigators have noted that efferocytosis likely has a number of features in common with cell motility (e.g., Elliott & Ravichandran 2016).

The abovementioned tether-and-tickle concept (**Figure 1**) has an additional implication, namely that soluble stimuli to the phagocyte, unrelated to the apoptotic cell recognition process, could initiate macropinocytic uptake of tethered apoptotic cells, even if the tethering process did not initiate activation of the ingestion. This possibility has been shown in vitro, but its importance in vivo is not clear. A similar so-called bystander uptake stimulation of bacteria tethered to epithelial cells has been reported (Francis et al. 1993). These unrelated stimuli (termed inducers in **Figure 1**) may include a variety of inflammatory mediators or growth factors if they are able to activate pathways leading both to appropriate localized Rac:Rho activation at the point of target cell tethering and to induction of macropinocytosis or some other set of endocytic processes. Once again, the ability of the tethering process to include a set of "don't eat me" signals (likely tipping the Rac:Rho balance toward the inhibitory Rho effect) would be an important regulator to avoid inappropriate cell removal. Additional environmental stimuli—termed enhancers and inhibitors in

Figure 1—may mediate a more general proefferocytic (or antiefferocytic) state within the potential phagocyte. A potential example of an enhancer is the well-established ability of corticosteroids to increase macrophage efferocytic potential (e.g., Giles et al. 2001).

An even more specialized uptake process may be required for the important part played in phagocyte (especially microglial) sculpting of neuronal connections in the CNS, wherein neuronal terminals and synapses are selectively removed in the synaptic pruning process. Recent studies have suggested an important role for complement and C3 in these physiological processes (see Hong & Stevens 2016), but the physical mechanisms underlying chewing off pieces of such cells, or indeed underlying what is likely similar pruning of other cells in the body, are unclear. Possible related processes are trogocytosis (nibbling), in which T cells obtain membrane patches from antigen-processing cells, and amoeboid trogocytosis, which involves *Entamoeba* taking "bites" out of host target epithelial cells (see Guillen 2014).

Finally, examples of close cell interaction without subsequent ingestion abound, including lymphocyte binding to DCs during their normal costimulatory interactions and leukocytes crawling over the surfaces of blood or lymphatic endothelial cells. What prevents the uptake of such cells, especially in circumstances in which bystander stimulation of various forms of endocytosis is also present?

RESPONSES TO RECOGNITION AND INGESTION OF CELLS UNDERGOING PROGRAMMED CELL DEATH

As noted above, a key element to efferocytosis and the constant removal of effete cells within multicellular organisms is that the processes are normally effectively silent, i.e., do not initiate either local or systemic inflammatory (or, for the most part, immunological) reactions. This characteristic is clearly diametrically opposite to the responses to recognition and removal of foreign organisms or particles. In fact, extensive studies have shown that efferocytosis is actively anti-inflammatory and suppressive of normal innate and adaptive immune responses. This effect is due to both intracellular suppression of inflammatory pathways in the ingesting cell and their production of a variety of potent anti-inflammatory mediators (reviewed in Birge et al. 2016, Elliott & Ravichandran 2016). In particular, stimulation of the anti-inflammatory and anti-immunogenic responses seems to be an important property of the recognition receptors and signaling pathways associated with PS exposure, although probably not exclusively limited to these elements. Hence the noninflammatory nature of the constant cell and cell fragment removal in the normal animal is associated with, for example, the normal turnover and limited life spans of the circulating blood cells. Another notable example is the outer rod cellular segments released nightly from the retina, in this case ingested by retinal pigment epithelial cells, suggested by some to be the most phagocytic cells in the body (Mazzoni et al. 2014). All these events are ongoing and, in the normal animal, are unrecognized in terms of initiation of inflammation or immune responses.

However, as might be expected, the actual situation is substantially more complex and represents a balanced response to dying cells and cell debris. Among the number of elements that determine the extent and strength of the anti-inflammatory response, the keys are the mode of cell death and the contents of the cell that is dying. Necrotic cells have long been recognized as being able to initiate inflammation, as have cells that are dying from intracellular infection due to either viruses or other parasites. These observations raise the issue that necrosis, like apoptosis and its family of PCD mechanisms, is a heterogeneous process, ranging from frank physical disruption of the cell (e.g., by heat, cold, pH extremes) to a variety of programmed necrosis mechanisms (Galluzzi et al. 2012) as well as cell disruption by parasites and viruses. Key characteristics of necrosis relevant here are more rapid disruption of cell integrity and therefore a wider variety of

available stimuli to the ingesting phagocyte. However, by definition, such disruption will also expose PS, and thus the stimuli involved will likely induce both anti- and proinflammatory responses, with an overall effect that represents a balance between them. This complexity suggests that the simplistic dichotomy between the effects of apoptosis and necrosis needs to be reconsidered for each specific condition and cell type (e.g., the type of dying cell and the potential phagocyte).

For immunologists, clearance of the excess, unselected lymphocytes as they undergo apoptosis in lymphoid organs such as the thymus represents an interesting conundrum. Removal, as noted above, is highly efficient and appears to be carried out primarily by stromal cells. However, the precise characterization of the stromal cells with regard to this function, and the likely participation by local macrophages, has not received much investigation. Likewise, open areas for investigation include the effects of this ongoing clearance and intracellular disposition on subsequent actions of the engulfing cells, on the local immunological environment, and on the substantial clearance of excess hematological cells that occurs in the bone marrow as a part of normal hematogenesis. A specific example is the constant efferocytic clearance of extruded membrane-bound nuclear contents during erythrocyte formation (Toda et al. 2014).

INTRACELLULAR DISPOSITION OF INGESTED DYING CELLS

Key questions here in the context of intracellular disposition of ingested dying cells address the processes of efferosome (LAPasome) maturation and how they may differ between the different receptors and ingestion mechanisms involved in the uptake of cells undergoing PCD (see, for example, Kinchen et al. 2008, Yin et al. 2016). In professional phagocytes such as macrophages, maturation of the efferosomes and digestion of the apoptotic cells appear to be extremely rapid, and thus observing the overall processes of cell removal in vivo is difficult. As with other endocytic processes, the rates of digestion of apoptotic targets appear to be slower in DCs than in macrophages (Erwig et al. 2006), with enhanced opportunity for the association of antigens with MHC molecules for antigen presentation. Much less is known about efferosomal functions and digestion in nonprofessional efferocytes, although in these cells too the processes may be presumed to be highly efficient—another area ripe for future investigation.

Metabolic Effects

The broad extent of these efferocytic processes clearly leads to the concept of conservation of resources, i.e., the reutilization of the ingested cell constituents (see, for example, Theurl et al. 2016). By contrast, it also raises questions about potential overload within the ingesting cell, such as ingestion of too much cholesterol (see Kiss et al. 2006). These issues have so far not received much attention, although they are likely important in the context of the metabolic regulation of the efferocytic cell. For example, the role played by changes in cellular metabolism in functional programming by many different cell types, including phagocytic cells such as macrophages, is a current hot topic. The impact on these effects of a huge load of potential cell constituents derived from the ingested apoptotic cells will likely be significant.

Immunological Effects

The ability of some efferocytic cells to process ingested materials for immunological presentation on MHC structures to the adaptive immune system is another important outcome from the uptake process. Intriguingly, classical DCs appear to be selective in their ability to recognize and ingest apoptotic cells in that those dependent on the Batf3 transcription factor ($CD8a^+$

DCs and CD103$^+$ DCs) are much more effective than those dependent on IRF4 (CD11b$^+$ DCs) (e.g., Desch et al. 2011). The mechanisms underlying this selectivity are not clear but likely include selective expression of apoptotic cell recognition receptors, although no particular receptor has been identified. Functionally important is the concurrent selective ability of these efferocytic DCs to cross-present and cross-prime to induce cytotoxic CD8 T lymphocytes. Critical to this process is the ability of immunogens to escape the endosome to gain access to the MHC1 molecules for the cross-presentation step, raising once again the potential special properties of the efferosome. High levels of endosomal TLRs (e.g., TLR3 and TLR7) are key players in these processes, including the subsequent induction of CD8 T cell differentiation to gain their cytotoxic effector properties. Again, these specialized processes are ripe for detailed investigation, with the added impetus of their importance in the natural mechanism for disposition of cells undergoing cell death after infection with viruses or in neoplasia and the possible implications for vaccine development.

EFFECTS OF DYING CELL INGESTION ON THE PROPERTIES OF THE PHAGOCYTE

In addition to the likely effects of the ingested cell and its contents, the processes of efferocytosis can initiate changes in the phagocytic cell. These changes will differ by type of efferocyte, as noted by a recent publication by Cummings et al. (2016). Thus, ingestion of apoptotic cells by an immature Batf3-dependent DC leads not only to the processes of antigen presentation, but also to maturation of the DC to upregulate its migratory properties, allowing for transit to the lymph nodes. The effects of efferocytosis on tissue cells that become involved in this process, such as the epithelial cells or stromal cells mentioned above, are unclear and need investigation.

Effect of Efferocytosis on Macrophage Programming States

Macrophage (and monocyte) programming is a topic of huge interest in terms of innate and adaptive immunity, inflammation, host-parasite interactions, wound healing and tissue repair, and a wide variety of disease processes. The topic has tended to evolve into a dichotomy between so-called proinflammatory and reparative states (type 1 immunity versus type 2 immunity, or M1 versus M2 macrophages). Before discussing the effects of efferocytosis, I would like to express the cautionary note that, although these broad concepts may be useful in some contexts, they can also be substantially misleading. For one, the states are often termed phenotypes. But these macrophage programming states are highly sensitive and rapidly responsive to the environment and stimuli that the cells are being exposed to, so if a cell "phenotype" is considered to be relatively stable, then the term is inappropriate. More critical is the consistent observation that, especially in vivo, one almost never finds a pure population of macrophages exhibiting one or another standard set of the markers used to determine these different programming states. Moreover, the markers used are not necessarily directly responsible for, or even related to, the ongoing functional activities of the cells in vivo.

Given these issues, the macrophage programming states seem both to affect the ability of the cells to undergo efferocytosis and to be changed by the efferocytic process. Macrophages present early in an inflammatory process are usually proinflammatory and are less efficient at efferocytosis than those present during resolution (resolving or reparative macrophages). In fact, monocytes emigrating into an inflammatory site can mature to become macrophages and then go through the stages of low and then high efferocytic ability. These maturation steps occur under the influences of (*a*) the shifting pattern of mediators and growth/maintenance factors and oxygen tension,

(*b*) protein and lipids in the surrounding fluid, and (*c*) the understudied effect of the physical structures in the environment with which the cells are constantly interacting.

Effects of Efferocytosis

As noted above, the other side of this programming coin is the recognition that the efferocytosis process can lead to macrophage programming toward gaining resolving/reparative properties (e.g., Arnold et al. 2007). This development is highly connected with the aforementioned anti-inflammatory responses seen during the recognition and removal of apoptotic cells. Thus, the same secreted anti-inflammatory mediators (e.g., IL-10, TGFβ) not only reduce inflammation (through mediator production and inflammatory cell accumulation) and mediate reparative or even fibrotic repair sequelae, but also act on macrophages to alter their programming state in those anti-inflammatory directions. These efferocytosis-induced anti-inflammatory/reparative mediators can act in an autocrine or a paracrine fashion on the local monocytes and macrophages, as well as on local tissue cells. In addition, a host of intracellular signaling pathways, including the LAP pathway, are initiated by efferocytosis, altering the properties of the engulfing cell from within (for example, by suppressing NFκB pathways) to support changes in overall programming state (see, for example, Ipseiz et al. 2014 and Elliott & Ravichandran 2016). More specifically for the process of efferocytosis, the uptake of one dying cell has been suggested to alter (enhance) the ability for uptake of others, providing portals for future engulfment (Nakaya et al. 2008).

Monocytes as Efferocytes

In mammals, a possible additional consequence of monocytes interacting with apoptotic cells (and/or other types of dying cells and their fragments) is monocyte maturation, for example, toward becoming a macrophage. In the adult animal, circulating, bone marrow–derived monocytes can migrate into tissues during injury and inflammation and can there develop various functions, states, and morphologies. Some gain the immunological properties of antigen presentation and the ability to migrate to lymph nodes and initiate adaptive immune responses. Others mature into macrophages, with the subsequent ability to exhibit proinflammatory as well as reparative programming states—presumably sequentially. Monocytes can also ingest apoptotic cells (i.e., are efferocytic), although not as efficiently on a cell-to-cell basis as macrophages or DCs can (Larson et al. 2016). From these observations, we suggest that an additional potential consequence of efferocytosis is enhancement of the monocyte-to-macrophage maturation process. Which receptors and/or intracellular or extracellular signaling systems are involved is unclear, although mechanisms associated with PS recognition seem likely. Further maturation of macrophages to the intriguing multinucleated giant cells, a process that involves macrophage fusion, is also PS dependent (Helming et al. 2009, Milde et al. 2015).

In a broader perspective, the consequent impact on the engulfing cell of nonhematological tissue cells—such as stromal cells, epithelial cells, and near-neighbor cells in the nematode—carrying out efferocytosis (including the recognition steps) is not clear but is well deserving of investigation (e.g., Penberthy et al. 2014).

What Happens to the Efferocyte?

In the case of an inflammatory reaction, clearance of the emigrated inflammatory cells during resolution is largely due to their efferocytic removal by local macrophages, although some inflammatory cells may be lost to mucosal surfaces. Even if a few of the inflammatory cells migrate back

into the blood or down the afferent lymphatics, they are eventually removed (although by what efferocytic cell is not usually clear; see below). In addition, to restore homeostasis, the efferocytic macrophages must also be removed through the same routes, i.e., exiting the body from the mucosa; emigrating to the blood or lymph; or undergoing PCD, with local removal by cannibalistic efferocytosis. All three of these have been proposed and, to a limited extent, demonstrated. However, macrophages, in contrast to DCs, do not seem to have significant abilities to migrate back into the blood or down lymphatics. Macrophages can certainly be cleared on mucosal surfaces but for the most part seem to undergo apoptosis and local clearance by tissue macrophages (see, for example, Janssen et al. 2016). The role of uptake of apoptotic cells in the eventual promotion of apoptotic pathways in macrophages effecting efferocytosis is not clear. Alternatively, the successful efferocytic macrophage may succumb to the presence of appropriate apoptosis-inducing mediators, such as Fas ligand, in the environment at that time point in the resolving inflammatory reaction. Or the abovementioned metabolic changes, or even the efferocytic process itself, may prime the cell for its own eventual demise. Intriguingly, the tissue-resident macrophages (of embryological origin) do not seem to demonstrate the same response; i.e., they are not generally removed at the end of the inflammatory response and do not seem to be primed for their own apoptosis (Janssen et al. 2011) (but neither are they as effective efferocytes as the macrophages derived from emigrated monocytes). In contrast to inflammatory processes, the effects of efferocytosis on the fates of the many other cells that are carrying out this process in the normal animal or during, for example, tissue remodeling are largely unknown.

DEFECTS IN CELL CLEARANCE PROCESSES AND THEIR EFFECTS ON TISSUE HOMEOSTASIS AND DISEASE PROCESSES

Implicit in the anti-inflammatory effects of normal efferocytic recognition and removal of apoptotic cells is the opposite effect—absent, ineffective, or defective clearance—which leads to marked proinflammatory consequences. In addition to the lack of anti-inflammatory effects, apoptotic cells undergo secondary necrosis, with liberation of proinflammatory materials, such as DNA, RNA, mitochondria (having properties like bacteria), and lysosomal contents. Perhaps not surprisingly, abnormal enhancement of phagocytic (efferocytic) uptake of normal leukocytes and erythrocytes can also be a problem, as seen in hemophagocytic lymphohistiocytosis, a group of life-threatening, hyperinflammatory syndromes (see Brisse et al. 2015). Here, one must suppose some ability to overcome the normal action of the "don't eat me" signals to allow for excessive cell removal, although this topic has not been investigated.

Defective Efferocytosis and Dysregulation of Inflammation

The importance of efferocytosis in the normal resolution of inflammation is supported by extensive literature showing in experimental settings that, when the process is defective, inflammation is exacerbated and prolonged. Similarly, in related human inflammatory diseases, efferocytosis has in vivo often been shown to be diminished. However, as noted in the Introduction, because the process is normally so efficient, its identification and quantification are difficult. In this context, we have suggested that detecting significant numbers of cells undergoing PCD in tissues should be viewed with suspicion for the presence of defects in the clearance processes. Here, in lieu of a long list of inflammatory diseases for which defective efferocytosis has been shown and therefore implicated as contributing to pathogenesis, a few examples may be illustrative. In emphysema (Barnawi et al. 2017, Dehle et al. 2013, Mukaro & Hodge 2011) and in chronic bowel disease (Lacy-Hulbert et al. 2007), defective efferocytosis has been frequently noted and suggested to contribute to

persistent inflammation. As in numerous other animal models of inflammatory diseases, studies similarly support such observations in humans, usually by showing disease-promoting or exacerbating effects of the defectiveness or absence of one or more efferocytic receptors. The potential roles for efferocytic processes in cancer represent an important developing area of study and are addressed briefly below.

The presumption in most of these situations is that defective cell removal contributes to (*a*) secondary induction or prolongation of the inflammation due to proinflammatory cell content release and (*b*) loss of the anti-inflammatory and proresolving mediator production that accompanies normal efferocytic removal of inflammatory cells as the inflammation wanes. Thus, disruption of the normal progression in a self-resolving inflammatory response of first pro- and then anti-inflammatory mediators is delayed or prevented. In many cases, this effect has been clearly demonstrated experimentally and has often been shown to be specifically related to the efficacy of the efferocytic processes.

A special case reflects the abovementioned physiological process of nightly retinal outer rod segment clearance processes, which are essential for normal restitution of the daily process of vision. Thus, in experimental animals, the absence of efferocytic receptors such as αv integrins, MerTK, and CD36 leads to retinal degeneration (Mazzoni et al. 2014) and to defective expression of these molecules, and the efferocytic efficiency of the retinal pigment epithelium is associated with retinal dysfunction in human beings. Note that however we define the term efferocytosis, on the basis of, e.g., receptor usage and intracellular signaling pathways, this uptake of membrane-bound cellular fragments is so similar to whole apoptotic cell uptake that the latter should constrain the definition of this term (see also the context of neuron and synapse pruning).

Defective Efferocytosis and Autoimmunity

Defective efferocytosis and autoimmunity constitute an important subject that has received substantial experimental attention (Colonna et al. 2014, Kimani et al. 2014, K.W. Yoon et al. 2015). The inability to efficiently remove dying cells may also lead to immune recognition of normally hidden (and normally effectively removed and digested) intracellular constituents, i.e., to the initiation of autoimmune responses. In fact, defects in or losses of substantial numbers of efferocytic receptors or recognition molecules (such as MerTK, CD36, MFG-E8) lead to autoimmunity in mice and to autoimmune disease, especially systemic lupus erythematosus (SLE), in humans. Thus, SLE has been consistently associated with defective clearance of apoptotic cells (reviewed in Chaurio et al. 2009, Colonna et al. 2014, Munoz et al. 2008). Again, the underlying mechanisms are complex and include not only the release or exposure of normally internal and unrecognized autoantigens but also the substantial dysregulation of the pattern of anti-inflammatory and immune regulatory mediators normally seen after successful efferocytosis. More broadly, the ability of DCs (and monocytes) to undergo efferocytosis and, from these uptake mechanisms, present and cross-present antigens and induce T cell activation may play a key initiating role in many autoimmune processes.

Efferocytes and Efferocytic Receptors in Cancer

A special set of cases for understanding of, and then potential exploitation of, efferocytosis and its mechanisms is currently arising in the area of antitumor immunity. It has long been known that many tumor cells seem to expose PS on their surface (e.g., Shurin et al. 2009, Vallabhapurapu et al. 2015), although whether such exposure reflects inadequate rectification of the phospholipid asymmetry in an otherwise viable cell (see above) and/or incipient apoptosis is not always clear.

Targeting this PS by various approaches is therefore under consideration to remove the cells and/or to alter the inflammatory or targeted immune responses (see Ayesa et al. 2017, Birge et al. 2016, Blanco et al. 2015). In this context, removing the "don't eat me" signals by blockade of CD47 with anti-CD47 antibodies may enhance phagocytosis of viable and/or apoptotic tumor cells, both removing the cells and enhancing the T cell immune response (e.g., Tseng et al. 2013).

EXPERIMENTAL AND THERAPEUTIC EFFECTS OF CELL REMOVAL AND THEIR POTENTIAL EXPLOITATION

Experimental Cell Deletion and Its Potential Hidden Effects

Many therapeutic treatments and experimental approaches in animals either deliberately or inadvertently are designed to induce, or involve, the removal of cells in situ. Experimental cell deletion studies abound. Although often not considered in discussion of these approaches, this cell removal inevitably means cell death and/or ingestion by phagocytic processes, including efferocytosis, within animals, including humans. The extensive use of diphtheria toxin–induced selective cell removal is a classic example. All too often the investigator focuses on the impact of the cell loss without equal consideration of the effects mediated by the dead cells themselves and/or of the impact of their necessary removal. In this context, experimental deletion of cells in vivo with anticell antibodies, acting through Fc receptors and complement, would be expected to have quite different sequelae relative to those involving apoptotic or induced cell death and removal by efferocytic processes.

Potential Exploitation of the Effects of Efferocytosis

It has become customary to conclude a discussion such as this with a consideration of potential therapeutic implications with regard to human disease. Given the clear indication that cell removal is a normal and essential component of tissue and whole-body homeostasis, it would seem surprising to acknowledge that there has been little evidence of successful exploitation of these processes along these lines. Perhaps this paucity reflects the complexity, redundancy, and balancing checkpoints involved. Two examples highlight the possibilities and caveats. Deliberate administration of apoptotic cells can alter the outcome of an inflammatory response in animal models (e.g., Y.S. Yoon et al. 2015). In more directly clinical relevant systems, administration of what may be presumed to be apoptotic cells, achieved by treating blood leukocytes ex vivo with a light-sensitive dye and then UV light exposure (termed extracorporeal photopheresis), has been proposed, and is being used, for treatment in a variety of chronic inflammatory processes, with some reported success (see Del Fante & Perotti 2017 and Malagola et al. 2016 for two recent publications). This approach presumably exploits the anti-inflammatory properties of the efferocytic processes outlined above. However, the inherent crudity of the approach and the possibility of numerous secondary negative consequences (including possible autoimmune sequelae) do not seem to recommend its widespread use. Another example is the apparently obvious approach to exploiting the anti-inflammatory and proresolving consequences of efferocytic processes by direct therapeutic stimulation of one or more of the efferocytic receptors, for example, those involved in recognition of PS. Although a number of such attempts have been made, no clear effective candidate has emerged. Potential problems include the redundancy of the receptors and processes; the need to balance prostimulatory versus controlling inhibitory effects; and, perhaps most likely, the need for extensive cross-linking of the receptors (including combinations of receptors), as would be achieved by interacting with Patched ligands on an intact apoptotic cell or

cell fragments. The concept of an efferocytic receptor complex (depicted in **Figure 1**) reflects this possibility. In addition, a specific possibility lies in the two-step response of the efferocyte to apoptotic cell recognition and concurrent activation of "don't eat me" signals. Costimulation via PS-recognizing receptors coupled with blockade of the inhibitory pathways may be a more effective approach. These are but two examples of possible approaches to utilizing efferocytosis and its consequences for altering disease processes. There are surely many more to be considered in the future.

CONCLUSIONS

The major theme of this article is the critical roles played by cell turnover and removal in the maintenance of normal homeostasis in metazoans. One striking observation in the context of these processes is the highly conserved nature of the basic processes of dying cell recognition as well as that of the phagocytic (efferocytic) processes involved in cell removal—an homage to Metchnikoff's phagocytosis theories. Another general observation is that, despite the common mechanism underpinning the process of uptake, the actual detailed mechanisms and molecules show striking redundancy. How much of this redundancy reflects the overall essential nature of the processes and how much reflects adaptation of the necessary removal to many different cell types and local environmental situations is unclear. Over the last century, much scientific endeavor has been focused on sorting out the highly specialized processes whereby animals recognize foreignness as a potential threat to their integrity—the various forms of immunity. Here we have to acknowledge a comparable, and equally specialized, ability to recognize native cells that are dying and/or destined for removal. In this case, however, the response is not to mount inflammatory and immunological effector processes, including the ability to remember the nature of the insult, but rather to carry out the removal as quickly and with as little local or systemic effect as possible. Not surprisingly, such complex processes can become disrupted by external actions, local metabolic or environmental changes, or genetic/epigenetic dysregulation. Under these situations, altered quiet and efficient cell removal can result in loss of tissue homeostasis and in disease. Alternatively, by exploiting the efficient and silent processes of efferocytic cell removal, therapeutic modulation of inflammatory or perhaps particularly neoplastic disease processes might be achieved.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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