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Review

The innate immune system and the clearance of apoptotic cells

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ABSTRACT

Removal of unwanted, effete, or damaged cells through apoptosis, an active cell death culminating in phagocytic removal of cell corpses, is an important process throughout the immune system in development, control, and homeostasis. For example, neutrophil apoptosis is central to the resolution of acute inflammation, whereas autoreactive and virus-infected cells are similarly deleted. The AC removal process functions not only to remove cell corpses but further, to control inappropriate immune responses so that ACs are removed in an anti-inflammatory manner. Such "silent" clearance is mediated by the innate immune system via polarized monocyte/macrophage populations that use a range of PRRs and soluble molecules to promote binding and phagocytosis of ACs. Additionally, attractive signals are released from dying cells to recruit phagocytes to sites of death. Here, we review the molecular mechanisms associated with innate immune removal of and responses to ACs and outline how these may impact on tissue homeostasis and age-associated pathology (e.g., cardiovascular disease). Furthermore, we discuss how an aging innate immune system may contribute to the inflammatory consequences of aging and why the study of an aging immune system may be a useful path to advance characterization of mechanisms mediating effective AC clearance. *J. Leukoc. Biol.* 90: 447–457; 2011.

Introduction

Death is essential to life, throughout development to the latest point in our active lives. Estimates suggest that approximately 1 million apoptotic deaths per second occur within the human body—deaths that are "balanced" against cell birth to maintain homeostasis. However, there is often an imbalance between the rates of cell birth and cell death to permit accumulation of cells (e.g., leukocytes during infection) or to delete cells (e.g., postinfection to permit resolution of an immune response). Active cell death by apoptosis is a vital process to delete such unwanted, effete, or damaged cells. Intriguingly, AC clearance mechanisms share remarkable similarities with those involved in recognition and removal of pathogens. Despite such similarities, responses to the removal of pathogens versus ACs are diametrically opposed proinflammatory versus anti-inflammatory. Such differences likely arise from a variety of mechanisms, including the profound immunomodulatory effect that ACs exert upon their phagocytes. In the following sections, we review the molecular mechanisms by which ACs are removed by the innate immune system, highlighting mechanisms that may result in an anti-inflammatory response, and discuss how this may associate with inflammatory consequences of aging.

THE ULTIMATE PHASE OF APOPTOSIS

Apoptosis is often considered to be a simple process of cell inactivation and dismantling, where the functional endpoint of apoptosis—AC clearance—is ignored. However, the final cell clearance stage of apoptosis is arguably the most important in the apoptosis program and is necessary for removing cells in a variety of situations, from normal, physiological cell death (associated with tissue homeostasis) through to death associated with pathology (inflammation and infection).

The ultimate clearance phase is a complex, multistage process that comprises a number of steps that rely on a large range of phagocyte receptors and AC-derived ligands, which link directly or via soluble bridging/opsonizing molecules. Even at a most simple level, these steps include recognition of dying cells, binding, and phagocytosis. In vivo, however, as we will cover later in this review, the process may involve a number of cellular and molecular functions upstream of the simple "eating" of dying cells.

Abbreviations: AC=apoptotic cell, ACAMP=apoptotic cell-associated molecular pattern, AxI=annexin I, CLR=C-type lectin receptor, CX3CL1=fractalkine, DAMP=damage-associated molecular pattern, dRP S19=dimer of ribosomal protein S19, EMAP-II=endothelial monocyte-activating polypeptide II, Gas-6=growth arrest-specific 6, Lox-1=lectin-like oxidized LDLR-1, MBL=mannose-binding lectin, Mer=myeloid epithelial reproductive tyrosine kinase, NLR=NOD-like receptor, PS=phosphatidylserine, PTX=pentraxin(s), RLR=retinoic acid-inducible gene-I-like receptor, RP S19=ribosomal protein S19, SP-A/D=surfactant protein A/D, SR=scavenger receptor, TSP=thrombospondin, ttRS=tyrosyltRNA synthetase

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PHAGOCYTES OF DYING CELLS

A classification of AC phagocytes as "amateur/semiprofessional" or "professional" has been used for many years. Macrophages and neutrophils of the innate immune system are known as "professional phagocytes" because of their powerful and robust capacity to phagocytose particles, especially of microbial origin. Although macrophages are also proven phagocytes for ACs (**Fig. 1**), it is not clear that they are the routine phagocytes for dying cells. Additionally, neutrophils are extremely active in the phagocytosis of microbes with little role in dead cell clearance until activated [1]. Indeed, ACs actively repel viable granulocytes [2].

In most sites within higher organisms, cell death will occur in the absence of neighboring macrophages, a similar situation to that seen in the nematode *Caenorhabditis elegans*, where professional phagocytes are absent. In such situations, the usual phagocyte is likely to be a viable neighbor. Although the ability of amateur phagocytes (i.e., non-macrophages), such as epithelial cells, to eat ACs has long been noted [3–7], its importance has been under-valued. Given the strong conservation in clearance mechanisms (in terms of phagocyte receptors [8, 9], adaptor proteins [10, 11], and bridging molecules [12, 13]) and AC ligands [14] between *C. elegans* and humans, the "traditional" phagocytes of dying cells are almost certainly the "amateurs".

In higher organisms, macrophages are seen to clear dying cells at sites where there is a high incidence or persistence of ACs. This association suggests that macrophages are the professional phagocyte of ACs and has been clearly shown in Burkitt's lymphoma (for tumor-associated macrophages [15]), lymphoid follicles (for tingible-body macrophages [16, 17]), and experimentally, in animal models defective for certain phagocyte receptors (e.g., CD14 [18] and MBL [19]; see below). This contrasts with sites of low-level apoptosis, where dying cells are rarely imaged, as cell corpses rapidly disappear following highly efficient phagocytosis, which may even occur prior to any overt morphological features of apoptosis. However, given the efficiency with which dead cells are routinely cleared within tissue homeostasis and macrophage recruitment to persisting death (as a consequence of high-level death or failed/overwhelmed clearance), macrophages have been termed "professional scavengers" of dying cells [20].

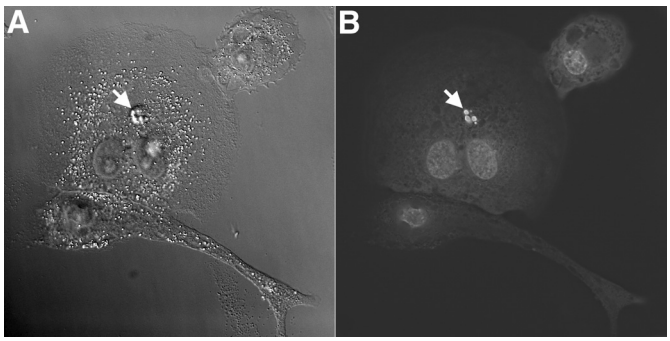

Figure 1. Macrophage recognition of an apoptotic B cell. Coculture of macrophages with ACs reveals clearance of ACs (arrows). (A) Differential interference contrast microscopy; (B) acridine orange revealing the intense punctate staining of the AC nucleus.

MOLECULAR MEDIATORS OF AC CLEARANCE

Removal of dead and dying cells requires different molecular functions at different stages. Many, largely in vitro, studies identified a wide array of phagocyte-associated receptors that are proposed to link to a more limited number of identified, AC-associated ligands. This link is known to be direct or via a soluble bridging/opsonizing molecule (**Fig. 2**).

The large number of molecules involved in dead cell clearance provides redundancy to ensure efficient clearance even in the face of inhibition of one or a number of pathways. Defects in AC clearance molecules have been linked to a range of diseases [21]. However, as deficiency of individual molecules (e.g., CD14, MBL, and C1q) may lead to defective clearance, redundancy may not be so extensive as often thought, and the superphagocyte, shown in Fig. 2, is unlikely to exist, as it is the simple corollary of all phagocyte studies. The clearance mechanisms depicted are expressed across different phagocytes (e.g., amateur vs. professional) and within different cell subpopulations (e.g., monocyte/macrophage subpopulations). Such phenotypic variation within the phagocyte "pool" may serve to tailor the nature of activity and response to ACs. As the phagocyte is decisive in the net response to cell death [22], further characterization of phagocyte phenotypes in different situations (i.e., aging, pathology, or normal tissue structure/function) is necessary.

These multiple mediators of clearance may work together, perhaps sequentially, to mediate firm adhesion and ultimate engulfment of ACs. This cooperative of molecules has been termed the "phagocytic synapse", as a result of its proposed similarity to the immune synapse formed between T cells and APCs [23, 24]. In both synapses, it is likely that low-affinity interactions become strengthened through activation of adhesion molecules (e.g., integrins) and the net effect of many low-affinity interactions to increase the overall avidity of binding. Such a model would require multiple molecular interactions and would explain the variety of molecular players.

To simplify the complexity, previous work has sought to characterize molecules by their function. The clearance process can be divided into a range of steps: recognition of, responses to, and engulfment of ACs, which can be subdivided further. Consequently, it has been possible to assign a function to certain molecules within this multistep process, and this has led to molecules being labeled, for example, as tethering, signaling, or engulfment receptors [18, 21, 25–28].

Pattern recognition and AC clearance

The fine detail of AC clearance mechanisms has been reviewed elsewhere and is beyond the scope of this review (reviewed in refs. [15, 20, 28]). Here, we consider a subset of molecules involved in clearance, the PRRs of the innate immune system. Many identified AC recognition molecules (Fig.

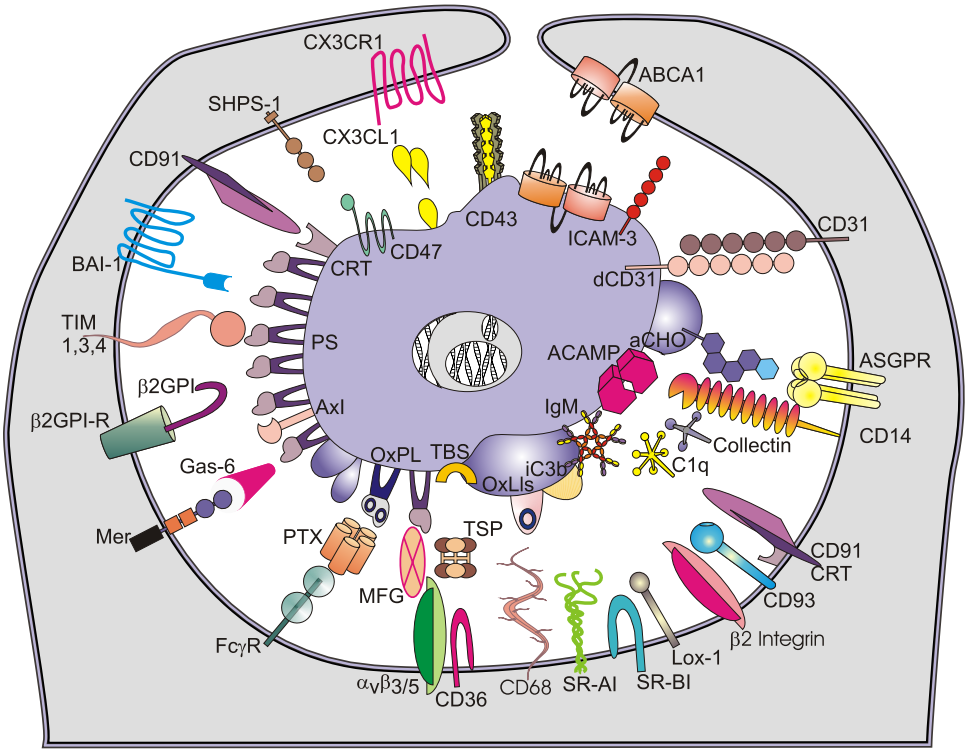

Figure 2. Molecules implicated in the binding and phagocytosis of ACs by phagocytes are shown on this cartoon of a "superphagocyte" and schematic AC. β2GPI-R, β2-glycoprotein I receptor; ABCA1, ATP-binding cassette transporter 1; aCHO, altered carbohydrate; ASGPR, asialoglycoprotein receptor; BAI-1, brain-specific angiogenesis inhibitor 1; CRT, calreticulin; dCD31, disabled CD31; MFG, milk-fat globule EGF-8; OxLls, oxidized LDL-like site; OxPL, oxidized phospholipid; SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1; TBS, TSP-binding site; Tim 1,3,4, T cell Ig and mucin domain-containing molecule 1, 3, or 4. Adapted from ref. [20].

2) are established, active components of the innate immune system and include SRs, complement components and their receptors, and soluble and cell-associated pattern recognition molecules. The concept of pattern recognition proposes that pathogens are identified by means of evolutionarily conserved patterns (PAMPs), which are recognized by specialized PRRs [29]. Given the strong, immune-activating function proposed for PRRs, it is intriguing that the innate immune system is involved in AC clearance.

Macrophage PRRs and AC clearance

CD14, the prototypic PRR, promotes noninflammatory AC recognition and phagocytosis [30], while mediating proinflammatory responses [31, 32] and septic shock [33, 34] to PAMPs (e.g., LPS). CD14 appears to tether ACs to macrophages [18], although a function for CD14 beyond the tethering stage (e.g., phagocytosis) remains possible, and its association with other molecules within the phagocytic synapse remains an important area for study, as this may influence the potential inflammatory outcome of CD14 ligation.

Notably, CD14 appears nonredundant (or only partially redundant) in its function. Knockout mice reveal a dramatic clearance defect in vivo with persisting ACs in many tissues, although such mice are healthy and fertile and do not suffer the devastating inflammatory consequences of failed AC clearance, suggesting that immunomodulatory functions were still intact [18].

Although CD14 was the first "defense" receptor implicated in AC clearance, it was not the first PRR to be implicated in this process. Early studies of AC clearance largely identified SRs [35–40], which comprise a family of apparently highly redundant receptors for modified forms of LDL [41] and demonstrate conservation of function throughout evolution. In 1992, the class B SR CD36 was reported to function for AC clearance in conjunction with the previously identified integrin αvβ3 (the vitronectin receptor) and a soluble bridging molecule TSP. Later, *Croquemort* was discovered in *Drosophila* as a CD36 superfamily member with equivalent capacity to clear dead cells in flies. Since these early studies, a variety of other SRs—Lox-1, CD68, SR-AI, SR-AII, and SR-BI—has been implicated in the removal of dead and dying cells. This is a family of receptors that unlike CD14, appears to exhibit redundancy. In relatively few cases do animals deficient for molecules implicated in AC clearance exhibit defective clearance of ACs. For example, SR-A-deficient mice appear to clear ACs effectively [42], despite a clear role for SR-A-mediated AC clearance in vitro [40].

Over recent years, different PRR classes have been identified, and understanding of the TLRs, NLRs, CLRs, and RLRs is expanding [43]. These receptor classes reside throughout the cell to detect ligands of microbial and nonmicrobial origin (e.g., DAMPs released from injured, non-ACs) and signal to promote immune responses appropriate to the ligand [43–45]. There is little evidence for the involvement of these receptor classes in AC clearance, although given similarities in ligands for CD14 and these receptors (e.g., muramyl dipeptide from peptidoglycan is bound by CD14 [46, 47] and NOD-2 [48, 49]), a role in binding and responses to ACs are possible.

Soluble factors

The involvement of pattern recognition in dead cell clearance is even more striking when one considers the extensive range of soluble, bridging molecules known to opsonize ACs and mediate their uptake (reviewed in refs. [20, 50]). These solu-

ble opsonins include complement components [51–56]. An in vivo deficiency of C1q results in defective AC clearance and severe pathology and autoimmune consequences [51, 57].

The collectins, a family of C-type lectins, including SP-A and SP-D (lung collectins), along with MBL, have all been shown to bind and opsonize ACs for uptake [19, 54, 58–60]. It is of note that an in vivo deficiency in MBL, such as CD14, results in deficient AC clearance without associated pathology [19].

A further family of soluble factors includes the PTXs, with C-reactive protein and serum amyloid protein, which can promote AC clearance [61–63]. The long PTX3 also binds ACs but has been suggested to moderate AC clearance [64–66]. The presence in this list of positive, acute-phase proteins suggests that these molecules may play an active role in the resolution of inflammatory responses [50].

Although CD14 was identified initially as a membrane receptor [67, 68], it also exists as a soluble plasma protein [69–71]. Its soluble counterpart has been proposed to function as a soluble pattern recognition molecule for the recognition of LPS and to promote responses in cells that do not express CD14 [72, 73]. Although involvement of soluble CD14 in binding to ACs has been shown experimentally, its role in mediating AC clearance is not proven.

DISTINGUISHING DEATH

Specific recognition of dead and dying cells is essential and during apoptosis, a loss of "don't eat me" signals and a gain of "eat me" signals to phagocytes. However, relatively little is known about the identity and structure of the AC-associated ligands for phagocyte receptors.

The most characterized of these specific surface changes is the exposure of the anionic phospholipid PS [74, 75]. The distribution of PS is poorly controlled in apoptosis, and its presence in the outer leaflet of the plasma membrane is prolonged, generating a negative charge at the cell surface, and can mediate clearance of ACs [76]. PS, also present in viable cell membranes, sets an important example of how molecule relocation (rather than fundamental structural changes) can underlie a novel function. Beyond PS, the identification of specific eat me signals has proved challenging. Other, less defined, changes indicate an involvement of sugars [77, 78], oxidation of PS [79], and exposure of intracellular components (e.g., AxI [80]).

ICAM-3 becomes functionally altered during apoptosis to mediate clearance of apoptotic leukocytes through suggested generation of neo-epitopes (i.e., eat me signals) [81]. However, the proposed changes in ICAM-3 that may underlie its change of function have remained elusive, although it may be structurally altered (e.g., glycosylation changes) or may relocate to support its change of function. The loss or gain of partner molecules at the cell surface during apoptosis may generate or alter receptor specificity for any given ligand on ACs.

The ability of phagocytes to clear dead cells is finely balanced, although the detail of this important balance is still to be fully elucidated. Coupled with the appearance of surface alterations on dead cells is an apparent loss of other (viable cell-associated) signals. The analysis of CD31 suggested that molecules on viable cells may provide so-called don't eat me (inhibitory) signals, which are lost during apoptosis, permitting viable cellular interactions (e.g., CD31-CD31 in trans) to become functional phagocytic interactions [82]. Further support for this has been provided through a report that disruption of CD47 on ACs removes inhibitory signals, thus allowing cell clearance [83]. It seems likely that gain of eat me signals and loss of don't eat me occur at the surface of the AC, and together, they facilitate cell removal.

Given the shortage of data describing structural alterations within specific molecules during apoptosis, it seems likely that careful analysis of the relative location and topology of molecules will prove important in underlying any change of function associated with a molecule on dying cells. For example, PS exposure on ACs has been noted to occur in patches, a redistribution that may be essential to its function [84, 85].

Similarity between pathogens and ACs

The significant involvement of the innate immune system in recognition of pathogens and dying cells raises questions relating to the structural similarity of pathogens (unwanted "nonself") and ACs (unwanted "self") and has led to the suggestion that ACs, like pathogens, carry conserved molecular patterns, which when recognized, promote their clearance; these are termed ACAMPs [86, 87]. A key question is whether there is structural overlap between ACAMPs and PAMPs. As CD14, lactoferrin, and anti-LPS antibodies reportedly bind ACs and LPS, one may speculate that ACAMPs appear "LPS-like" in structure [15]. The ligand repertoire of CD14, however, is large [88, 89], although it is unclear whether within these diverse ligands, there resides a small, conserved submolecular pattern that is recognized.

PHAGOCYTE RESPONSES TO ACS

Although AC clearance is considered anti-inflammatory (reviewed in ref. [90]), in part, through the release of immunomodulatory cytokines [91–93], evidence indicates that this is a far-from-simple system of control. ACs promote proinflammatory responses to PAMPs over early time-points, suggesting that multiple inflammatory control mechanisms are working together over the period of stimulation [94]. Given the extensive involvement of the innate immune system in AC clearance and protective inflammatory responses to pathogens, a key remaining question within this field is what dictates the net anti-inflammatory response following ligation of PRRs with ACs. Ligation of CD14 by different ligands (ACs or PAMPs) leads to opposing responses, where CD14 may represent an important decision point in the inflammatory responses of macrophages. The molecular basis for this dichotomy has yet to be defined and is similarly important for other implicated PRRs.

A number of possible models have been put forward to explain the divergence of response following ligation of PRRs by PAMPs or ACAMPs [20].

Most simply, structural similarity between PAMPs and ACAMPs may not exist, so their ligation of PRR may differ and signal via different mechanisms for pro- and anti-inflammatory

responses, respectively. The molecular basis for this may reside in precise residues within CD14, which are ligated by LPS and ACs, and detailed mapping of CD14 is required.

A further basis may be in the constitution of the phagocytic synapse and inherent signaling partners. Thus, the molecular composition of the phagocytic synapse is a further important area for study. CD14, a GPI-anchored glycoprotein, requires signaling partners to elicit cellular activation following ligation [95]. Studies suggest that following CD14 ligation with PAMPs, a range of signaling molecules and receptors, including TLRs, is recruited to generate a signaling complex [96, 97]. It seems likely that the nature of CD14 function will rely on the constitution of its signaling complex, which has been shown to alter in a ligand-specific manner [98]. An attractive hypothesis for testing is that the involvement of TLRs in the CD14 signaling complex may alter when ligated with ACs or PAMPs. Some support for this may be afforded by observations that TLR4 deficiency did not adversely affect the ability of macrophages to bind and engulf ACs [99].

Should structural similarities between ACAMPs and PAMPs exist, this would indicate that phagocytes do not discriminate ligands and subsequently, direct responses at the level of the PRR. In this situation, ligation of CD14 with ACAMPs may initiate proinflammatory signaling. The divergence of responses must then result downstream of the CD14-containing signaling complex. Again, the nature of the phagocytic synapse may dictate the outcome, with large complex ligands (e.g., ACs) recruiting receptors/signaling pathways, resulting in alternative, downstream signaling that may mitigate the inflammatory consequences of PRR ligation. Reports have highlighted a mechanism by which CD14-TLR inflammatory signaling may be inhibited with a negative cross-talk reported between receptor tyrosine kinases and TLRs. Mice defective in these three family members succumb to profound systemic autoimmune disease, resulting from a failure to induce members of silencer of cytokines, which inhibit TLR signaling in these mice [100, 101]. Interestingly, this family of tyrosine kinases has been shown to mediate binding ACs and mediate clearance [26, 102]. Ligation of CD14-TLR, in addition to ligation of Mer tyrosine kinase, may lead to a dominant anti-inflammatory effect and provide a crucial point of control in the inflammatory process within sites of inflammation. Gas-6 has been shown to activate an anti-inflammatory pathway to modulate LPS-induced proinflammatory cytokine responses in monocytes and macrophages [103].

WHICH UNDERTAKER FINDS YOU?

The decision as to which phagocyte undertakes dead cell clearance at any site may be a simple competition. With low-level death, local amateur phagocytes may remove the corpse, although should the phagocytic capacity of these cells be overwhelmed, macrophages will infiltrate to augment clearance by scavenging dying cells in the late stages of apoptosis [20].

Most phagocyte studies in vitro rely on gravity to associate ACs and phagocytes. In reality, the phagocyte will move to the corpse, a movement that has been an often-neglected part of the process until relatively recently. How do macrophages know where to scavenge? It is possible that in some cases, resident tissue macrophages simply patrol and chance upon dying cells in a stochastic manner. However, over recent years, there has been an increase in studies demonstrating a highly active recruitment of macrophages to ACs. Within these studies, a series of molecular mechanisms has been proposed, which has been reviewed extensively elsewhere [21, 104, 105].

Phagocyte recruitment to situations of high-level death is essential. This is clearly evident in, for example, models of sterile inflammation, where "waves" of cells infiltrate in a predictable manner to result, ultimately, in the resolution of this inflammation [106, 107]. In this context, a range of mechanisms has been proposed to function in this elegant and self-limiting inflammatory system [108–110]. **Fig. 3** shows a macrophage approaching a monocyte dying by apoptosis. This directional movement results ultimately in clearance of released blebs, followed by binding of the larger cell corpse.

Attractants released from dying cells include soluble proteins such as RP S19 [111], whose attractive function appears dependent on its dimerization (to form dRP S19), possibly through the action of tissue transglutaminse-2 [112], and it exerts its effect through CD88, the GPCR for complement component C5a [113]. EMAP II, following caspase-dependent processing, also mediates phagocyte recruitment [114], along with human ttRS, which following release from ACs and proteolytic cleavage, exerts its chemokine function to recruit phagocytes. Lysophosphatidycholine was the first lipid-based

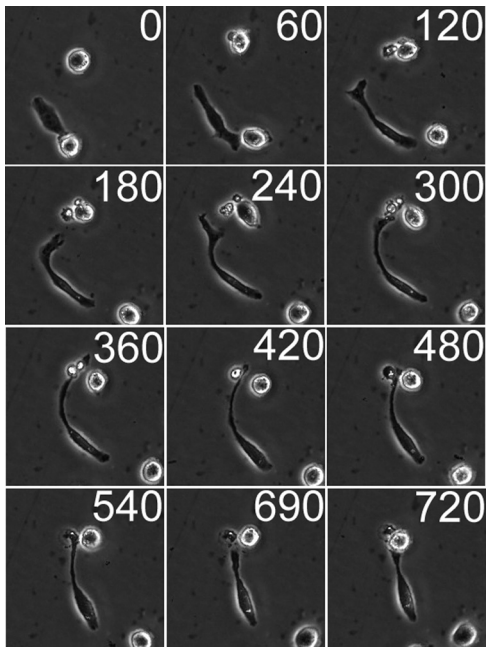

Figure 3. Movement of a macrophage toward an apoptotic monocyte. The images depict the active movement of a macrophage toward a dying monocyte. The dying monocyte is visible as the upper cell in all panels, and blebbing is visible from 60 min from start of image capture. The macrophage first binds and removes the apoptotic bodies and then turns its attention to the remaining AC body. The time in minutes from the start of image capture is shown.

chemoattractant identified from ACs [115]. Its caspase- and phospholipase-dependent release results in recruitment of monocytes via the phagocyte GPCR G2A [116]. Most recently, nucleotides released from ACs have been reported to act as phagocyte attractants [117]. In this study, ATP and UTP were released to attract monocytes and macrophages in a selective manner through the GPCR P2Y2. A number of other putative chemoattractants are also reviewed elsewhere, although the specificity of their action and the timing, source, and/or mechanism of release require further study [105].

Complex particulate material also exerts important phagocyte recruitment effects. Segundo et al. [118] characterized loss of membrane and associated surface proteins from B cells during apoptosis via release of "blebs" following zeiosis. These particulates (also known as microparticles) exert chemoattractive potential on monocytes. Despite this work highlighting a number of adhesion molecules present within the released microparticles from B cells, the actual molecular mediators of chemoattraction remained ill-defined until recently. Work by Truman et al. [119] identified CX3CL1 as a key chemokine released, at least in part, in microparticles from apoptotic B cells. This classical chemokine, the first described in this context, exerts its effects through its receptor CX3CR1, where it is proposed to also function as an adhesion molecule. Although the involvement of CX3CL1 has been limited to recruitment toward apoptotic B cells, the expression of CX3CL1 may not be so restricted as reported. CX3CL1 is expressed, in some cases following immune challenge in a wide range of cells, including smooth muscle cells [120], endothelial cells [121–123], fibroblasts [124], astrocytes [125], mesangial cells [126], renal tubular epithelial cells [127], and monocyte cell lines [128]. This raises the possibility that CX3CL1 may be a more general recruitment factor than anticipated initially, although further work is needed.

Many molecules will be released in microparticles from ACs, and Fig. 2 shows potential candidates already implicated in AC clearance. Preliminary work shows ICAM-3 is released in microparticles from apoptotic B cells and promotes chemoattraction of macrophages [129]. Furthermore, Hart et al. [130] demonstrated preferential loss of ICAM-3 over CD31 from dying neutrophils, suggesting ICAM-3, a known adhesion molecule on viable cells, may be released preferentially from dying cells to promote their chemoattractive capacity. Indeed, given the clear role for chemokines in recruitment to ACs, it is entirely reasonable that adhesion molecules are required to effect the chemoattraction. Such similar chemokine:adhesion molecule functional associations exist in other systems, e.g., leukocyte extravasation.

The surface constitution of microparticles and AC requires further study. However, AC surface protrusions (blebs) have been shown to contain relocated/concentrated components, including phagocytic markers, PS [84], C1q, and MBL [54, 131, 132]. Such protrusions are suggested to be the precursors to released microparticles and as such, may suggest that microparticles are smaller, tastier, more edible, and consequently, attractive to phagocytes. A key feature of ACs and bodies is surface charge, which is known to affect phagocytosis of yeast cells [133], and the exposure of PS generates a negative surface charge, which promotes cell sprouting toward ACs [134]. A flow of negatively charged microparticles from ACs may act to provide an electric current to attract macrophages in a manner similar to the effect of electrical signals in wound healing [135, 136].

The timing of attractant release will likely be crucial in the appropriate recruitment of phagocytes. EMAP II has been suggested to function late in the death program and perhaps constitute a back-up recruitment call should additional phagocytes be required to deal with persisting death [137]. Microparticle generation following zeiosis occurs early in apoptosis [138] and may therefore represent a key, early attractive event for early phagocyte recruitment.

The cellular site of action of these attractants will also be important. Certainly, EMAP II and ttRS exert effects on different populations, recruiting granulocytes and monocytes [139, 140]. Lactoferrin is an important factor preventing granulocyte recruitment, essential if AC clearance is nonphlogistic [2, 141]. Nucleotides selectively recruit monocytes and macrophages, although whether there is any subtype specificity is not known [117]. The extent to which different attractants recruit different subpopulations of phagocytes may impact on the inflammatory outcome of any site of death and suggests a point of intervention where modulation of recruitment may be sought for therapeutic benefit.

Characterization of these functionally important, AC-derived microparticles remains an important focus for research. Although we know a little about their constitution and function, it seems likely that they hold many other important molecular agents, for example, the lipid mediators lipoxins, resolvins, and protectins, so important in control of many inflammatory situations [108, 110, 142]. It seems likely that they may have an important role to play in the modulation of phagocyte recruitment toward dying cells in situations other than acute inflammation and thus, help prevent inflammation associated with failed AC clearance. Consequently, it will be important to characterize the lipidomics and proteomics of AC-derived microparticles.

PROFESSIONAL PHAGOCYTE POPULATIONS

Phagocyte heterogeneity can be confusing and may arise from inherent plasticity within macrophages. Different subpopulations of macrophages undertake different functions, and varied macrophage receptor expression from different anatomical locations has been clearly demonstrated [143]. Polarity within macrophages is well-established (reviewed in refs. [144, 145]). "M1"- and "M2"-tyPE macrophages have been functionally defined as "classically-activated" (Proinflammatory, cytotoxic) macrophages and "alternatively-activated" (reparative, scavenger) macrophages, respectively [145]. Clearance of ACs and possibly microparticles by macrophages may skew the phenotype toward M2 and lead to deactivation through the effects of the anti-inflammatory cytokines TGF-β1 and IL-10 (reviewed in ref. [90]). In reality, of course, the precise macrophage phenotype may be any one of a huge number along a continuum of phenotypes altered by the microenvironment, in which the

phagocyte finds itself. It is unclear whether in vivo ACs recruit different subpopulations of phagocytes, although it has been proposed that ACs within tumors may recruit macrophages and drive their phenotype toward an anti-inflammatory M2 type, such that they may support tumor growth [15].

Monocytes also exhibit subpopulations, characterized by surface receptor expression, e.g., CD14 and CD16 [146], which exhibit different functions [147]. The monocyte subsets recruited to sites of apoptosis may be defined by chemokine receptors carried, and further work is needed here. However, when considering the chemokine receptor CX3CR1, there are interesting associations, as monocytes positive for this receptor are preferentially recruited to atherosclerotic plaques. Given the nature of the plaque, a nonresolving inflammatory site with abundant ACs and failed clearance [148, 149], it is possible that CX3CL1, in apoptotic microparticles, may act to recruit monocytes to this pathological site. In atherosclerosis, CX3CL1 is found on foam cells and is important in recruitment of macrophages to the vessel wall during atherogenesis (see review, ref. [150]). It will be of interest to assess if apoptotic foam cells release CX3CL1 in microparticles and if this is a mechanism used to recruit phagocytes to plaque. Interestingly, the clearance of ACs within plaque appears defective, despite in vitro models, suggesting no fundamental defect. This failure to adequately deal with apoptosis promotes plaque growth and instability through secondary necrosis [148, 149]. Phagocyte recruitment to the plaque is significant, but the nature of the recruited phagocyte may well impact on the inflammatory outcome.

FUTURE DIRECTIONS—AN AGING PERSPECTIVE

The innate immune system, vital to detection and proinflammatory control of infection, also mediates noninflammatory clearance of dying cells. Although apparently counterintuitive, this highlights the powerful control mechanisms preventing unwanted inflammatory consequences of AC clearance. As such, an improved understanding of AC clearance will provide novel opportunities for therapeutic modulation of inflammatory conditions.

Our understanding of pattern recognition of microbial PAMPs and the orchestration of inflammatory responses is expanding rapidly with the analysis of different classes of PRRs, including TLRs, RLRs, CLRs, and NLRs (reviewed in refs. [43, 151]). Ligation of these different receptor classes in different cellular locations (e.g., cell surface, endosomal, cytoplasmic) initiates proinflammatory consequences and ultimately activates the adaptive immune system (reviewed in ref. [152]). These receptors are also activated by ligands of nonmicrobial origin, i.e., damaged cells. However, our understanding of the inflammatory control of AC-PRR ligation is not so comprehensive, and there are many outstanding questions over the control of inflammatory responses to ACs. It is reported that ACs don't activate these PRR classes, possibly through sequestration of stimulatory DAMPs [153], but it is unclear if AC ligands fail to ligate these PRRs or additionally exert a dominant, anti-inflammatory signaling. The role that these different receptor classes may play in supporting tolerogenic antigen presentation is unclear and is an important area for study.

Although molecular deficiency can inhibit AC clearance and result in inflammatory consequences (e.g., complement deficiency and autoimmunity [53]), it is unclear how defective removal of or responses to ACs may impact on inflammation in "normal" individuals. It is noteworthy that inflammatory diseases associated with consequences associated with AC clearance genes (reviewed in ref. [21]) are conditions strongly associated with aging (e.g., atherosclerosis, cancer, autoimmunity, arthritis) and suggest that clearance of and responses to AC may be impaired during aging, which is an inescapable, proinflammatory phenomenon (termed "inflamm-aging" [154]), yet remarkably little is known about the effect of aging on the immune system and how this may affect AC clearance, its consequences, and microbial defense.

Aging may exert profound effects on the innate immune system (reviewed in refs. [155, 156]), including increased phagocyte number and decreased cellular proinflammatory cytokine production, coupled with increased total plasma level of the cytokines, as a result of prolonged phagocyte activation. These effects point to a dysregulation of inflammation associated with age. Such age-associated dysregulation may also occur within those molecular mechanisms, which ensure that AC clearance is efficient and dominantly anti-inflammatory. A detailed analysis of the innate immune system in aging may provide valuable insights to the mechanisms involved in responses to ACs.

In this review, we have highlighted the importance of recruiting the most appropriate phagocyte to mediate the desired ligand-dependent responses, and the nature of cell death signals has been suggested to dictate recruitment [45]. Whether inappropriate subpopulations of phagocytes are recruited to cell death, leading to inflammation, or whether during aging, phagocyte subpopulations change is an important area for study. In aging, neutrophils exhibit defective chemotaxis [157]. To assess chemotaxis of aged macrophages to a range of stimuli, including ACs and microparticles, will be important. However, in atherosclerotic plaques, monocyte recruitment is high, suggesting normal chemotaxis, although detailed study of recruited-cell phenotype will address whether a subpopulation of monocytes (possibly age-modified) or the plaque environment is key to failed resolution of inflammation. Nevertheless, the phenotype of a recruited phagocyte will often be pivotal in deciding the outcome [22].

Aging modifies innate immune receptor expression and function. Neutrophil FcγRs and phagocytic capacity are reduced on neutrophils from aged individuals [158]. FcγRs promote apoptotic neutrophil opsonization and consequent clearance [159, 160], and CD14 levels are reduced on aged macrophages [156]. Such age-associated changes may lead to age-related persistence of ACs and exemplify how an age-compromised innate immune system may impact on the ability of phagocytes to effectively deal with ACs. Should AC persistence lead to necrosis, released DAMPs would be proinflammatory [153]. It will be important to assess the competence of aged phagocytes to raise appropriate immunomodulation in response to ACs. This raises the possibility that aging may

modulate our ability to clear or respond appropriately to dying cells.

Although our knowledge of AC clearance has expanded rapidly over the past 25 years, there is a need to more fully understand the mechanisms by which dying cells orchestrate anti-inflammatory clearance by phagocytes, a process that comprises an important and relatively unexploited natural resource that will provide novel insights into mechanisms of physiology and disease while raising the possibility of novel therapies through modulation of this process (e.g., for inflammation, cardiovascular disease, and autoimmunity). The study of normal and diseased states has proved invaluable, but here, we propose that the study of aging immune systems may further promote this path of discovery.

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