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Phagocytosis: Elegant Complexity

Review

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Phagocytosis requires receptor-mediated recognition of particles, usually in the guise of infectious agents and apoptotic cells. Phagosomes fuse with lysosomes to generate phagolysosomes, which play a key role in enzymatic digestion of the internalized contents into component parts. Recent findings indicate that a simple paradigm of a single cognate receptor interaction that guides the phagosome to phagolysosome formation belies the complexity of combinatorial receptor recognition and diversity of phagosome function. In fact, phagosomes are comprised of hundreds of proteins that play a key role in deciphering the contents of the phagosome and in defining host response. In this review we discuss how the challenge of recognizing diverse molecular patterns is met by combinatorial interactions between phagocytic receptors. Furthermore, these combinations are dynamic and both sculpt the balance between a proinflammatory or anti-inflammatory response and direct phagosome diversity. We also indicate an important role for genetically tractable model organisms in defining key components of this evolutionarily conserved process.

Phagocytes, Phagocytosis, and Innate Immunity

Phagocytosis was popularized at the end of the nineteenth century by the Russian embryologist Ilya Metchnikoff, who observed that amoeboid-like cells in transparent sea star larvae contained ingested cells. He hypothesized that these cells would be able to recognize and internalize foreign material. Metchnikoff proved this idea in a simple experiment in which he observed that these cells moved toward and engulfed a thorn that he had introduced into a larva. Based on these findings, he extrapolated that these so-called phagocytes were capable of ingestion and might play a key role in host defense and tissue homeostasis, which intricately linked them with inflammation in man (Metchnikoff, 1905). Remarkably, over the next century and a quarter, many of Metchnikoff's ideas as to the origins of inflammation have been validated. What he could not have foreseen, however, is the enormous heterogeneity and complexity of phagocyte biology.

In mammals professional phagocytes (i.e., macrophages, dendritic cells (DCs), and granulocytes) derive from a common myeloid progenitor cell. Specific combinations of inductive events instruct differentiation of these common progenitors to their mature progeny. It is pertinent to note that during early embryogenesis, coincident with the establishment of a circulation, myeloid cells constitutively populate newly formed organs (Gordon et al., 1986). These tissue macrophages have a distinct surface phenotype (CX3CR1^{hi}, CCR2^{-},Gr1^{-}) and appear to traffic constitutively from the blood to the tissues throughout adult life (Geissmann et al., 2003). In the mouse these cells are able to differentiate into DCs and, together with mast cells, NKT cells, B1 B cells, and γδT cells, form the sentinels at the potential portals of microbial entry. Diverse inflammatory signals rapidly mobilize polymorphonuclear leukocytes and a short-lived subset of inflammatory macrophages together with other plasma components, which all serve as key components of the innate immune response (Hoffmann et al., 1999). Phagocytes are required to continually sense and edit the extracellular environment. This constant surveillance requires a set of distinct cell surface receptors that have redundant and nonoverlapping repertoires for recognizing and responding to infectious and noninfectious injury. Janeway proposed that these canonical, germline-encoded, and invariant receptors directly recognize pathogens and popularized the concept of "pattern recognition receptors" (Janeway, 1989).

In this review we will first focus on the complexity of pattern recognition receptors and subsequent phagosome diversity in mammalian phagocytes and then describe the potential role for model systems in deconstructing phagocytosis down to the essential nonredundant components. We will not discuss the regulation of the cytoskeleton in detail, as it is extensively covered in other specialist reviews (Aderem and Underhill, 1999; Greenberg and Grinstein, 2002; Underhill and Ozinsky, 2002) but, rather, present a conceptual overview using certain selected examples to illustrate our points.

How You Eat

Phagocytic Receptors: Direct Recognition of Targets

The surface of the phagocyte is adorned with many receptors that are able to recognize and decode their cognate ligands expressed on the surface of infectious agents and apoptotic cells and trigger engulfment (Figure 1). These receptors either directly recognize the particle or recognize targets coated in opsonic molecules (see below). Although these ligands were originally referred to as pathogen-associated molecular patterns or PAMPs, this definition neither includes the recognition of commensal bacteria nor apoptotic and necrotic cells. For this reason we propose "molecular pattern" (MP) as a more inclusive term and will use it in this review.

Early experiments, constrained by the tools available at the time, were by necessity reductionist and aimed at identifying and defining the structure and function of individual phagocytic receptors. The macrophage mannose receptor (MMR) was one of the earliest phago-

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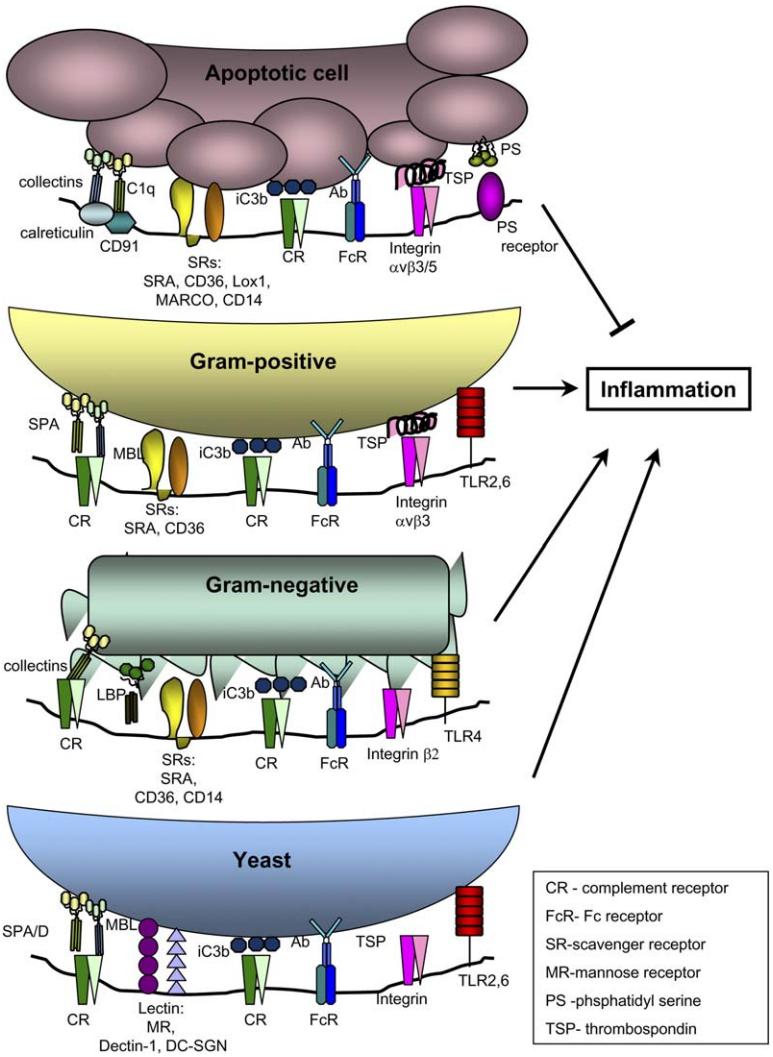

Figure 1. Commonality of Phagocytic Recognition of Apoptotic Cells and Pathogens

Particles are recognized by a combination of scavenger receptors, integrins (complement receptors), and lectins binding directly or via opsonins such as LBP, TSP, or collectins. Most of these receptors are able to recognize both pathogens and altered self-ligands such as apoptotic cells. In addition, phagocytes have specific receptors that discriminate pathogen-associated components causing inflammatory responses. As an example, recognition of gram-positive bacteria by TLR2 and TLR6 or gram-negative by TLR4 is shown. In contrast, anti-inflammatory signals are triggered after binding of apoptotic cells that expose phosphatidyl-serine (PS) on their cell surface. Ligation of receptors for apoptotic cells (especially the receptor for PS) causes liberation of TGFβ and other immunomodulatory cytokines. Hence, multiple receptors are involved in recognition with certain common receptors mediating internalization of both pathogens and self-ligands. Together these receptor complexes contribute to discriminating and initiating appropriate responses.

cytic receptors cloned and represents a paradigm in this regard (Ezekowitz et al., 1990; Taylor et al., 1990). MMR was first defined as an endocytic receptor that recognized mannosyl- and fucosyl-containing neoglycoproteins (Stahl et al., 1978). The molecular characterization of this protein revealed that it was the first member of an ever-growing family of C type lectins that require calcium for ligand binding to the multiple carbohydrate binding domains. The mannose receptor contains a tandem array of eight prototypic lectin folds that consists of two antiparallel β strands and two α helices. Transient overexpression of the MMR in Cos7 cells revealed that MMR was able to recognize yeast, certain bacteria, and *Pneumocystis carinii* (Ezekowitz et al., 1990, 1991). However, it was clear even at that time that preincubation of macrophages with the soluble yeast wall product mannan, a high-affinity MMR ligand, resulted in only partial inhibition of phagocytosis, thus providing circumstantial evidence for the existence of additional receptors that recognize yeast. The original reductionist view has been superseded as knowledge has advanced and many other receptors that are capable of recognizing similar MPs as those recognized by MMR have been characterized. These include other members of the family of C type lectin transmembrane proteins such as DC-SIGN, L-SIGN, DEC-205, Endo-180, Langerin, DCAL-1, BDCA-2 and Dectin-1. Each of these molecules has the potential to recognize subtle differences in the structure of displayed carbohydrate ligands that define their cognate MP. The array of these carbohydrate ligands, while generally conserved on the surface of micro organisms, have the potential to undergo subtle alterations that are predicted to redefine the binding affinity of any one lectin receptor for that particular infectious agent. In order to combat this potential evasive strategy adopted by infectious agents, phagocytes do not rely on any one receptor for recognition of a pathogen. An illustration of the cooperative action of two lectin receptors is best illustrated by experiments that demonstrate colocalization of MMR and DC-SIGN in a *Candida albicans*-containing phagosome (as discussed in the review Cambi et al. [2005]). In this study the presence of only these two receptors was probed, and it is likely that other candidate molecules

that all have the potential to recognize this ligand, including Toll-like receptor (TLR) 2, other multi-lectin receptors, and a class of receptors called scavenger receptors, might also be found within these phagosomes.

Phagocytic Receptors: Defining Response

Importantly, receptors not only trigger engulfment but also act to define the consequences of phagocytosis as either proinflammatory or anti-inflammatory. The TLRs play a key role in mediating sensing and signaling of downstream effectors in response to a number of well-defined ligands. TLR ligands are highly diverse but demonstrate a key common feature: these ligands are invariant and necessary components of pathogens that are absent from host cells and include bacterial derivatives (such as lipoteichoic acid [LTA], lipopolysaccharide [LPS], flagellin, peptidoglycan, and CpGDNA) and components associated with viral replication (ss and dsRNA). However, TLRs are not phagocytic receptors and for many TLRs, ligand engagement does not occur on the cell surface but after internalization into the endolysosomal or phagocytic compartment (Latz et al., 2004; Underhill et al., 1999).

However, a consensus is emerging that TLRs function not only in combinations with one another (reviewed in depth in Akira and Takeda [2004]; Underhill and Ozinsky [2002]) but also with a number of other pattern recognition and phagocytic receptors, thereby adding to the diversity of recognition. One such receptor is Dectin-1, a β-glucan receptor that recognizes the yeast cell wall product zymosan in the context of TLR2 and TLR6, as surmised from colocalization of these three molecules in the contact points around the zymosan particle (Gantner et al., 2003). Recent work has begun to decipher the complex signaling cascades that allow Dectin-1 to trigger diverse responses to yeast (Rogers et al., 2005). Upon ligation by zymosan, the protein tyrosine kinase Syk is recruited to the immunoreceptor tyrosine-based activation motif (ITAM) contained within the intracellular domain of Dectin-1 and stimulates production of IL10. This cytokine profile contrasts with that produced by DCs when costimulated via Dectin-1 and TLR2 to induce IL12 and TNF. These divergent outcomes are similar to those of Fc receptors (FcR) that also signal via immunoreceptor tyrosine-based motifs (see below). This interesting study provides important information concerning the molecular basis for the diverse responses possible after ligation of a single receptor and it is likely that other receptors that act to fine tune responses to pathogens will also have similar ligand-dependent intracellular signaling cascades. However, it is important to put the recognition of these particles in physiological context, and it should be noted that both *Candida albicans* and zymosan are excellent targets for three potential opsonins: complement, mannose binding lectin (MBL), and, in the primed host, antibody (see below). It is likely that these also contribute to the complexity of ligand recognition.

To provide specific immunological meaning to phagocytosis is a particular challenge for multi-ligand receptors (including scavenger receptors) because of their broad specificity and capacity to bind a wide variety of pathogens. The ligands for these receptors are varied and include pathogen-derived LTA, LPS, and other lipopeptides. In addition, and a common emerging theme amongst multi-ligand receptors is their ability to recognize "modified self" such as β-amyloid, oxidized and acetylated lipid, and apoptotic cells. The immunological outcome of engagement of these receptors is diverse. It is not fully understood whether this depends on the receptor engaged, the cargo internalized, or whether phagocytic receptors subtly modify "hard-wired" signaling from other receptors, such as TLRs, that are also engaged during phagocytosis. CD36 is a prime example of such diversification of response from a single receptor; it is required for nonphlogistic recognition of apoptotic cells (Savill et al., 1992) but has recently been shown to also contribute to LTA-dependent triggering of TLR2 (Hoebe et al., 2005). A similar paradoxical role is also true for scavenger receptor A (SRA), which is required for both response to pathogen invasion (Suzuki et al., 1997; Thomas et al., 2000) and recognition of dying cells (Platt and Gordon, 1998). How CD36, CD14, SRA, and other such molecules can be involved in both proinflammatory and anti-inflammatory recognition remains to be defined, but it's likely that it is a result of distinct combinations of signaling molecules that associate with these receptors with different ligands (as discussed for Dectin-1 and Fc receptors) or due to events that occur after internalization such as signaling initiated from within the phagosome or the cytosol as discussed below.

Opsonic Phagocytic Receptors: Adding Value to the Meal

Opsonization coats the target and allows generic receptors to mediate engulfment, thereby increasing efficiency and diversifying the recognition repertoire of the phagocyte (Ezekowitz et al., 1984). This is of particular importance for particles that are not immediate ligands for phagocytic receptors. In this regard the MBL is an important circulating opsonin that has a carboxy-terminal lectin domain and associates as multimers of trimers. This structure allows MBL to function as a canonical circulating pattern-recognition molecule, able to recognize a broad range of infectious agents ranging from bacteria, yeasts, parasites, and the envelope glycoproteins of certain viruses as well as apoptotic cells. The recent generation of MBL null animals has confirmed the in vivo importance of this molecule in recognition of *S. aureus* and apoptotic cells (Shi et al., 2004; Stuart et al., 2005). MBL ligand interactions trigger activation of the complement cascade, and, thus, clearance of MBL ligand complexes by phagocytes may occur either via complement receptor CR3 or via so-called collectin receptors. The engagement of complement receptors triggers a distinct form of Rho-dependent phagocytosis, characterized by the "sinking" of the particle into the cell without triggering proinflammatory mediators (Aderem et al., 1985). What is not clear is whether a critical density or distribution of the cleaved third complement component, C3bi (the ligand for CR3), is required to trigger a predominantly CR3-mediated effector function.

It is likely that the same particle or pathogenic agent that fixes complement might also trigger an antibody response and thus become opsonized and engage one of the FcRs. The ligation of FcR induces Rac activation and pseudopodia formation (Caron and Hall, 1998). The

diversity of Fc-mediated phagocytosis and signal transduction has been well defined and is the subject of several excellent reviews (Aderem and Underhill, 1999; Ravetch and Bolland, 2001). It is clear from these extensive studies that for certain classes of FcRs, proinflammatory signaling is initiated via an ITAM whereas others signal via an inhibitory motif (ITIM) to downregulate responses. Importantly, the regulation of this system provides a paradigm as to how receptors ligated by the same ligand (antibody) might evoke divergent responses.

The ability of complement and antibody to define the response to phagocytosis is an important feature for many opsonins. This is exemplified by thrombospondin, which acts to bridge malaria-infected erythrocytes, apoptotic cells, and other ligands to its receptors, CD36, CD47, and integrin $\alpha v\beta 3$ (CD51/CD61). This multifunctional molecule has numerous, distinct structural domains, providing it with a broad spectrum of biological activities including the ability to activate the important immunoregulatory cytokine, TGFβ (Crawford et al., 1998). Similar functions have recently been ascribed to another family of opsonins, the lung collectins SPA and SPD. These molecules engage either the ITIM-containing molecule SIRPα via their globular heads to downregulate response or, via the collagenous "tail," activate phagocytes through a CD91-calreticulin complex (Gardai et al., 2003).

There is further complexity as numerous other molecules including the pentraxins, MFGE8, Gas6, matrix components like mindin, and coagulation factors such as protein S and fibrinogen mediate the humoral arm of phagocytosis (Anderson et al., 2003; Hanayama et al., 2002; He et al., 2004). Interestingly, the phagocytes that utilize them actively secrete many opsonins. It is reasonable to suggest that proteomic analysis of serum is likely to reveal many more potential opsonins. The vast arrays of opsonins may not only facilitate engulfment but also define the responses after phagocytosis. However, several critical questions remain as to the mechanism of this "editing" of response. For instance, how does the phagocyte come to reconcile multiple ligands with similar methods of molecular recognition but apparently contrasting downstream consequence? In addition, what determines the destination of the phagocytosed cargo and how is this linked to the secretion profile that ensues?

The "Phagocytic Synapse"

It seems unlikely that a particle engages only one receptor on the cell surface, and, normally, an array of receptors will interact with a specific pathogen. The involvement of a number of receptors is consistent either with sequential recognition or simultaneous recognition by a multimolecular complex. A precedent for cooperative, sequential recognition of a ligand has been established for LPS, the strongly proinflammatory component shed from the outer wall of gram-negative organisms. LPS is recognized by a low-specificity but high-affinity interaction with LPS binding protein (LBP) and CD14, which in turn act to deliver the ligand first to MD2 and then TLR4 to trigger signaling (Figure 2A). The second (and not mutually exclusive) model is that a phagocytic synapse, broadly analogous to the T cell synapse (Figure 2B), might associate numerous molecules into a complex to mediate recognition. The T cell synapse is the point of contact of the T cell with an antigen-presenting cell (APC) and consists of low-specificity, high-affinity interactions between LFA-1 and ICAM-1 that mediate initial attachment, surrounded by a ring of lower affinity interactions between the TCR and its cognate MHC-peptide complex. Maturation of the synapse rearranges the position of these molecules and provides a scaffold for optimal TCR stimulation. It is conceivable that similar events occur during phagocytic recognition and would be important in coordinating intracellular signaling cascades (Figure 2B). In support of this, many molecules that cooperate in LPS signaling including CD11b/CD18, CD14, CD16, and CD36 exist in close proximity in the cell membrane, as indicated by fluorescence resonance energy transfer (FRET) between molecules upon ligation with LPS (Pfeiffer et al., 2001). Using similar techniques, it has also been shown that hsp70, hsp90, CXCR4, GDF5 and TLR4 localize to the site of CD14-LPS ligation within the lipid rafts (Triantafilou et al., 2002), further emphasizing the potential complexity of the phagocytic interface. Together these data provide evidence for a model in which low-specificity receptors such as scavenger receptors and integrins "scan" the targets and mediate the initial interaction before more specific but lower affinity signaling receptors, such as TLRs, are recruited to the core of the phagocytic synapse. Importantly, although we have alluded to a complex of receptors, it is important to appreciate that it is likely to be a highly dynamic structure, undergoing constant remodeling during the process of internalization.

In considering these models, it is important to appreciate that another level of complexity exists: not all receptors are expressed by all phagocytic cells and, dependent on their tissue origin and activation state, phagocytes may have only a limited repertoire of potential receptors. As an example, CD14 is highly expressed by monocytes and macrophages, but not DCs. This "editing" of innate immune receptors provides combinatorial variations between different phagocytes and contributes to the enormous diversity in host response that occurs. In addition, these complexities of recognition also explain the partial redundancy that is evident for many phagocytic receptors when studied in knockout systems.

Apoptotic Cells Recognition: You Eat What You Are

During morphogenesis and embryogenesis, millions of cells undergo programmed cell death, and large numbers of cells are generated and then die during certain normal physiological processes. These include the deletion of effector lymphocytes after antigen challenge, removal of inflammatory cells recruited to injured or infected tissue, and the remodeling of tissues such as the involuting mammary gland. It is apparent, therefore, that for a multicellular organism, the majority of phagocytosed material will be derived from self-cells and not invading pathogens. Recognition of effete cells is mediated by a variety of receptors and opsonins, most of which also recognize pathogen determinants (Figure 1). However, phagocytosis of apoptotic cells is unique. Unlike pathogens, apoptotic cells are actively anti-inflammatory, limiting production of proinflammatory cytokines from macrophages (Fadok et al., 1998; Voll et al.,

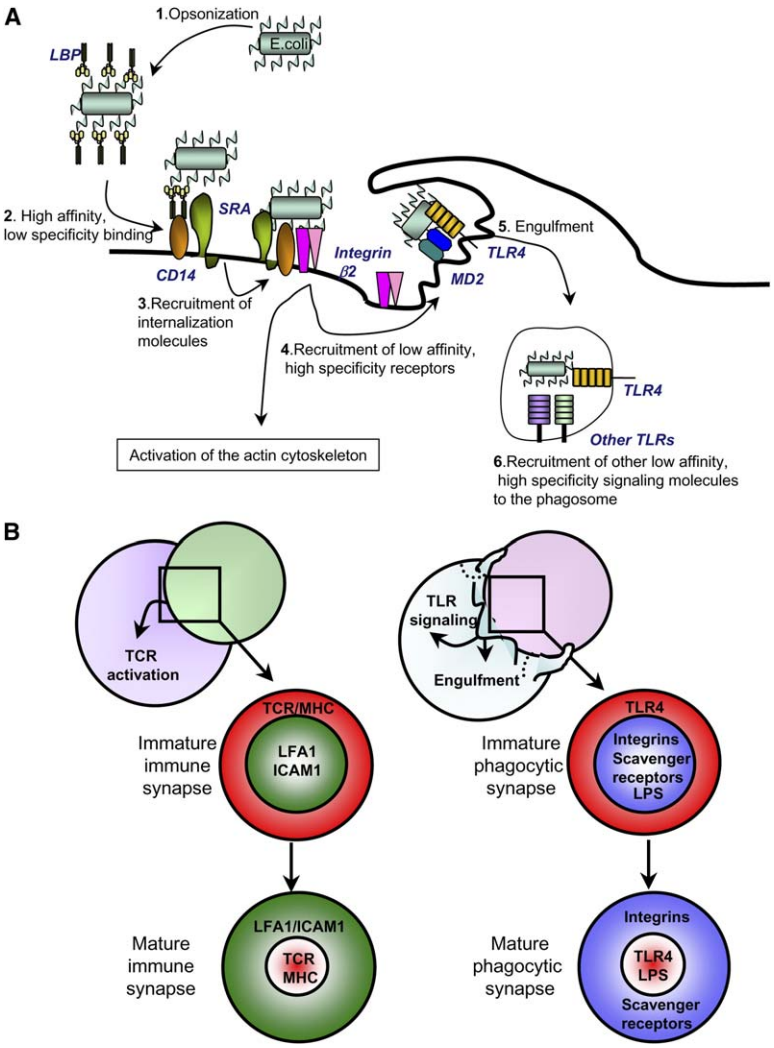

Figure 2. The Phagocytic Synapse

(A) Sequential Recognition and engulfment of *E. coli* is initially mediated by high-affinity, low-specificity interaction with LBP and the scavenger receptors, CD14, and SRA. These molecules then recruit regulators of the cytoskeleton such as integrin β2 to trigger engulfment. In addition, reorganization within the plasma membrane delivers LPS to lower affinity but high-specificity molecules (MD2 and TLR4) to initiate signaling. Signaling occurs either at the cell surface or within the phagosome.

(B) The membrane reorganization events during recognition of *E. coli* may bear similarities to the T cell synapse. The T cell synapse is characterized by an initial high-affinity, low-specificity interaction between LFA-1/ICAM-1 that mediates noncognate attachment. Subsequent reorganization of the T cell synapse then delivers the MHC-peptide-TCR complex to a central "bulls-eye" for signaling. Similar events may occur within the phagocytic synapse in which the low-specificity but high-affinity recognition by scavenger receptors and integrins initiate binding and then subsequent reorganization both triggers engulfment and delivers LPS and other ligands to their cognate TLR to initiate signaling.

1997) and DCs (Stuart et al., 2002; Urban et al., 2001) and actively encouraging cell regeneration (Golpon et al., 2004). This self-regulation protects the organism from uncontrolled inflammation and self-reactivity that might otherwise be induced by phagocytosis of dying cells. The mechanisms by which apoptotic cells modulate macrophages have not been fully defined but are thought to be linked to the complex events that mediate binding and engulfment of the dying cell (reviewed by Savill et al. [2002]). What is apparent is that the downstream consequences are defined by early binding events that act both directly and via an autocrine regulatory loop involving phosphatidylserine recognition and release of TGFβ to modulate subsequent proinflammatory response.

DCs also phagocytose dying cells, and their potent function as antigen-presenting cells (see below) allows them to present antigens derived from them to T cells (Albert et al., 1998), a process thought to be vital for viral and tumor immunity. However, apoptotic cells are also a source of self-antigen, creating the dilemma that clearance of dying cells by DCs may contribute to autoimmunity and raising the question of how phagocytes distinguish constitutive cell death from that induced by infection or malignant transformation. Recent work is beginning to address the mechanism for this discrimination. It has emerged that not all dying cells are equivalent; viral-infected dead cells act as "Trojan horses," delivering TLR3 ligands to induce T cell priming (Schulz et al., 2005), whereas constitutive apoptosis does not activate DCs (Sauter et al., 2000). These experiments provide important evidence that even subtle differences in the nature of the internalized dying cell have far-reaching consequences for the adaptive immune response. Fully understanding the regulation of the response of DCs after phagocytosis of various particulate material and apoptotic cells will be an essential step toward understanding both tolerance and priming to phagocytosed antigen.

Tasting Your Meal

The Phagosome: A Highly Specialized Organelle

After receptor engagement and engulfment the internalized particle is delivered to a de novo membrane-limited organelle, the phagosome (Figure 3). When a well-described technique used to isolate phagosomes

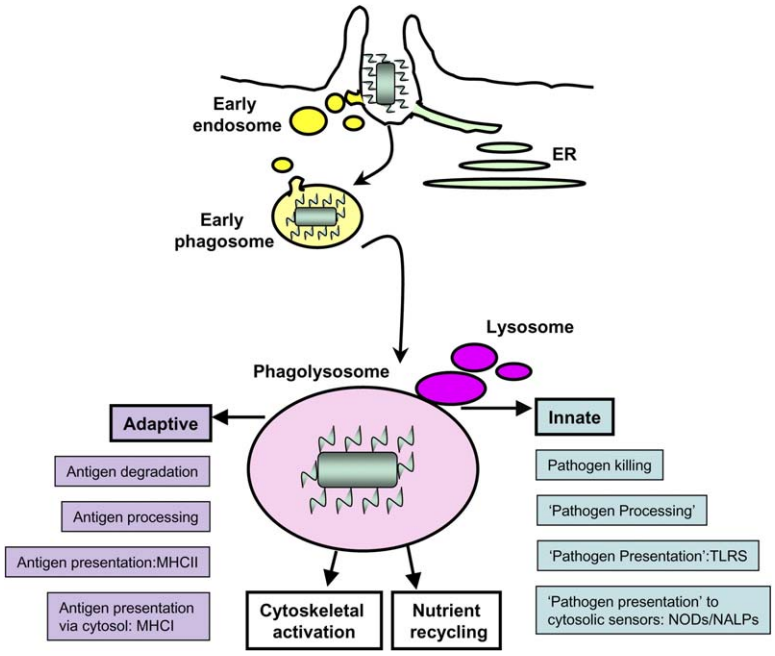

Figure 3. Generation of the Phagosome, an Important Organelle in Innate and Adaptive Immunity

During generation of the phagosome, both early endosomes and endoplasmic reticulum (ER) fuse with the phagocytic cup to provide excess membrane required to engulf large particles. After formation, the phagosome undergoes maturation by fission and limited fusion with early endosome and then lysosomes to generate a mature phagolysosome. The fully mature phagolysosome has important functions in both innate and adaptive immunity.

containing latex beads (by utilizing the relative buoyancy of this cargo to facilitate separation) was combined with proteomic analysis of the constituents, insights into the complexity of this organelle were made (Desjardins, 2003; Garin et al., 2001). The phagosome contains hundreds of proteins that provide the necessary molecular machinery required for it to fulfill numerous functions within the cell (Figure 3). One such role is in killing internalized pathogens, which requires maturation of the phagosome by a series of fission and limited fusion events with endosomes and lysosomes. The resulting phagolysosome is highly hydrolytic and directly limits pathogen replication. In addition, the NADPH-oxidase assembles within the phagosome with concomitant release of reactive oxygen intermediates. Furthermore, it has been recently proposed that the NADPH-oxidase also, through regulation of pH, provides an optimal environment for activation of proteases that directly kill the internalized organism (Reeves et al., 2002).

To internalize large or numerous particles, phagocytes must rapidly replenish their cell membrane. Early work demonstrated that some of this membrane was provided by endosomes, and this has been confirmed in more recent studies using real-time imaging. This model is further supported by evidence of the involvement of molecules important for endosome trafficking (such as VAMP3 and Rab11) in particle internalization (Bajno et al., 2000; Cox et al., 2000). More recently, the proteomic analysis of latex bead-containing phagosomes has led to the proposal of an additional, but not mutually exclusive, source of membrane. Preparations of early phagosomes contain many constituents of the endoplasmic reticulum (ER), leading to the suggestion that ER is a source of some of the excess membrane required to internalize large particles (Gagnon et al., 2002). More recent studies have supported these ideas and suggested a mechanism involving the ER SNARE Sec22 in ER:phagosome fusion (Becker et al., 2005). However, isolation of highly pure phagosomes is technically challenging and the relative abundance of ER membranes makes them likely contaminants, raising the possibility that the detection of ER proteins in phagosome preparations may be artifact. However, numerous other lines of evidence support a role for ER in phagocytosis. First, ER components were isolated only from early, but not late, phagosomes, indicating that ER is not a uniform constituent of all preparations. Second, labeled ER proteins calreticulin and calnexin can be localized to ER and to the phagocytic cup in the model organism *Dictyostelium discoideum* (Fajardo et al., 2004; Muller-Taubenberger et al., 2001). Finally the ER can be seen in close proximity to the base of the phagocytic cup when imaged by electron microscopy. Together these data provide compelling evidence to support the suggestion that ER, either directly or indirectly, contributes membrane to phagosomes. Unfortunately, the limitation in definitively establishing the relative contributions of ER and endosome membranes remains technical and will rely on developing further methods that allow isolation of highly pure phagosomes. Nonetheless, it is important to appreciate that these models are by no means mutually exclusive, and it is probable that both early endosomes and ER contribute membrane to the early phagosome, creating a hybrid organelle with characteristics acquired from plasma membrane, endosomes, and ER (for a more detailed discussion see Gagnon et al., 2005; Touret et al., 2005). Importantly, the relative roles of these membranes in different forms of phagocytosis will need to be defined, particularly in the context of biologically relevant cargo rather than latex beads.

Phagocytosis and Antigen Presentation: At the Heart of Adaptive Immunity

An important function of phagocytes in higher organisms is to provide antigenic ligands to stimulate clonal

expansion of T and B cells. In this regard DCs are unique in their capacity to initiate naive immune responses by acting as potent APCs. Immature DCs are highly phagocytic but, upon receipt of a maturation stimulus (such as TLR ligands), lose phagocytic/endocytic capacity and become potent antigen-presenting cells. However, relatively little is known concerning the details of the regulation of DC phagocytosis in the context of their maturation program, and further work in this area will be essential to deciphering certain aspects of their role to link the innate and adaptive immune response. Mature DCs present phagocytosed antigen on class II MHCs to activate CD4 T cells and also efficiently "crosspresent" internalized material on class I MHCs to activate CD8 T cells. These characteristics of DCs intrinsically link phagocytosis to the adaptive immune response. Although a detailed discussion of the role of phagocytosis in antigen delivery is beyond the scope of this review, we will discuss briefly one aspect that is of particular relevance: crosspresentation.

The efficiency of presentation of exogenous antigens on class I MHC (i.e., crosspresentation) is greatly increased if the antigens are delivered by phagocytosis. There appear to be at least two mechanisms for crosspresentation, which can be defined by their requirement for the TAP transporter. The requirement for TAP suggests that exogenous antigens access the endogenous pathway in the cytosol before entry into the ER (Kovacsovics-Bankowski and Rock, 1995). For this to occur, antigens must traverse the phagosome membrane to enter the cytosol by a mechanism that remains poorly understood. Subsequent to the proposal of ER-mediated phagocytosis, a model has emerged that may provide some explanation. It has been suggested that antigen-processing machinery is recruited along with the ER membrane to the nascent phagosome, allowing it to act as an organelle sufficient for antigen presentation (Ackerman et al., 2003; Guermonprez et al., 2003; Houde et al., 2003). In this model peptides would use conventional ER transporters such as Sec61 or Derlin-1 (Lilley and Ploegh, 2004; Ye et al., 2004) to exit the phagosome and enter the cytosol. However, alternative, but not necessarily mutually exclusive, models are possible. As an example it is possible that phagosome contents are "shared" with endosomes during transient fusion events and may then escape into the cytosol from this organelle. Unfortunately, the identity of the putative but necessary "phagosome/endosome-to-cytosol transporter" essential for both of these models of TAP-dependent crosspresentation has remained elusive, and identification of the molecular machinery that allows antigens to transverse from phagosomes or endosomes to the cytosol will be vital to validate these proposals. A second model is that endosomes that have captured some of the contents from phagosomes or directly from the cell surface may be retrieved to the ER to deliver antigen directly into this compartment (Ackerman et al., 2005). In this model antigens do not enter the cytosol, and, hence, is it consistent with TAP-independent crosspresentation. Undoubtedly, exactly how certain phagocytes and, specifically, DCs (den Haan et al., 2000), crosspresent remains to be fully defined, and the diverse possibilities remain the subject of much debate (for more detail see Trombetta and Mellman [2005]).

Innate and Adaptive Processing and Presentation

An important observation is that like MHC, certain TLRs are actively recruited to the phagosome (Latz et al., 2004; Underhill et al., 1999) and suggests that this organelle provides an important site for "sampling" of cargo and initiation of both adaptive and innate immune signaling. To further understand this, it is interesting to compare the fates of internalized material from both an innate and adaptive immune perspective (Figure 4). The highly hydrolytic environment of the phagosome releases peptides from internalized proteins for presentation on MHC II. This process also releases bacterial components and TLR ligands into the phagosome at high concentration, allowing them to efficiently interact with the cognate recruited TLR. Importantly, disruption of phagosome acidification with chloroquine perturbs TLR9 (Leadbetter et al., 2002) and TLR3 (Schulz et al., 2005) signaling in a manner reminiscent of its effects on antigen presentation and indicates that the phagosome environment is optimized both for antigen loading and certain ligand:TLR interactions.

However, can we make a similar comparison between pathogen sensing and MHC class I presentation, in which the peptides presented are derived from within the cell? The existence of phagosome-to-cytosol crosspresentation suggests that communication between these compartments must exist. In this regard, another family of important innate immune sensors, the NODs and NALPs, are of great interest. Unlike TLRs, these pathogen sensors are located not on the cell surface but within the cytosol, where they assemble a complex known as the "inflammasome," which initiates caspase-mediated cleavage of the prototype inflammatory cytokine pro-Il-1β to the active form (reviewed in Martinon and Tschopp [2004]). In addition, these molecules also directly activate NFκB. Thus, as pathogen ligands must first cross the cell membrane to activate these molecules, the presentation of bacterial ligands to NODs and NALPs bears certain similarities to that of antigen crosspresented on class I MHC (Figure 4). For *Helicobacter pylori* it is known that a bacteria-derived type IV secretion system "injects" the NOD1 ligand GM-tri-DAP muropeptide across the membrane (Viala et al., 2004). However, it remains unknown how (or indeed if) products from those pathogens that do not have specialized secretory apparatus or induce lysis of the phagosome membrane access these molecules in the cytosol. One possibility is that the phagosome actively "leaks" pathogen-derived ligands into the cytosol to activate these cytosolic sensors. Importantly, these new insights may be critical in providing a direct link between the contents of the phagosome and the ultimate strength of the inflammatory response, and the possibility that fine-tuning of response could be regulated by these series of cytosolic sensors remains to be explored.

Phagosome Autonomy and Sensing of Cargo

It is important to appreciate that not all phagosomes are created equal, and the phagosome around a latex bead is not equivalent to that formed after bacteria or apoptotic cells are internalized (Griffiths, 2004). Important advances have been made in this area by studying the dynamic interaction between the pathogen mycobacterium tuberculosis (mTB) and the phagocyte. The pioneering work by D'Arcy Hart that described the abil-

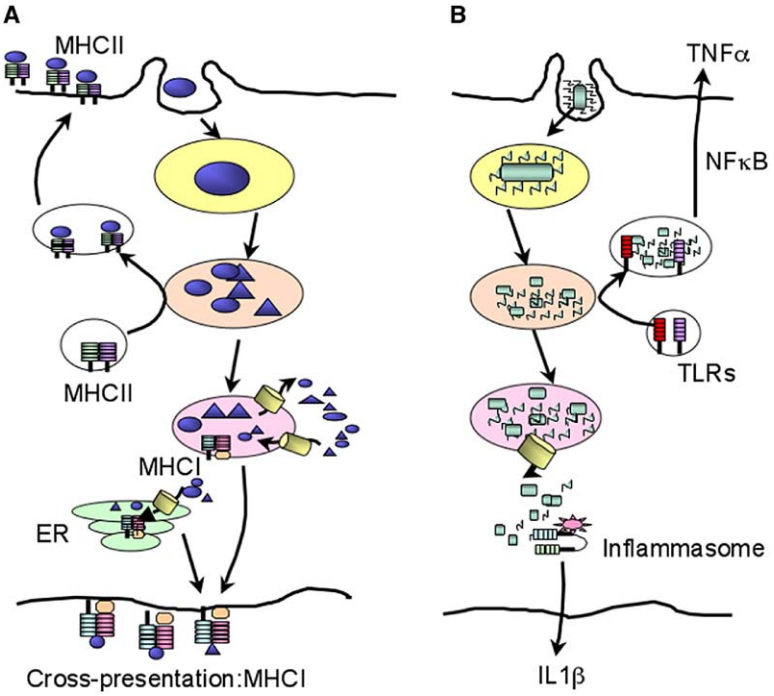

Figure 4. Antigen Processing and a Proposed Model of "Pathogen Processing"

(A) Internalized antigen is degraded within the phagosome and peptides loaded onto class II MHC. In the case of crosspresentation, proteins "leak" from the phagosome and are degraded to peptides in the cytosol. These peptides are either returned to the same phagosome for loading on class I MHC or enter the endogenous pathway of antigen processing and presentation.

(B) By comparison, whole pathogens are internalized and killed within the phagosome. In this model of "pathogen processing" phagosome degradation liberates pathogen-derived by-products at high concentration where they can interact with their recruited cognate TLRs. In addition certain pathogen-derived products escape from the phagosome to activate cytosolic sensors such as NODs and NALPs in a manner analogous to crosspresentation.

ity of ingested mTB to inhibit phagosome maturation (Armstrong and Hart, 1975) has been confirmed and extended. Furthermore, there is a growing literature on the interaction of other intracellular bacteria with phagosome biology. We cite three simple examples to illustrate a general conceptual point: that the cargo influences the dynamic architecture and fate of the phagosome. Using comparative analysis it was shown that tryptophan-aspartate-containing-coat protein (TACO) was recruited to pathogenic, but not heat-killed, TB phagosomes and that this protein was important for pathogenic TB to subvert the normal maturation of the phagosome (Ferrari et al., 1999). More recent work by Pethe et al. has focused on the pathogen rather than the host by isolating a number of mTB mutants whose phagosomes fuse normally with lysosomes. To do this they recovered mTB clones from the lysosomal compartment that they had preloaded with iron-dextran to facilitate separation (Pethe et al., 2004) and, using this strategy, identified a number of candidate genes that may be important for mTB to subvert phagosome maturation. In contrast to mTB, maturation of phagosomes containing bacteria is accelerated, and the rate is determined by the ability of bacteria to engage TLRs recruited to the phagosome (Blander and Medzhitov, 2004). Moreover, in these experiments the apparent rate of maturation of phagosomes containing bacteria differed from those with apoptotic cells even within one phagocyte, suggesting that the fate of an individual phagosome is modulated in an organelle autonomous manner by their cargo. These examples confirm that individual phagosomes are autonomous, able to sense, and signal their constituents from within.

Autophagosomes

Autophagy is a process of cellular autophagocytosis that is initiated in times of nutritional stress (reviewed in Levine and Klionsky [2004]). During autophagy, controlled fusion of subcellular organelles with lysosomes generates a distinct, double-membrane structure, the autophagosome. Autophagosomes have many shared features with phagosomes but are unique; the internalized "target" is a subcellular organelle, and the origin of membrane that engulfs it is unknown. An important function of autophagy is to specifically harness intracellular sources of nutrition from macromolecules and whole organelles and, by self-digestion, recycle limited resources from within. Over the past few years, a role has emerged for controlled generation of autophagosomes in host defense. For pathogens such as mTB that escape degradation in conventional phagosomes by preventing maturation, induction of autophagy induces fusion of the TB-containing phagosomes with lysosomes, killing the pathogen (Gutierrez et al., 2004). This method of limited autophagocytosis is regulated by IFN-γ, known to enhance macrophage killing of certain intracellular pathogens. Thus autophagy is in many ways analogous to phagocytosis and has functions in nutrition, deletion of unwanted cells, and provides an alternative and regulated mechanism of host defense, of particular relevance for pathogens that evade phagosome-mediated killing to exist in intracellular compartments. It is likely that more parallels between autophagy and phagocytosis will emerge.

In summary, phagosomes are highly complex organelles. It appears that one of the numerous functions of the phagosome is to provide a site for antigen and pathogen processing, presentation, and signaling. In this regard, it is possible to define both exogenous and endogenous pathways for presentation of pathogen-derived products, in a manner loosely analogous to those for class I and class II MHC presentation. Intriguingly, these innate functions predate or coevolved with

adaptive immunity and raise the possibility that antigen processing is simply an adjunct to evolutionarily more ancient functions of phagocytosis. However, it is evident that our understanding of this de novo organelle is relatively limited, and further work will be needed to expand our knowledge of its organization and regulation to fully appreciate its role in the biology of the cell and in the context of immunity.

Why to Eat: Lessons from Phagocytosis in Model Systems

Burying the Corpses and Sensing Invaders

The roots of phagocytosis in lower organisms probably lie in the internalization of nutrients from their surrounding milieu. However, in multicellular organisms, phagocytosis has adopted yet an additional role, that of tissue remodeling and homeostasis of cell number. Apoptosis, or programmed cell death, is an essential process by which excess unwanted or damaged cells are removed during development. One model organism, the nematode *Caenorhabditis elegans*, has provided enormous insight into these events. Sulston and Horvitz, (1977) detailed description of apoptosis in this simple system has allowed mutations that perturb this to be formally defined (Ellis and Horvitz, 1986; Hedgecock et al., 1983). Using complementing mutants, it was possible to identify two partially redundant pathways of uptake of apoptotic cells (Ellis et al., 1991) that converge to induce activation of Ced-10 (homologous to the Rho-GTPase Rac1) and the polymerization of actin (Kinchen et al., 2005). Importantly, in an organism with only 1090 cells, the existence of two pathways to remove 131 cell corpses suggests that "backup" systems are in place to ensure efficient engulfment even in this simple system and demonstrates the essential nature of this process. Intriguingly, failure of phagocytosis also impinges on the number of cells that die, indicating that engulfment is also required for completion of the death program (Hoeppner et al., 2001; Reddien et al., 2001). However, it is important to appreciate that although cells deleted by apoptosis in *C. elegans* are rapidly removed by their neighbors (demonstrating that many, if not all, cells are able to phagocytose), they lack circulating phagocytes, greatly limiting their usefulness as a model organism in which to study professional phagocytosis.

In contrast to *C. elegans*, higher organisms such as *Drosophila* have certain cells that specialize in phagocytosis, possibly reflecting the need for mobile phagocytes to remove the massive number of dying cells generated during morphogenesis (Tepass et al., 1994). Over the past decade, we and others in the field, have established the validity of *Drosophila* phagocytes as a model for professional mammalian phagocytosis (Pearson et al., 2003). In support of this, during *Drosophila* development apoptotic cells are recognized by evolutionarily conserved scavenger receptors, *Croquemort* (Franc et al., 1999) and *Draper* (Manaka et al., 2004), whose mammalian paralogs, CD36 and LRP, also bind apoptotic cells, confirming a conserved function for these molecules in professional phagocytes.

However, although it is obvious that phagocytosis of apoptotic cells is important in development, less clear is the in vivo role of phagocytosis in host defense in *Drosophila*. Absence of blood cells or deliberate blockade of phagocytosis by saturating the phagocytic machinery by injection of latex beads compromises the ability of flies to fight systemic infection (Elrod-Erickson et al., 2000), suggesting that phagocytosis acts in parallel with a potent humoral antimicrobial peptide response to contribute to host defense. Study of *Drosophila* cells has also been informative. *Drosophila* S2 cells are amenable to in vitro RNA interference (RNAi), providing a tractable system to test the function of known genes. Relevant to host immunity, it has been possible to identify other phagocytic receptors such as dSR-C1 that mediate bacterial uptake (Ramet et al., 2001). Using S2 cells and available genome-wide RNAi libraries (Boutros et al., 2004), significant advances have been made in the cell biology of actin regulation (Kiger et al., 2003) and phagocytosis (Ramet et al., 2002) including the identification of PGRP-LC, a peptidoglycan recognition protein that mediates uptake of *E. coli* by *Drosophila* blood cells (Ramet et al., 2002). In addition, PGRP-LC has also been shown to activate the *imd* pathway (analogous to the mammalian TNF pathway) to induce production of antimicrobial peptides (Choe et al., 2002; Gottar et al., 2002), indicating that a receptor can both mediate internalization and define the response after pathogen encounter. It is likely that combining in vivo and in vitro approaches with forward and reverse genetics will continue to facilitate identification of novel genes involved in phagocytosis, whose in vivo function can readily be assessed.

Importantly, recognition by *Drosophila* phagocytes does not appear to be rudimentary. The observation that multiple receptors recognize common ligands suggest a level of redundancy reminiscent of mammalian cells, thus providing an opportunity to utilize this genetically tractable system to further probe the complexity of phagocytosis. Furthermore, the similarities of the immortalized *Drosophila* S2 cell line with mammalian granulocytes and macrophages (Pearson et al., 2003) should allow an ideal opportunity to begin to dissect not only the complexity of ligand recognition and signaling but also to explore the cell biology that underlies the diverse fate of the internalized cargo as well as the generation and function of the phagosome.

Future Directions

Phagocytosis is a fascinating dynamic and critical biological system that plays a vital role throughout the life cycle of multicellular organisms. The basic templates that define this process appear to have their origins in simple life forms. The tools of modern biology have enabled a glimpse into the dynamic complexity of phagocytosis. A very recent study identified 85 proteins associated with the phagosome of amoeba (Okada et al., 2005), and our unpublished findings indicate that there maybe as many as 600 proteins that comprise a *Drosophila* phagosome. Hence, we are beginning to explore the intricate organization of the phagosome, and it is likely that studies that embrace its complexity will reveal novel insights into its many functions. It is clear, too, that extrapolation of the role of individual receptors in phagocytosis is problematic and that phagocytes

approach their task of antigen recognition by employing combinations of receptors. The new tools of biology will continue to provide a plethora of new information that leaves us with the reality that we cannot ignore the elegant complexity of phagocytosis.

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