

Phagocytosis and comparative innate immunity: learning on the fly

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Abstract | Phagocytosis, the engulfment of material by cells, is a highly conserved process that arose before the development of multicellularity. Phagocytes have a key role in embryogenesis and also guard the portals of potential pathogen entry. They discriminate between diverse particles through the array of receptors expressed on their surface. In higher species, arguably the most sophisticated function of phagocytes is the processing and presentation of antigens derived from internalized material to stimulate lymphocytes and long-lived specific immunity. Central to these processes is the generation of a phagosome, the organelle that forms around internalized material. As we discuss in this Review, over the past two decades important insights into phagocytosis have been gleaned from studies in the model organism *Drosophila melanogaster*.

Phagocytes

Cells such as macrophages and neutrophils that internalize particulate material in an actin-dependent manner. Derived from the greek phagein (to eat) and kytos (cell).

Apoptotic cell

A cell that has died by a genetically regulated programme of cell death. It is characterized by cell shrinkage, chromatin condensation, cell-membrane blebbing and DNA fragmentation. Eventually, the cell breaks up into many membrane-bound apoptotic bodies, which are phagocytosed by neighbouring cells.

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Phagocytosis is the process by which particles are recognized, bound to the surface of cells and internalized into a 'phagosome', the organelle that forms around the engulfed material (FIG. 1). This carefully orchestrated cascade of events begins with the engagement of phagocytic receptors that activate numerous signalling pathways. These signals coordinate an orderly progression of changes, including the rearrangement of the cytoskeleton that guides the circumferential movement of membrane to internalize the bound particles. Phagocytosis is an essential cellular process, performed by unicellular organisms and many cell types found in metazoans. In simple organisms, such as the slime mould *Dictyostelium discoideum*, the motile phagocytes patrol the cellular aggregates (slugs) and have a role in host defence¹. In higher organisms, engulfment is a particular feature of professional phagocytes, such as macrophages and neutrophils. These cells demonstrate a remarkable ability to internalize material and are able to engulf particles larger than their own surface area.

Two main classes of targets are removed by phagocytosis: microorganisms and 'altered self' particles, exemplified by apoptotic cells. Engulfment of apoptotic cells is prominent during tissue remodelling and embryogenesis when excess cells undergo programmed cell death and removal. An illustrative example of the crucial role for macrophages in development is in holometabolous insects, such as the fruit fly (*Drosophila melanogaster*), in which haemocytes have a crucial role in morphogenesis (BOX 1). Apoptosis begins by late stage 10 of embryogenesis and the removal of these effete cells by phagocytes that appear at the beginning of late

stage 11 of embryogenesis is essential for development^{2,3}. Likewise, in mice, macrophages populate remodelling tissues early during embryogenesis and remove dying cells^{4,5}. During pathogen invasion, phagocytosis is also the cornerstone of the early innate immune response and host-defence mechanisms of many species⁶. For unicellular organisms, such as amoeba, bacteria internalized from the extracellular milieu are an essential nutrient source⁷. However, the intracellular growth of these internalized microorganisms must be limited to prevent them overwhelming the host, and it is likely therefore that pathogen sensing has evolved along with the most fundamental role for phagocytosis — that is, nutrient acquisition. In higher species, phagocytes also function as important antigen-presenting cells and are required to prime effective adaptive immunity. The central role of phagocytes in host defence is emphasized by observations that many pathogen virulence factors directly manipulate the process of engulfment or functions of the phagosome.

Despite progress in understanding the complexity of phagocytosis since its first description by Metchnikoff a century ago (BOX 2), our knowledge remains incomplete. The study of phagocytosis is complicated by the partial redundancy of key components and it appears that many cell-surface receptors and components of the phagocytic machinery often have multiple overlapping functions. This high level of redundancy may reflect the essential role of phagocytosis and the need for alternative back-up mechanisms to remove dying cells during development, and invading pathogens during infectious challenge. Furthermore, examining the relative contribution of

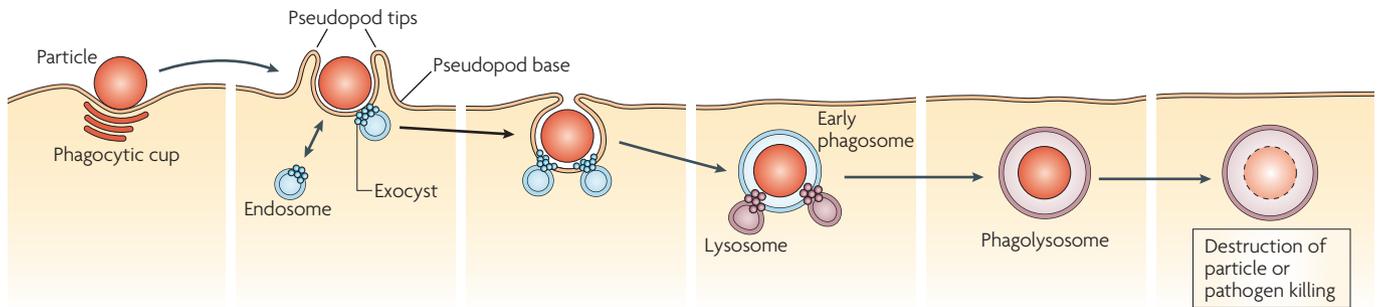


Figure 1 | Phagocytosis delivers the bound particle from the cell surface into the phagosome. Receptor ligation triggers an orderly progression of cellular changes leading to rearrangement of the actin cytoskeleton and membrane remodelling. Once formed, the phagosome undergoes maturation by fission and limited fusion events with endosomes and lysosomes to generate the acidic and hydrolytic environment of the mature phagolysosome. During phagocytosis, the membrane undergoes areas of marked curvature, in the base of the phagocytic cup and at the origins and tips of extending pseudopods. Studies of fly cells have suggested that this membrane curvature may be stabilized by coat proteins, including clathrin and the coatamer protein complex. To internalize large or numerous particles, phagocytes must recruit additional membrane from intracellular stores, such as endosomes and the endoplasmic reticulum. During this process the exocyst, an octodimeric complex that associates with *Drosophila melanogaster* phagosomes, is suggested to tether the recruited endosomes to the base of the phagocytic cup. The exocyst and the coatamer protein complex may also have roles in the docking of vesicles with the phagosome during maturation.

individual components of this system is difficult as primary mammalian phagocytes are not readily amenable to genetic manipulations, such as cDNA overexpression or knockdown of expression of candidate receptors by RNA interference (RNAi). Investigators have therefore relied on classic cell biology and microscopy techniques, as well as exploring the role of individual receptors that have been overexpressed in heterologous, non-professional phagocytes such as COS cells^{8,9} or by using cells isolated from knockout mice. These approaches have provided important insights into Fc receptor (FcR)- and complement-mediated phagocytosis and signalling^{10,11}. However, the limitations of the mammalian system have hampered the study of phagocytosis in intact organisms and have prevented the use of more exploratory and unbiased approaches. Therefore, building on the work of developmental biologists, some researchers in the field of innate immunity and macrophage cell biology have turned to genetically tractable model organisms that have been used successfully to study host defence pathways and the complex cell biology of phagocytosis. In this regard, work in both *D. discoideum* and *Caenorhabditis elegans* has been instructive but is beyond the scope of this Review (for recent reviews, see REFS 12, 13) and we focus instead on recent work in the fruit fly, *D. melanogaster*.

Professional circulating phagocytes were identified in *D. melanogaster* several years ago (BOX 1) and their identification suggested that this genetically tractable system might be well suited for the study of professional phagocytes. Over the past decade the crucial components of engulfment in these cells have been defined. The initial approaches used the amenability of *D. melanogaster* to genetic manipulation *in vitro*, *ex vivo* and *in vivo*^{14,15}, and recently an *in vivo* screen has been used to identify a new E3-ubiquitin-ligase-dependent mechanism of uptake of apoptotic cells¹⁶. However, although informative, these *in vivo* screens are also labour intensive. Therefore, to take advantage of the emerging power of RNAi, an *in vitro* system was developed using a variant of the well-known

embryonic S2 (Schneider line 2) cell line^{15,17}. These cells demonstrate properties similar to mammalian macrophages and efficiently ingest particles and bacteria in a temperature- and actin-dependent manner. In addition, S2 cells adopt a morphology after phagocytosis that is identical to that observed by primary larval plasmatocytes and reminiscent of the typical electron micrographs of professional phagocytes¹⁸. This *in vitro* system has been readily amenable to high-throughput screening with RNAi and has established S2 cells as a powerful platform for RNAi screens¹⁹. Subsequently, S2 cells have been used in many RNAi screens to identify molecules involved in host interactions with pathogens, such as *Escherichia coli*, *Staphylococcus aureus*^{19–21}, *Mycobacterium fortuitum*²², *Candida albicans*²³ and *Listeria monocytogenes*^{24,25} (reviewed in REF. 26). Moreover, much of this work has been carried out in parallel with studies revealing a more extensive understanding of the innate immune defence system in this organism^{27,28}. In addition, the experimental tractability of *D. melanogaster* has facilitated the testing of the role of phagocytosis, and some of the phagocytic machinery, in host defence *in vivo*^{29–34}. Using various models of pathogenesis, it is now clear that phagocytes are required to regulate some, but not all, bacterial infections. Here we discuss these advances and place recent findings in the broader context of mammalian phagocytosis. In addition, we examine how findings from this model system have provided insights into the evolutionary origins of immunology, adding value to this approach.

A phagosome-centric view of engulfment

After internalization, particles are delivered into a *de novo*-generated organelle, the phagosome. This simple definition of this organelle belies its complexity and importance in immune defence. Phagosomes have a central role in both innate and adaptive immunity. It has become evident that the nature and composition of each phagosome is determined by factors such as the cell-surface receptors

Holometabolous insects
Insects, such as *Drosophila melanogaster*, that have a pupal stage during which they undergo complete reorganization and metamorphosis.

Haemocytes
Cells found within the haemolymph of an insect that are equivalent to the blood cells in vertebrates.

RNA interference
The silencing of gene expression by the introduction of double-stranded RNAs that trigger the specific degradation of a homologous target mRNA and often subsequently decrease production of the encoded protein.

Opsonin

A soluble molecule that, when bound to a particle, enhances uptake of the particle by phagocytosis.

Pattern-recognition receptors

Receptors that recognize molecular patterns often associated with pathogens, such as lipopolysaccharide found in the bacterial cell walls of Gram-negative microorganisms. They also recognize molecular patterns expressed by non-pathogenic bacteria.

engaged by the particle, the mechanism of entry used, the origins of the contributing membrane and the nature of the cargo^{35,36}. In most circumstances, the nascent organelle undergoes a process termed ‘maturation’ by fusion and limited fission events with endosomes and lysosomes to generate the mature phagolysosome³⁷ (FIG. 1). The mature phagosome is highly hydrolytic, being able to limit the replication of bacteria and in many cases can kill the internalized microorganism. However, the fate of the newly formed phagosome can be actively subverted by certain pathogens that have developed mechanisms to hijack or escape the natural maturation process³⁸. Therefore, each phagosome is uniquely tailored for, and by, its cargo. For these reasons phagosomes are heterogeneous, quasi-stable and highly complex organelles, making the study of the underlying cell biology challenging.

Recent work using proteomics and computational modelling of *D. melanogaster* has made a significant advance in this area and provided a novel framework that accommodates the emerging complexity of the phagosome²¹. Building on previous studies in which the protein content of mammalian phagosomes was determined³⁵, proteomic analysis of generic latex-bead-containing phagosomes from *D. melanogaster* S2 cells has revealed many similarities between the phagosomes from these evolutionarily distant species. It is evident that, even in this simpler organism, the phagosome is a highly complex organelle (FIG. 2). Approximately 600 *D. melanogaster* proteins were identified to be associated with phagosomes, 70% of which had mammalian orthologues, validating this as a model system for mammalian phagocytosis. Using a new computational approach, it was predicted that hundreds of protein–protein interactions exist within the phagosome, which contain numerous subcellular biomodules or complexes including the vacuolar-ATPase (V-ATPase), the exocyst and the coat-amer protein complex²¹. Furthermore, it identified the potential for numerous signalling pathways that originate from this subcellular structure such as the nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signalling pathways²¹ (FIG. 2).

Box 1 | *Drosophila melanogaster* development and phagocyte function

D. melanogaster development is complex and comprises embryonic, three larval, pupal and adult stages. The entire developmental cycle from fertilization to adulthood is completed in 9 days. Embryonic development takes 20 hours and is divided into 17 stages that are defined by precise morphological events. It includes gastrulation, neurogenesis and generation of parasegments, and is terminated by larval hatch (see [The Interactive Fly](#) website). At the end of the three larval stages, pupariation takes place. Over the ensuing 4 days, metamorphosis occurs entailing widespread tissue remodelling, resorption of larval tissues and growth of adult organs. The adult fly hatches on day 9. Phagocytic blood cells or haemocytes can first be observed at the end of embryonic stage 11, shortly after the onset of apoptosis². The first studies of insect haemocytes were done by Rizki and Rizki in the 1960s; they showed the phagocytic capacity and the integral role that haemocytes have in defence against microorganisms¹⁰⁰. Similar to mammals, *D. melanogaster* haematopoiesis occurs in two distinct waves at two different sites — in the case of *D. melanogaster*, one embryonic, the other one larval. Both lineages of haemocytes persist to the adult stage. Depending on the developmental stage, the predominant function of *D. melanogaster* phagocytes may either be the removal of effete cells and larval tissues (embryos and pupae) or in pathogen surveillance and clearance (larvae and adults).

It is evident therefore, that there is amazing complexity to this organelle. Thus, although phagocytosis might be considered simply as a mechanism of waste disposal, an alternative view is that its function is to generate the phagosome, an organelle that is required for the effective induction of host defence and other important homeostatic processes. We discuss what has been learned from *D. melanogaster* about how phagosomes form and the multiple roles this has in the cell biology of phagocytes.

Cell-surface recognition

Phagocytosis is initiated by the ligation of cell-surface receptors that either directly bind to the particle or to opsonins that are deposited on the particle's surface. Both forward and reverse genetics, as well as RNAi screens, in *D. melanogaster* cells have been successfully used to identify potential phagocytic machinery^{19–25}. Some of the receptors have direct mammalian orthologues and demonstrate conservation of function, whereas others appear to be unique to insects (TABLE 1). These data indicate that there are four main classes of molecules involved in recognition: complement-like opsonins, scavenger receptors, a newly emerging family of epidermal growth factor (EGF)-like-repeat-containing receptors, and a highly variant receptor and opsonin, Down syndrome cell-adhesion molecule ([DSCAM](#)).

Complement-like opsonization. Complement components have multiple functions in mammalian host defence, including chemoattraction, formation of the membrane-attack complex and opsonization. The best-characterized opsonins in *D. melanogaster* are a group of proteins that are related to mammalian α_2 -macroglobulin and C3, the thioester-containing proteins (TEPs). Similar to mammalian acute-phase proteins, the transcription and secretion of TEPs is upregulated after bacterial challenge³⁹. Insight into the function of *D. melanogaster* TEPs was derived from an S2-cell RNAi screen, in which a member of the TEP family, macroglobulin-related protein ([MCR](#); also known as [TEPVI](#)), was found to bind and increase phagocytosis of *C. albicans*²³. Other TEPs also bind *E. coli* (TEPII) and *S. aureus* (TEPIII) and enhance phagocytosis²³. In addition, an *in vivo* RNAi strategy confirmed the role of the TEPs in phagocytosis in the mosquito *Anopheles gambiae*⁴⁰, indicating that other insects also use these opsonins. However, the functional equivalent of complement receptors in *D. melanogaster* remains to be defined.

Scavenger receptors. The scavenger receptors are structurally unrelated multi-ligand receptors that are defined by their ability to bind polyanionic ligands. They bind a wide range of pathogens and modified self ligands, and have emerged as important pattern-recognition receptors (PRRs)⁴¹ in many species. Some mammalian scavenger receptors have additional roles, acting not only as phagocytic receptors but also in host defence⁴²; these include CD14 and [CD36](#), which regulate Toll-like receptor 4 (TLR4) and TLR2 signalling, respectively^{20,43,44}. Supporting their importance in host

Box 2 | The discovery of phagocytes and phagocytosis

Elie Metchnikoff was a Russian embryologist whose seminal work on phagocytosis and 'physiological inflammation' was inspired by the observation of these processes in a simple metazoan, the sea-star larva⁹⁷. Metchnikoff observed the accumulation of cells he termed phagocytes around a rose thorn that he used to induce an inflammatory insult in the larva. This observation was the nidus from which his theories of cellular immunity were generated and for which he won a Nobel Prize in 1908. Importantly, his work emphasized the value of comparative embryology and established the validity of studying phagocytic engulfment in model organisms.

defence, scavenger-receptor families are expanded in many species, particularly those that lack classic adaptive immunity. As an example, only three CD36-like scavenger receptors exist in humans but there are ten in *D. melanogaster*. It is likely that this expansion is driven by the ability of these receptors to bind pathogens and hence expansion increases the repertoire of receptors for bacterial recognition and provides a strong survival advantage.

The class B scavenger receptors, CD36 and the CD36-like protein known as Croquemort, are noteworthy examples of multifunctional receptors in mammals and flies, respectively. In mammals, CD36, in association with integrins $\alpha_v\beta_3$ -integrin and thrombospondin, mediates the engulfment of apoptotic cells⁴⁵. Similarly, Croquemort is a receptor for apoptotic cells in *D. melanogaster*^{14,46}. Apoptosis is prominent in the embryo² and Croquemort-deficient flies demonstrate persistence of uncleared dying cells, a defect that is rescued by transgenic expression of Croquemort. So, Croquemort appears to be a member of an evolutionarily conserved, CD36-like family of receptors for apoptotic cells and was the first example to validate the use of *D. melanogaster* as a model to study mammalian phagocytic receptors.

Croquemort is also expressed in adult flies when apoptosis is not prominent, an observation that raised the possibility that it has additional functions beyond the clearance of dying cells. Consistent with a role as a multi-ligand receptor, it is now known that Croquemort binds *S. aureus*, and this led to the identification of mammalian CD36 as a phagocytic receptor for *S. aureus*²⁰. Contemporaneous to this discovery, an *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis strategy in mice identified a non-sense mutation in *Cd36* (*oblivious*) that resulted in defective host defence against *S. aureus*⁴⁴. CD36, and presumably Croquemort, recognize *S. aureus* through diacylated lipopeptides that are found on the bacterial cell surface. Interestingly, not only does CD36 act as a phagocytic receptor but it also cooperates with TLR2 and TLR6 to increase signalling in response to lipoteichoic acid (LTA) and diacylated lipopeptides in mammals^{20,44}. It remains to be determined whether Croquemort functions similarly in Toll signalling in *D. melanogaster*.

Another class B scavenger receptor, *Peste*, which was identified by an RNAi screen, is a receptor for bacteria in *D. melanogaster*. It can bind *M. fortuitum*²², suggesting that mammalian class B scavenger receptors may be involved in the recognition of mycobacteria. Although not yet tested *in vivo*, this possibility is supported by

overexpression experiments in which the class B scavenger receptors SR-BI and/or SR-BII, but not CD36, conferred the ability to bind *M. fortuitum in vitro*²². In contrast to these two examples of functionally conserved class B scavenger receptors, *D. melanogaster* has a structurally unrelated insect-specific class of scavenger receptors, SR-C (scavenger receptor class C)⁴⁷. *SR-CI* is a type I membrane protein that contains domains that are related to complement control proteins and mucins, and binds bacteria¹⁷. It belongs to a small gene family of four proteins, two of which are predicted to be secreted proteins. Consistent with a role in host defence, SR-CI expression is upregulated in fly larva after exposure to bacteria⁴⁸ and a high level of naturally occurring polymorphism exists in *Sr-CI* that has been associated with varying levels of resistance to bacterial infection⁴⁹. Unlike SR-CI, SR-CII is not thought to have a role in immune defence⁴⁹.

Receptors with multiple EGF-like repeats. Accumulating evidence from numerous species indicates the existence of a new family of PRRs that use EGF-like repeats to recognize diverse ligands^{30,50} (FIG. 3). Members of this family are particularly well defined in *D. melanogaster*, although they are found in many insect species, in *C. elegans* and in mammals, in which they might have similar functions⁵⁰.

A *D. melanogaster* protein, *Eater*, is perhaps one of the best characterized proteins of this newly emerging family of EGF-like-repeat-containing PRRs. It is a type I membrane protein that contains 32 characteristic EGF-like repeats in the extracellular domain. *Eater* acts as a bona fide PRR that directly binds microbial surfaces through its four N-terminal EGF-like repeats³⁰. *Eater* was identified as a putative target of *Serpent*, a *D. melanogaster* GATA transcription factor that had been found to be essential for bacterial phagocytosis by an RNAi screen¹⁹. Silencing *Eater* expression in S2 cells decreases bacterial binding and uptake, and macrophages from flies that lack *Eater* are impaired in their ability to phagocytose bacteria, leading to increased susceptibility to certain infections³⁰. *Eater* therefore appears to be a major receptor for a broad range of pathogens in *D. melanogaster*.

A similar *D. melanogaster* molecule, *Nimrod C1*, was identified as the protein target of a haemocyte-specific antibody⁵⁰. It is a 90 kDa transmembrane protein with 10 EGF-like repeats that are similar to those found in *Eater*. RNAi-mediated silencing of *Nimrod C1* expression decreases the uptake of bacteria, and overexpression of this protein increases the adherence of S2 cells to tissue-culture plates⁵⁰, suggesting that it acts both as a phagocytic receptor and a potential adhesion molecule. The gene encoding *Nimrod C1* is part of a cluster of 10 related *Nimrod* genes that are found in *D. melanogaster*. Similar proteins are also found in silk moths and the beetle *Holotrichia diomphalia*⁵¹, in which they serve as secreted opsonins that are involved in bacterial clearance. Orthologues also exist in flesh flies, mosquitoes and honeybees but not in mammals.

N-ethyl-*N*-nitrosourea (ENU) mutagenesis

An alkylating agent and potent mutagen that when given to mice efficiently induces random mutations.

Draper, originally identified as being regulated by the transcriptional factor glial cells missing, is expressed on the surface of glial cells and *D. melanogaster* macrophages⁵². It is required for the removal of apoptotic cells in the central nervous system⁵² and embryonic macrophages and S2 cells⁵³. *Draper* also contributes to axon pruning⁵⁴ and the removal of severed axons in models of Wallerian degeneration⁵⁵. *Draper* is the *D. melanogaster* orthologue of the *C. elegans* apoptotic-cell receptor, *CED-1* (REF. 13), and similar to *CED-1*, *Draper* triggers engulfment through engagement of *CED-6* by an NPXY (where X denotes any amino acid) motif on its intracellular tail⁵⁶. This motif is conserved in the mammalian apoptotic-cell receptor *CD91* (also known as LRP), which suggests that *Draper*, *CED-1* and *CD91* trigger similar engulfment machinery in *C. elegans*, *D. melanogaster* and mammals, respectively. However, *CD91* and *Draper* differ in their extracellular domain and the closest mammalian homologues of *Draper* are multiple EGF-like domain 10 (*MEGF10*) and *MEGF11* (REF. 57). The essential role of *Draper* in altered-self recognition in *D. melanogaster* strongly suggests that *MEGF10* and *MEGF11* might also have a role in the recognition of dying cells in mammalian systems⁵⁷.

DSCAM and receptor diversification. A key feature of immune receptors is the enormous repertoire required to accommodate a large and rapidly evolving pool of potential pathogens. In mammals, antigen receptor diversity is generated somatically through gene rearrangement and, in the case of B cells, is further increased by somatic hypermutation. By contrast, these classic features of adaptive immunity were thought to be absent in *D. melanogaster*, raising the question of how *D. melanogaster* generates the required repertoire to deal with emerging pathogens. The recent discovery of the role of an immunoglobulin superfamily member, DSCAM, in host defence in *D. melanogaster* might provide the answer. DSCAM was first described in neuronal development and, through homophilic and heterophilic interactions, determines the correct wiring of neurons⁵⁸. To accomplish the necessary diversity to accommodate many different neurons, it is predicted that DSCAM may have more than 38,000 potential splice variants that are generated by combining constant and variable regions⁵⁹. Interestingly, it is possible that *D. melanogaster* immune tissues (comprised of haemocytes and fat-body cells) can express more than 18,000 different extracellular domains of DSCAM⁶⁰ (FIG. 4a), and soluble forms of DSCAM have also been identified in cell supernatants and in the haemolymph.

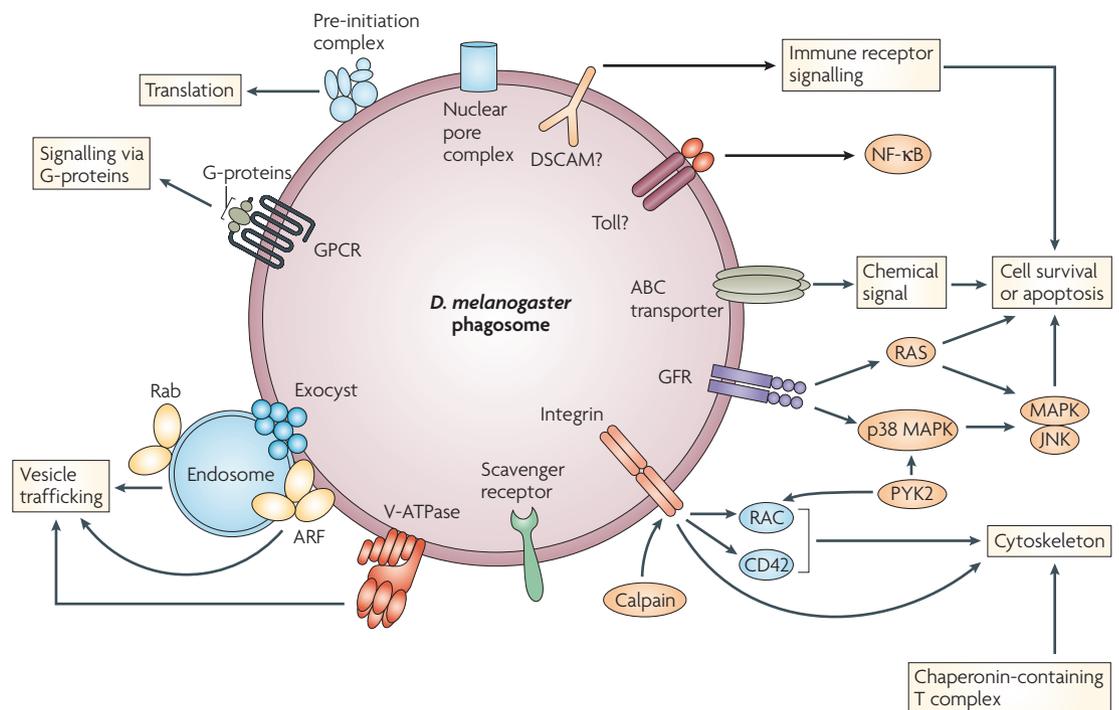


Figure 2 | The phagosome is a multifunctional organelle. Proteomic analysis has identified more than 600 proteins that are associated with the phagosomes of *Drosophila melanogaster*. Bioinformatic analyses of these proteins suggest that numerous protein–protein interactions and macromolecular complexes associate with this organelle. These include the vacuolar-ATPase (V-ATPase), the exocyst and the chaperonin-containing T complex. In addition, components of multiple signalling pathways are present in association with phagosomes, suggesting this as a point of initiation of signal transduction. These pathways are likely to be downstream of transmembrane proteins found in the phagosome such as G-protein-coupled receptors (GPCRs), scavenger receptors, integrins, the Toll receptor and growth-factor receptors (GFRs). ABC, ATP-binding cassette; ARF, ADP-ribosylation factor; MAPK, mitogen-activated protein kinase; DSCAM, Down syndrome cell-adhesion molecule; NF-κB, nuclear factor-κB; PYK2, protein tyrosine kinase 2.

Gene rearrangement
The ordered rearrangement of variable regions of genes encoding antigen receptors that contributes to increase receptor diversity.

Somatic hypermutation
The process by which antigen-activated B cells in germinal centres mutate their rearranged immunoglobulin genes. The B cells are subsequently selected for those expressing the 'best' mutations on the basis of the ability of the surface immunoglobulin to bind antigen.

Table 1 | Cell-surface recognition receptors and opsonins

<i>Drosophila melanogaster</i> receptors	Ligands	Related mammalian molecules	Refs
TEPs			
TEPVI (MCR)	<i>Candida albicans</i>	Complement components	23
TEPIII	<i>Staphylococcus aureus</i>		
TEPII	<i>Escherichia coli</i>		
Scavenger receptors			
Croquemort*	Apoptotic cells and <i>S. aureus</i>	CD36*	14, 20, 46
Peste*	<i>S. aureus</i> , <i>Listeria monocytogenes</i>	SR-BI*	22
SR-CI	<i>S. aureus</i> , <i>E. coli</i> and dsRNA	ND	17
EGF-like-repeat-containing Nimrods			
Eater	<i>S. aureus</i> , <i>L. monocytogenes</i> , <i>E. coli</i> and dsRNA	MEGF10; MEGF11; CD91 (LRP); SREC;	30
Draper	Apoptotic cells, axon pruning and severed axons	Stabilin 1 and Stabilin 2	52–55
Nimrod C1	<i>E. coli</i> and <i>S. aureus</i>		50
Others			
PGRP-LC	<i>E. coli</i>	Mammalian PGRPs [‡]	19
PGRP-SA (soluble)	<i>S. aureus</i>		

*Direct mammalian orthologues. †Soluble molecules with no role in phagocytosis. MCR, macroglobulin-complement related protein; LRP, lipoprotein receptor-related protein; MEGF, multiple epidermal growth factor (EGF)-like domain; ND, not determined; PGRP, peptidoglycan-recognition protein; SR, scavenger receptor; TEP, thioester-containing protein; SREC, scavenger receptor expressed by endothelial cells.

DSCAM binds *E. coli* and potentially acts as both a phagocytic receptor and an opsonin⁶⁰ (FIG. 4b). This suggestion is supported by the identification of DSCAM in the phagosome proteome²¹. The crystal structures of two DSCAM isoforms reveal each one with two distinct surface epitopes, one on either side of the receptor, which are generated by its hypervariable amino-acid residues. This configuration allows a given DSCAM isoform to form a homodimer (via a homophilic interaction through one hypervariable epitope) and retain the ability to recognize, opsonize and crosslink pathogens (via a second hypervariable epitope)⁶¹. DSCAM therefore has many similarities to antibodies and might have similar functions in *D. melanogaster* as immunoglobulins in mammals.

Individual cells have been predicted to express between 15 and 50 DSCAM isoforms⁶², and data from the mosquito *A. gambiae* suggest that there is increased secretion of pathogen-specific isoforms after infectious challenge⁶³. However, although these observations raise the possible preferential use of splice variants after infection, they have not yet been confirmed in studies of DSCAM in other species. Nonetheless, it is tempting to compare DSCAM and immunoglobulin receptors. DSCAM exists in a secreted form and an intriguing possibility is that DSCAM-secreting cells are functionally similar to mammalian immunoglobulin-secreting plasma cells (FIG. 4b). DSCAM provides a potential example of convergent evolution in the immune system of these two distant species and suggests that highly variable receptors (such as immunoglobulins, highly variable lymphocyte receptors (VLRs) of lamprey⁶⁴ and DSCAM splice variants) are examples of ‘optimal design’ and are a desired feature found in many effective immune systems.

The machinery of uptake

Over the past 20 years, an extensive body of work has defined the machinery that functions downstream of mammalian complement and of Fc receptors^{65–68} (reviewed in REFS 69–71), although much less is known about how other phagocytic receptors function. Over the past 3 years, genome-wide RNAi screening strategies in *D. melanogaster* S2 cells have facilitated the identification of the components of the machinery required for the uptake of *E. coli*, *S. aureus*, *M. fortuitum*, *L. monocytogenes* and *C. albicans*^{19–25}. These studies have shown that much of the machinery triggered by complement receptors and FcRs in mammals is also used for pathogen uptake in *D. melanogaster*, an organism that does not express these receptors. In addition to validating many known regulators of uptake, these screens have also provided novel insights into the process of internalization. In particular, RNAi screens in *D. melanogaster* S2 cells have been the first to implicate the coatamer protein complex^{19,21–25} and the exocyst^{19,21,22,24} in bacterial uptake.

The coatamer complex. During phagocytosis marked changes occur in the plasma membrane, which must bend significantly to form the phagocytic cup and during pseudopod extension (FIG. 1). Although such membrane remodelling is vital during engulfment, the molecular mechanisms that stabilize the areas of membrane curvature are poorly understood. Three well-recognized mechanisms exist for the generation of membrane curvature and vesicle budding, which are mediated by the protein-coat complexes clathrin, coat-protein complex I (COPI) and COPII (REF. 72), and it is likely that some or all of these contribute to maintain the curvature of the membrane that is required for phagocytosis. Clathrin-mediated endocytic uptake is probably the best understood

Convergent evolution

The development of similar characteristics in organisms that are unrelated as a consequence of how each adapts to a similar evolutionary pressure but which occurs after evolutionary divergence.

Pseudopod

A transient protrusion from the cell during cell movement or to envelop a particle that is to be internalized.

and serves as a paradigm for how coated vesicles form. Clathrin acts to both concentrate adaptor proteins and cargo and, through polymerization into a lattice, stabilizes the budding membrane. Clathrin is implicated in the uptake of certain pathogens⁷⁵. COPI and COPII are best known for their role in vesicle trafficking from the Golgi to the endoplasmic reticulum (ER) and from the ER to the Golgi, respectively^{73,74}. Although, the possible involvement of the COPI and COPII in engulfment has not been extensively investigated in mammals, several *D. melanogaster* RNAi screens have implicated them in particle internalization^{19,21–25}. The association of COPI and COPII with the *D. melanogaster* phagosome²¹ support a role for these proteins in phagocytosis and one can speculate that the COPI and COPII might help to establish the curvature that is required for membrane invagination (FIG. 1). An alternative, but not mutually exclusive, possibility is that COPI and COPII stabilize the curvature of the membrane of the vesicles (endosomes and lysosomes) with which phagosomes fuse during maturation⁷⁶ (FIG. 1).

The exocyst. The exocyst is an octodimeric complex consisting of the proteins Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84, and was first identified as being assembled between the plasma membrane and secretory vesicles during neurotransmitter exocytosis⁷⁷. This complex tethers the docking vesicle to the plasma membrane and hence facilitates membrane fusion mediated by soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) and soluble NSF attachment protein receptor (SNARE) complexes⁷⁸. The exocyst is now known to be involved in diverse processes, such as the delivery of adhesion or signalling molecules to targeted sites on the plasma membrane and mobilizing membrane fragments from intracellular compartments to areas of rapid membrane remodelling, such as the leading edge of migratory cells⁷⁹. Proteomic analysis of the *D. melanogaster* phagosome identified six of the eight known exocyst components, suggesting that the complex is also assembled on phagosomes, and this has been confirmed in mammalian cells²¹. Silencing of the exocyst components has been reported to decrease bacterial internalization and affect intracellular survival in several independent RNAi screens^{19,21,22,24}, which supports the functional contribution of this complex to particle engulfment. The current model suggests that the exocyst is required to tether endosomes to the phagocytic cup²¹ or lysosomes to the maturing phagosome (FIG. 1), but it requires further validation.

Nutrient acquisition and immune sensing

The highly hydrolytic and digestive capacity of the phagosome is evident even in unicellular organisms, in which the phagosome is required to degrade bacteria and derive energy from the internalized microorganisms. To prevent them from being overwhelmed by the bacterial 'meal', the phagosome also destroys the internalized organisms, which suggests a primordial link between nutrient acquisition, pathogen destruction and innate

immune sensing (FIG. 5a,b). *D. melanogaster* has provided insight into how bacterial by-products are generated after phagocytosis and sensed by the immune system.

Digestion of pathogens and PRR ligand generation.

Recent work in *D. melanogaster* has suggested that exocytosis of bacterial-degradation products from the phagosome contributes to activation of the systemic innate immune response⁸⁰. This has been suggested as a haemocyte-expressed lysosomal protein, Psidin,

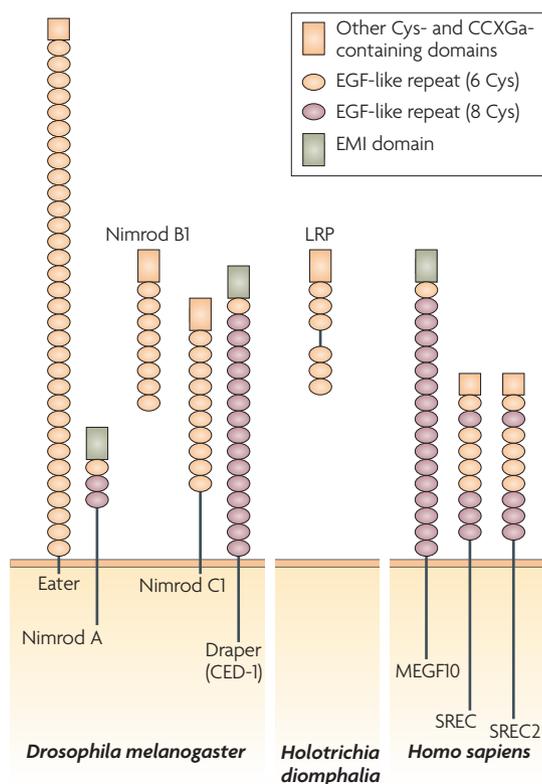


Figure 3 | An emerging superfamily of EGF-like-repeat-containing phagocytic receptors and opsonins. This figure depicts selected members of a superfamily of receptors, the extracellular domains of which contain an N-terminus with a characteristic, cysteine-flanked CCXGa (where X denotes any amino acid and a denotes an aromatic amino acid) motif followed by multiple epidermal growth factor (EGF)-like repeats. The Emilin (EMI) domain is a dimerization domain¹⁰¹ that was first described in *Caenorhabditis elegans* CED-1, a putative phagocytic receptor for apoptotic cells. Several members of this emerging superfamily of scavenger receptors have been implicated experimentally in the phagocytosis or clearance of microbial pathogens (Eater and Nimrod C1 in *Drosophila melanogaster*, lipopolysaccharide-recognition protein (LRP) in the large Korean beetle, *Holotrichia diomphalia*) or of apoptotic cells (*D. melanogaster* Draper and *Homo sapiens* MEGF10 (multiple EGF-like domain 10), the respective homologues of *C. elegans* CED-1). Direct recognition of the microbial ligand lipopolysaccharide was demonstrated for LRP⁵¹ and for the four N-terminal EGF-like repeats of Eater (C. Kocks and R.A.E., unpublished observations). SREC, scavenger receptor expressed by endothelial cells.

Exocytosis

The release of material contained within vesicles by fusion of the vesicles with the plasma membrane.

was identified as contributing to systemic immune activation. One interpretation is that Psidin is required to generate bacterial degradation products that are then exocytosed from the phagolysosome into the haemolymph to amplify the innate immune response systemically by activating fat-body cells, the main source of circulating antimicrobial peptides. This

model suggests a potentially new mechanism for generating immunogenic ligands that are sensed by the innate immune system. Mammals also have a homologue of Psidin, and it will be of interest to determine whether phagosome-generated pathogen-associated degradation products also amplify systemic immune responses in mammals.

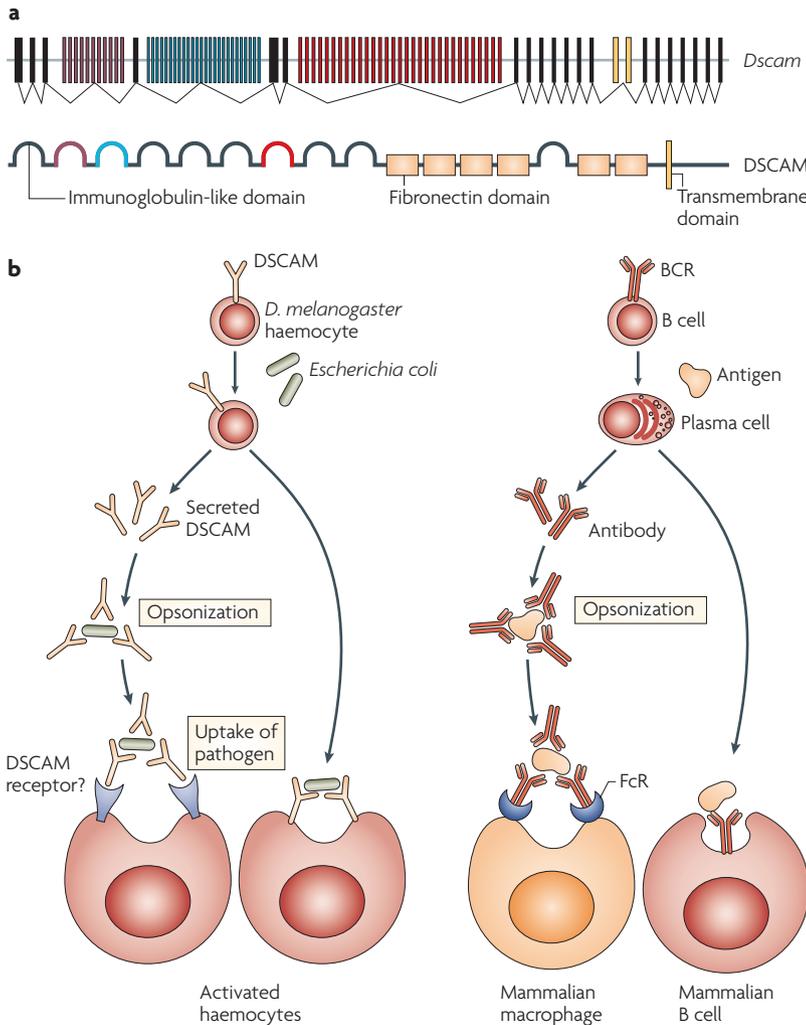


Figure 4 | Insect DSCAM and mammalian immunoglobulins display common functional characteristics. **a** | Gene and protein structure of *Drosophila melanogaster* DSCAM (Down syndrome cell-adhesion molecule). Reminiscent of immunoglobulins, mutually exclusive alternative splicing occurs for exon clusters 4, 6, 9 and 17 to generate DSCAM receptor diversification with greater than 30,000 predicted splice variants, approximately 18,000 of which could be expressed by haemocytes and fat-body cells. A smaller 170 kDa form of DSCAM has been identified and is believed to represent the secreted form of the protein. Variation occurs in the second, third and seventh immunoglobulin domains. **b** | DSCAM and B-cell receptors (BCRs) in *D. melanogaster* and mammals, respectively, recognize foreign antigen and transmit signals to immune cells resulting in cell activation or cell survival. These signals also stimulate the production of secreted forms of DSCAM from activated *D. melanogaster* fat-body cells or haemocytes or, in mammals, immunoglobulin production by plasma cells. These molecules in turn act as opsonins, facilitating uptake through receptors on phagocytes. In mammals, antibody is recognized by Fc receptors (FcRs) but in *D. melanogaster* haemocytes it is unclear whether DSCAM-opsonized particles are recognized by a dedicated receptor or through homotypic interactions with membrane-bound DSCAM. In addition, both the BCR and DSCAM can act directly as endocytic or phagocytic receptors to internalize antigens or pathogens.

PGRP-LE and cytosolic innate immune sensing. In addition to releasing bacterial derivatives back into the circulation, it is possible that the phagosome might also deliver them into the cytosol⁸¹. In mammals, cytosolic bacterial fragments, such as muramyl dipeptide (MDP), are sensed by members of the nucleotide-binding domain, leucine-rich-repeat-containing (NLR) family^{82–84}, which also recognize intact intracellular pathogens or bacterial components that are introduced by the secretory apparatus associated with certain microorganisms. In *D. melanogaster*, a structurally unrelated member of the peptidoglycan-recognition protein (PGRP) family, **PGRP-LE**, in addition to functioning as an extracellular soluble receptor, can also function as an intracellular receptor that senses the bacterial derivative tracheal cytotoxin (TCT)⁸⁵. How TCT accesses the cytosol is unknown and one possibility is that phagocytosis delivers the ligand to PGRP-LE. The functional equivalence between *D. melanogaster* PGRP-LE and mammalian NLRs suggest convergent evolution and it appears that these distantly related organisms have both evolved mechanisms to sense pathogen invasion into the cytosol. Interestingly, although a secreted soluble form of PGRP-LE exists, soluble NLRs have not been described. Secreted PGRP-LE can function in a cell-autonomous manner and may have a role as a co-receptor for PGRP-LC, which is expressed on the cell surface⁸⁶, and hence function in a manner analogous to CD14 that cooperates with TLR4 to respond to lipopolysaccharide in mammals.

Lessons pertaining to adaptive immunity

The adaptive immune response is characterized by the establishment of immunological memory made possible by clonal expansion of effector lymphocytes and somatic mutation and germline recombination of antigen receptors. Antigen presentation by antigen-presenting cells drives the clonal expansion of B and T cells and the retention of memory cells. Although *D. melanogaster* does not have a conventional adaptive immune response as defined by these characteristics (for a recent debate, see REF. 87), evidence suggests that the immune system of *D. melanogaster* may have greater complexity than previously appreciated²⁸ and that studies in flies might even provide some insights into mammalian adaptive immunity.

Immune adaptation. Although the specializations of the immune system that define adaptive immunity were believed to be unique to jawed vertebrates, recent studies in lamprey have identified an alternative adaptive immune system⁶⁴. However, studies in

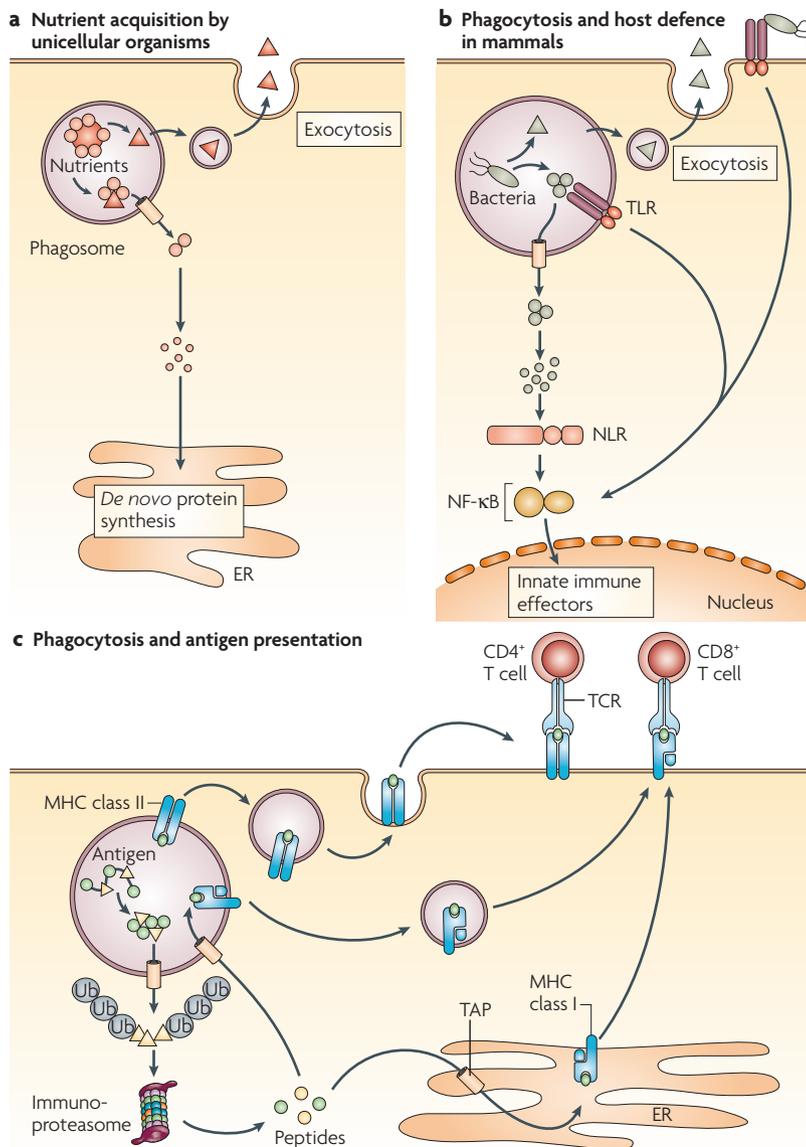


Figure 5 | Nutrient acquisition is possibly the evolutionary origin of the link between phagocytosis and bacterial recognition. **a** | Phagocytosis is linked to nutrient acquisition by unicellular organisms. Internalized nutrients are degraded in a multistep process: enzymatic degradation; export of desired components from the phagosome into the cytosol; exported material may be further modified by proteasomal degradation; component amino acids are then used for protein synthesis; and undesired components are exported out of the cell by exocytosis. **b** | In higher species, phagocytosis has an essential role in innate immune sensing and degraded products of internalized bacteria may traffic and be modified as for nutrients. In host defence, the degraded products are sensed by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), in the phagosome lumen. Hypothetically, phagocytosis may also deliver ligands to cytosolic receptors, such as the nucleotide-binding domain, leucine-rich-repeat-containing (NLR) family in mammals and the structurally unrelated peptidoglycan-recognition protein (PGRP-LE) in *Drosophila melanogaster*. **c** | In mammals, phagocytosis is essential for antigen presentation and adaptive immunity. Internalized antigens follow a similar path from the phagosome as nutrients. The antigenic peptides are then transported by transporter associated with antigen processing (TAP) either into the endoplasmic reticulum (ER) or back into the phagosome, and cross-presented on MHC class I molecules. *D. melanogaster* phagosomes contain much of the machinery required for antigen processing but lack the antigen-presentation machinery (that is, MHC and related molecules such as tapasin). These models suggest that the basic template of both innate immune sensing and antigen processing may be built on primordial functions of phagocytosis in nutrient absorption that existed before the acquisition of adaptive immunity. NF- κ B, nuclear factor- κ B; TCR, T-cell receptor; Ub, ubiquitin.

invertebrates, such as *D. melanogaster*, have failed to conclusively demonstrate immune adaptation⁸⁷. That said, it has been reported that resistance to certain pathogens can be transferred from mother to progeny in *D. melanogaster*, suggesting inheritable resistance⁸⁸. In addition, recent data indicate that fruit flies may demonstrate immunological memory in response to certain pathogens⁸⁹. This memory is sustained for the life of the flies and is specific, protecting only against the pathogen from the original infection. Importantly, this innate immune memory requires Toll but occurs independently of antimicrobial peptides, the key effectors induced by this pathway. Pertinent to this Review, the effector memory response requires phagocytes and can be blocked by paralysis of the phagocytic machinery. However, why memory is induced only by certain pathogens and the exact role of phagocytes in this context is unclear. Future work will need to define the molecular mechanism of this resistance before it can be conclusively said that *D. melanogaster* has the capacity for immune adaptation and whether this primitive adaptive immunity is the immunological forerunner of our own or whether it is independently evolved.

Antigen presentation. In mammals, the phagosome is important for antigen presentation. Phagocytosed material is processed into antigenic peptides that are either loaded onto MHC class II molecules for presentation to CD4⁺ T cells or, by a currently poorly defined mechanism, access the endogenous pathway and are cross-presented on MHC class I molecules to activate CD8⁺ T cells. Intriguingly, the efficiency of cross-presentation is greatly increased in circumstances in which the antigen has been phagocytosed^{90,91}. Although a subject of ongoing debate, it has been suggested that the contribution of the ER to the phagosome membrane facilitates this process, as machinery that is normally used for the dislocation of newly synthesized proteins from the ER is co-opted for phagosome-associated cross-presentation^{92–95} (FIG. 5c). Analysis of *D. melanogaster* phagosomes also identified ER components and fly orthologues for many of the mammalian proteins required for antigen processing²¹. However, as this approach was identical to that used to isolate mammalian phagosomes and that originally implicated the ER as a source of phagosome membrane, we do not consider this independent verification. Nonetheless, this intriguing observation suggests that the basic machinery required for antigen processing might have been associated with phagocytosis before the evolutionary acquisition of adaptive immunity. If this is the case, it is likely to reflect a role for this machinery in nutrient acquisition (FIG. 5). Thus, the nidus for adaptive immunity may not simply have been the acquisition of recombination-activating genes and immunoglobulin rearrangement but rather evolved in a modular way, with the early step of antigen processing made possible by building on an already existing function of phagocytosis in peptide and amino-acid absorption⁹⁶.

Concluding remarks and future directions

Phagocytes have emerged as crucial guardians and central effector cells in host defence. More than a century ago, Metchnikoff's work emphasized the value of comparative embryology and established the validity of studying phagocytic engulfment in model organisms⁹⁷. What is perhaps surprising is that similar model systems to those used by Metchnikoff in the nineteenth century have remained invaluable tools for the study of phagocytosis a 100 years later. These systems continue to provide insights into the complex cell biology of phagocytes and their importance in the evolutionary origins of immunity.

We expect to see yet more advances from studies of *D. melanogaster* phagocytes by the application of cutting-edge technologies and systems-based approaches,

including genomics and proteomics. *D. melanogaster* S2 cells will continue to be a preferred cell type for the high-throughput study of the complex cell biology of engulfment and to rapidly dissect the innate immune signalling pathways that are triggered by encounters with pathogens. In addition, using advanced imaging techniques in *D. melanogaster* embryos, it is now possible to obtain subcellular resolution that is sufficient to monitor organelle trafficking and to study phagosomes *in vivo*^{98,99}. Understanding how the process of phagocytosis is tailored for and by its numerous and varied cargo remains a challenge. We anticipate that this model system will remain pivotal and, through its simplicity and genetic tractability, will continue to facilitate comparative innate immunity and provide new insights into the complex cell biology of phagocytosis and evolutionary origins of host defence.

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DATABASES

Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>
 CD36 | CD91 | CED-1 | MEGF10 | MEGF11
 FlyBase: <http://flybase.bio.indiana.edu/>
 Croquemort | Draper | DSCAM | Eater | MCR | Nimrod C1 | Peste | PGRP-LE | SR-C1

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