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REVIEW

Phagocytosis, a cellular immune response in insects

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Abstract

Insects like many other organisms are exposed to a wide range of infectious agents. Defense against these agents is provided by innate immune systems, which include physical barriers, humoral responses, and cellular responses. The humoral responses are characterized by the production of antimicrobial peptides, while the cellular defense responses include nodulation, encapsulation, melanization and phagocytosis. The phagocytic process, whereby cells ingest large particles, is of fundamental importance for insects' development and survival. Phagocytic cells recognize foreign particles through a series of receptors on their cell membrane for pathogen-associated molecules. These receptors in turn initiate a series of signaling pathways that instruct the cell to ingest and eventually destroy the foreign particle. This review describes insect innate humoral and cellular immune functions with emphasis on phagocytosis. Recent advances in our understanding of the phagocytic cell types in various insect species; the receptors involved and the signaling pathways activated during phagocytosis are discussed.

Key Words: insect; phagocytosis; hemocyte; innate immunity; signal transduction

Introduction

Multicellular organisms possess a series of systemic, cellular and molecular mechanisms, which allow them to protect themselves from infection by viruses, bacteria, fungi and protozoa. These mechanisms are collectively known as immunity. Among the defense mechanisms taking place at the onset of an infection there are early systems, such as constitutive expression of antimicrobial peptides, recognition of microorganisms by pattern-recognition receptors (PRRs), and activation of phagocytic cells, involved in detecting and eliminating pathogens, through a wide variety of cellular responses. These responses are the innate immune systems. In vertebrates, such as mammals, the recognition of antigens by specific receptors of T- and B-lymphocytes is yet another, more precise layer of the defense system, known as adaptive immunity. This adaptive response is activated at later times during the course of an infection. Normally, leukocytes circulate in a resting state, not showing antimicrobial properties. Upon recognition of microorganisms via PRRs or via specialized phagocytic receptors, leukocytes develop a fully active antimicrobial and proinflammatory phenotype. This change is known as activation of phagocytic cells. The signals delivered to the cell by the various receptors determine the final activation stage of the leukocyte. At later times, these activated leukocytes can process and present antigens to specific T- and B-lymphocytes to develop an adaptive immune response, which provides a much better and faster response to the same pathogen during a second challenge. For insects, the view is that they, like other invertebrates, depend only on its innate immune response to fight invading microorganisms; by definition, innate immunity lacks adaptive characteristics. However, there are some reports showing that priming *Drosophila* with a sublethal dose of *Streptococcus pneumoniae* protects against an otherwise-lethal second challenge of *S. pneumoniae* (Pham *et al.*, 2007). This protective effect has loose specificity for *S. pneumoniae* and persists for the life of the fly. While not all microbial challenges induced this specific primed response, a similar specific protection could be elicited by the fungus *Beauveria bassiana*, a natural fly pathogen (Pham *et al.*, 2007). These results point out that insect immune responses can indeed adapt and suggest that insect hemocytes may also present an activation response similar to the one known in mammalian leukocytes.

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Millions of insect species live in practically every known habitat and ecological niche, though marine environments are an important exception. This diversity exposes insects to all sorts of infectious agents, such as viruses, bacteria, fungi and protozoa. Insects have evolved an effective innate immune system that permits rapid and efficient responses against infectious agents. The innate immune system of insects consists of physical barriers, humoral responses and cellular responses (Lavine *et al.*, 2002; Kanost *et al.*, 2004). In addition, recent evidence suggests that insects also have adaptive immune responses as mentioned before, although these responses are not similar as those traditionally defined in mammals with B- and T-lymphocytes.

Physical barriers include the integument and the peritrophic membrane. Integument, the outer surface of an insect, is formed by a single layer of cells covered by a multi-layered cuticle (Ashida *et al.*, 1995). The peritrophic membrane or peritrophic matrix is a chitin and glycoprotein layer that lines the insect midgut. It is functionally similar to the mucous secretions of the vertebrate digestive tract and hence it acts as a physical barrier, protecting the midgut epithelium from abrasive food particles, digestive enzymes and some digestive pathogens (Lehane, 1997; Hegedus *et al.*, 2009). However, due to its semipermeable nature, this matrix is not an efficient barrier to infection, particularly to viruses. Together these structures form the first line of protection for the hemocel (the insect body cavity) and the midgut epithelium from invading microorganisms. In the case these barriers are breached, the humoral and cellular immune responses are activated. Humoral immune responses include biosynthesis of antimicrobial peptides, activation of enzymes, such as lysozyme and the prophenoloxidase (proPO) system, to regulate coagulation of hemolymph and production of reactive oxygen species (Jiang, 2008; Tsakas *et al.*, 2010). Cellular immune responses include nodulation, encapsulation, and phagocytosis (Strand, 2008; Tsakas *et al.*, 2010).

Hemolymph, the liquid that fills the hemocel of an insect, has an analogous function to both blood and lymph in mammals. It is involved in transport of nutrients and waste products, although not transport of respiratory gases. In addition, it contains several types of free-moving cells or hemocytes. Hemocytes originate from mesodermally derived stem cells that differentiate into specific lineages. The most common types of hemocytes are granulocytes, plasmatocytes, spherulocytes, and oenocytoids (Lavine *et al.*, 2002; Meister *et al.*, 2003). However, it is important to emphasize that not all these hemocyte types exist in all insect species (Meister, 2004; Michela *et al.*, 2005; Manachini *et al.*, 2010; Wang *et al.*, 2010). Hemocytes are essential for insect immunity, as shown in *Drosophila melanogaster* larvae where plasmatocytes, making up approximately 95 % of circulating hemocytes, decrease in numbers during an infection (Williams, 2007). Also, by genetic ablation (Defaye *et al.*, 2009) or mechanical ablation (Charroux *et al.*, 2009b; Nehme *et al.*, 2011) of phagocytic hemocytes in *Drosophila*, it was observed that in adult flies there is an increase in infection susceptibility to various bacteria including *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*.

Cellular immune responses are immediate after an invasion of the hemocel, while humoral responses appear several hours after an infection. Based on data from coleopterans, although not shown in other insects yet, it is thought that humoral responses have the function of finishing up the invading microorganisms that escaped the initial cellular immune responses (Haine *et al.*, 2008). Together, physical barriers, humoral, and cellular immune responses provide an effective defense system for the insect. These elements, however, do not work in isolation and in an orderly fashion. There is a complex interplay among them. For example, hemocytes and other insect cells produce molecules that increase hemocyte-microorganism binding (Mohrig *et al.*, 1979; Wiesner *et al.*, 1996; Brivio *et al.*, 2010; Kim *et al.*, 2010). These molecules are similar to the opsonins that increase phagocytosis of leukocytes in mammals. In addition, *Drosophila* plasmatocytes, which are essential for phagocytosis, are also required during larval stages to induce fat-body (insect equivalent of the liver) cells to produce antimicrobial peptides after a bacterial infection (Charroux *et al.*, 2009b). In addition, in adult flies, plasmatocytes contribute to reduce the infection susceptibility to various bacteria including *E. coli*, *B. subtilis* and importantly *S. aureus* (Defaye *et al.*, 2009; Nehme *et al.*, 2011). These findings clearly indicate that there is an effective cross-talk between humoral and cellular immunity in insects.

Here, I will describe insect cellular immune functions with emphasis in phagocytosis and review recent findings on phagocytic hemocyte types, the receptors involved and the signaling pathways activated during this cellular response.

Insect cellular immunity

Hemocytes are responsible for a variety of defense responses in insects. Many variations in insect immune responses exist due to the presence of millions of insect species, and we are just beginning to understand these variations (Schmid-Hempel, 2005). However, a number of frequent innate immune responses have been described in most insects studied. These responses include nodulation, encapsulation, melanization, and phagocytosis. These general processes share common elements in terms of pathogen recognition, biochemical signals, and final clearance of the invading microorganism from the hemolymph. Next, I briefly describe our current understanding on these insect innate immune responses.

Nodulation

Nodulation is a predominant cellular response in insects to large bacterial infections. It consists of formation of multicellular hemocyte aggregates that entrap large numbers of bacteria. It begins when hemocytes, together with antimicrobial molecules in the hemolymph, surround bacteria. Hemocytes with their entrapped bacteria form small aggregates that grow by joining with additional hemocytes to form large nodules. The nodule is completed by covering

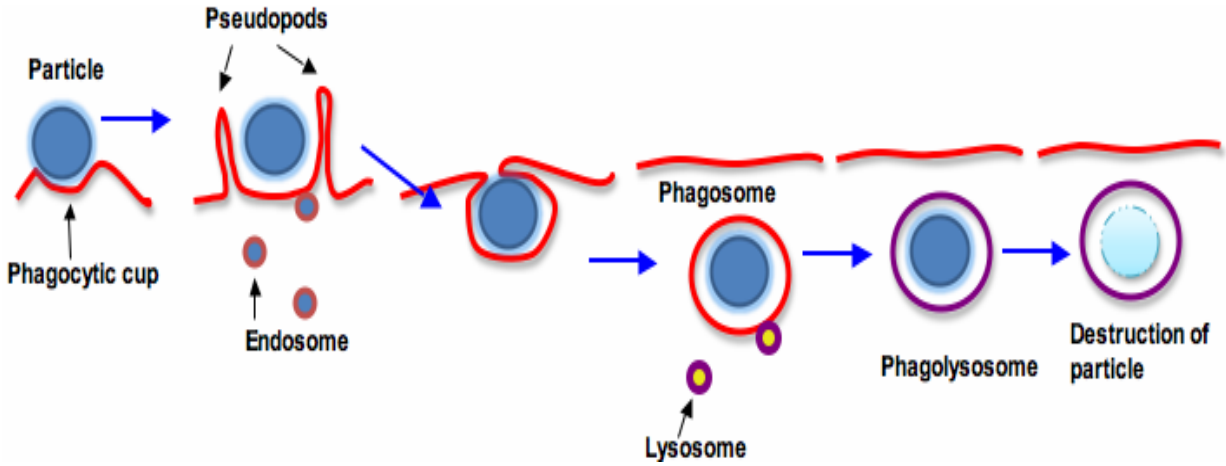

Fig. 1 Phagocytosis. The process of ingesting and destroying a particle begins with the particle recognition by special receptors on the cell. This initial interaction triggers a series of cellular signals that induce rearrangement of the actin cytoskeleton and membrane remodeling. The particle sits on a membrane depression, the phagocytic cup, and then it is surrounded by pseudopods. The membrane fuses around the particle and separates into the cell in the form of a new vesicle, the phagosome. In order to internalize large or numerous particles, the phagocyte recruits addition membrane from intracellular vesicles such as endosomes and the endoplasmic reticulum. The phagosome "matures" to form a phagolysosome by fusing with other vesicles such as lysosomes and undergoing acidification. Inside the phagolysosome, the ingested particle is finally destroyed.

it with layers of flattened hemocytes. In some cases, but not always, nodules are also melanized. These melanin-covered nodules are an effective way to isolate the invading bacteria from the hemolymph, since they have an impermeable wall between the nodule and the rest of the insect organism. The process of nodule formation is not completely characterized, but clearly eicosanoids are likely to be important for nodule formation in many insect species (Miller *et al.*, 1999; Shrestha *et al.*, 2009; Zhao *et al.*, 2009; Shrestha *et al.*, 2010) and prophenoloxidase (proPO) and dopa decarboxylase (Ddc) are also involved in this process at least in Mediterranean fruit fly (medfly) *C. capitata* hemocytes (Sideri *et al.*, 2008). In addition, screenings for novel immune genes from an Indian saturniid silkmoth (*Antheraea mylitta*) larvae, and from the silkworm (*Bombyx mori*) larvae, identified two proteins, Noduler (Gandhe *et al.*, 2007) and Reeler1 (Bao *et al.*, 2011) respectively, with a characteristic reeler domain in the fat-bodies of these insects. These proteins were essential in mediating nodulation response against *E. coli* K12 and *B. subtilis* bacteria challenge.

Encapsulation

Encapsulation is the response of hemocytes to large targets such as parasites, protozoa, and nematodes. Hemocytes bind to the target in multiple cell layers until they form a capsule around the invader. The capsule is normally melanized at the end (Ling *et al.*, 2006). Inside the capsule the invading organism is killed by reactive cytotoxic products or by asphyxia (Carton *et al.*, 2005; Nappi *et al.*, 2009).

Melanization

Melanization is the process of melanin formation. It is activated during wound healing and also in nodule and capsule formation against large pathogens or parasites in some lepidopteran and dipteran insects, such as *Manduca*, *Pseudolusia* and *Drosophila* (Lavine *et al.*, 2001; Lavine *et al.*, 2002; Kanost *et al.*, 2004; Nappi *et al.*, 2009). The enzyme phenoloxidase (PO) is key in this process. Activation of proPO to PO is mediated by a serine proteinase cascade (Cerenius *et al.*, 2008; Eleftherianos *et al.*, 2011) in adult *Drosophila*, but also in lepidopterans and requires PRRs such as peptidoglycan recognition protein (PGRP) (Yoshida *et al.*, 1996; Ochiai *et al.*, 1999) or beta-1,3-glucan recognition protein (betaGRP) (Ochiai *et al.*, 1988, 2000). Interaction of these recognition proteins with microbial peptidoglycan or beta-1, 3-glucan molecules initiates the activation of the proPO cascade. Then PO binds to foreign surfaces including hemocyte membranes (Ling *et al.*, 2005), where it initiates melanin formation. PO acts on tyrosine and converts it to dopa (Marmaras *et al.*, 2009). Dopa can then be decarboxylated by Ddc to dopamine or further oxidized by PO to dopaquinone. Both products are then further metabolized to eumelanin and finally melanin (Marmaras *et al.*, 2009).

Phagocytosis

Phagocytosis is the process by which cells recognize, bind and ingest relatively large particles (usually larger than 0.5 μm in diameter). Phagocytosis is an evolutionary conserved cell response that was first described 130 years ago by Elie Metchnikoff (1845-1916) in sea-star larva;

where he observed the accumulation of cells he named phagocytes, around a rose thorn that he inserted into the larva to provoke an inflammatory injury (Metchnikoff, 1884; Kaufmann, 2008; Tan *et al.*, 2009). Phagocytosis is probably the oldest defense mechanism against microorganisms. During phagocytosis the target particle is first recognized by phagocytic receptors that activate various signaling pathways in the cell interior (Jones *et al.*, 1999). These signals lead to dramatic changes in the dynamics of the plasma membrane and the cytoskeleton. The membrane extends pseudopods around the particle, forming a cup that moves into the cell. Within few minutes the membrane closes at the distal end, leaving a new plasma membrane-derived phagosome (Yeung *et al.*, 2006) (Fig. 1). Phagocytosis requires a rapid replenishment of plasma membrane. In mammalian leukocytes, endoplasmic reticulum-derived endosomes have been shown to be the source of this newly added membrane (Bajno *et al.*, 2000). In the next 40 minutes, the lumen of the phagosome becomes an environment capable of destroying the ingested particle. This process is called "phagosome maturation" and is the result of changes in the phagosome membrane through fusion with other membranous organelles including lysosomes (Yeung *et al.*, 2006) (Fig. 1).

Phagocytosis is a fundamental cellular process performed by unicellular organisms and by particular cell types in multicellular organisms. In simple organisms such as *Amoeba* and the slime mold *Dictyostelium discoideum*, phagocytosis is used both in feeding and in defense (Chen *et al.*, 2007; Cosson *et al.*, 2008). In vertebrates, phagocytosis plays an essential role in embryogenesis and also in host defense mechanisms through the uptake and destruction of pathogens (Greenberg *et al.*, 2002). Phagocytosis also contributes to inflammation and the immune response (Rosales, 2005). In mammals, a subset of specialized cells, named professional phagocytes, is responsible for rapidly and efficiently ingesting invading microorganisms at sites of inflammation. These phagocytes are neutrophils, monocytes and macrophages (Rabinovitch, 1995). Neutrophils and monocytes circulate in the blood, while macrophages reside in tissues.

In insects, phagocytosis is performed by a subset of hemocytes in the hemolymph (Strand, 2008). Professional phagocytes in Diptera and Lepidoptera have also been described as plasmatocytes and granular hemocytes, respectively (Lavine *et al.*, 2002). In agreement with this, plasmatocytes or granulocytes are the main phagocytic cells in most insects (Meister, 2004; Castillo *et al.*, 2006; Lamprou *et al.*, 2007; Lemaitre *et al.*, 2007; Garcia-Garcia *et al.*, 2009). However, there is clearly a great deal of variability among different insect species. Our knowledge of insect phagocytosis comes mainly from studies on the fruit fly *D. melanogaster* (Lemaitre *et al.*, 2007; Stuart *et al.*, 2008), on mosquitoes *Anopheles* (Blandin *et al.*, 2007), which are vectors of the human malaria parasite *Plasmodium falciparum*, or on the medfly *C. capitata* (Lamprou *et al.*, 2005; Sideri *et al.*, 2008). This however will change in the future, as more and more reports are coming out describing the phagocytic process by hemocytes from other insect species (Kanost *et al.*, 2004; Costa *et al.*, 2005; Mylonakis *et al.*, 2005; Borges *et al.*, 2008; Castro *et al.*, 2009; Garcia-Garcia *et al.*, 2009; Amaral *et al.*, 2010; Manachini *et al.*, 2010; Wang *et al.*, 2010).

Phagocytosis eliminates mainly two types of targets: microorganisms and "altered self" particles, represented by apoptotic cells. Ingestion of apoptotic cells is important during tissue remodeling and embryogenesis when excess cells undergo programmed cell death (apoptosis) and removal (Hopkinson-Woolley *et al.*, 1994). In insects, phagocytosis of dying cells is fundamental during embryogenesis, especially in the development of holometabolous insects such as *D. melanogaster*. In this fly, hemocytes eliminate the many apoptotic cells that appear during the process of metamorphosis (Neufeld *et al.*, 2008). Despite the importance of this process in insect development, the mechanisms of phagocytosis of apoptotic cells in insects are still poorly known (Sass, 2008). Ingestion of microorganisms is also fundamental for insect defense against infections. However, it is becoming clear that hemocyte phagocytosis can control some, but not all bacterial infections. For example, it has been reported that *E. coli* (a Gram-negative bacteria) is more readily phagocytosed than *S. aureus* (a Gram-positive bacteria) by hemocytes from the mosquito *A. gambiae* (Levashina *et al.*, 2001; Hillyer *et al.*, 2003), the fruit fly *D. melanogaster* (Rämet *et al.*, 2002), and the medfly *C. capitata* (Lamprou *et al.*, 2007). Similar results were found for the phagocytosis of *E. coli* by cricket *Acheta domesticus* hemocytes (Garcia-Garcia *et al.*, 2009), but opposite results for phagocytosis of *Streptomyces lividans* (a Gram-positive bacteria) by the beetle *Zophobas morio* hemocytes (Garcia-Garcia *et al.*, 2009). Also in *A. aegypti* the main response of hemocytes to *E. coli* was phagocytosis, while the response to *M. luteus* was melanization (Hillyer *et al.*, 2003). These results should be taken with caution because nonvirulent bacteria are compared with pathogenic bacteria, therefore we cannot generalize the phagocytic response observed will be similar for all Gram-positive or Gram-negative bacteria. Also, bacteria have been previously killed to perform these phagocytosis assays. Thus, the phagocytosis response in vivo might also be different. Together these reports suggest that indeed in insects several distinct molecular mechanisms controlling phagocytosis must exist.

Our knowledge of phagocytosis comes mainly from studies with mammalian cells and from these studies it is clear that there is much redundancy in this cellular response. Different phagocyte membrane receptors and various elements of the phagocytic machinery seem to have similar and many times overlapping functions. Despite this, much has been learned about phagocytic receptors for opsonins, such as antibodies and complement. Opsonins are substances that cover the particle to be ingested and promote its phagocytosis. The best-studied phagocytic receptors are the receptors for antibody, termed Fc Receptors (Garcia-Garcia *et al.*, 2002; Swanson *et al.*, 2004), and the complement receptors (Jones *et al.*, 1999; Rosales, 2007).

However, our knowledge on the function of individual receptors of phagocytosis in professional phagocytes remains incomplete because these cells are not suitable for many genetic and molecular biology techniques, such as cDNA overexpression or knockdown expression of molecules by RNA interference (RNAi). Thus, studies of phagocytosis by *D. melanogaster* hemocytes have become very attractive because this is a genetically tractable system. In addition, the power of RNAi analysis has been used to develop a molecular screen system for phagocytosis in the *Drosophila* embryonic S2 (Schneider line 2) cell line. These fruit fly cells present characteristics similar to mammalian macrophages and efficiently ingest bacteria in an actin-dependent manner (Pearson *et al.*, 2003). This system has also been adapted for high-throughput screening with RNAi leading to identification of many phagocytosis-related genes (Rämet *et al.*, 2002; Cheng *et al.*, 2005). Moreover, the system has also identified molecules involved in hemocyte interaction with microorganisms such as *E. coli* (Rämet *et al.*, 2002), *S. aureus* (Stuart *et al.*, 2005a), *Mycobacterium fortuitum* (Philips *et al.*, 2005), *Listeria monocytogenes* (Agaisse *et al.*, 2005; Cheng *et al.*, 2005), and *Candida albicans* (Stroschein-Stevenson *et al.*, 2006; Stroschein-Stevenson *et al.*, 2009). Similarly, the power of RNAi has also been used in studies of phagocytosis by mosquito *A. gambiae* hemocytes (Levashina *et al.*, 2001; Blandin *et al.*, 2002). Despite the fact that *Drosophila* S2 cells present characteristics similar to mammalian macrophages (Pearson *et al.*, 2003) the similarity of cultured cells to hemocytes remains an open question. Thus the findings from the studies mentioned above still require in vivo validation. The gold standard is an increased susceptibility to specific infections of mutant insects, coupled with in vitro and in vivo data demonstrating an impaired phagocytosis. Such an in vivo system has recently been described. A genome-wide in vivo *Drosophila* RNA interference screen was used to uncover genes involved in susceptibility or resistance to intestinal infection with the bacterium *Serratia marcescens* (Cronin *et al.*, 2009). This system, in turn requires in vitro validation of the genes potentially involved in phagocytosis. But, things look promising since some of the identified genes overlap with those identified in a systems biology Proteomic analysis of phagosomes isolated from cells derived from *D. melanogaster* (Stuart *et al.*, 2007) (see below).

At the other end of the phagocytosis process, it is the destruction of the ingested particle within the phagosome. In mammals, phagosomes are not only important in innate immunity but also in adaptive immunity since they are active participants of the process of antigen presentation (Houde *et al.*, 2003). Phagosomes vary in nature depending on the cell-surface receptors involved to recognize the target particle, the type of membrane used in their formation, the mechanism of internalization and the nature of the particle. Once formed, the new phagosome undergoes a process known as phagosome maturation (Kinchen *et al.*, 2008). In this process the phagosome fuses with internal vesicles such as endosomes and lysosomes to become a mature phagolysosome (Fig. 1). This mature compartment has an acid environment with highly active hydrolytic enzymes, which stop replication of bacteria and can kill many microorganisms (Kinchen *et al.*, 2008). The importance of the phagosome is also made evident by the fact that some microorganisms can alter the maturation process escape from being killed (Fratti *et al.*, 2001). Thus, there is a great interest in understanding the complex biology of phagosomes. Insect hemocytes and new techniques such as proteomics and computational modeling have also been helpful in elucidating the complexity of the phagosome (Stuart *et al.*, 2007). Proteomics analysis of latex beads-containing phagosomes from *D. melanogaster* S2 cells has confirmed the complexity of this organelle. Close to 600 *D. melanogaster* proteins were identified to be associated with phagosomes, and many of them had mammalian orthologs, validating this as a model for mammalian phagocytosis. Computational analysis has predicted that hundreds of protein-protein interactions take place in the phagosome and has identified several signaling pathways that could be initiated from this organelle including activation of the nuclear factor κB (NF-κB) and activation of mitogen-activated protein kinases (MAPK) (Fratti *et al.*, 2001).

Together RNAi and proteomics approaches with insect hemocytes have identified new genes and molecules important for phagocytosis, specially the putative receptors that insect hemocytes use to bind different microorganisms and signaling pathways activated during phagosome maturation. The relevance of these molecules for phagocytosis and host defense can now be tested *in vivo*.

Insect phagocytic receptors

Commonly for mammalian leukocytes, phagocytosis is initiated after the interaction of opsonins, on the surface of the particle to be internalized, with specific receptors on the phagocyte membrane (Swanson *et al.*, 1995). Phagocytosis can also be triggered, in the absence of opsonins, through the interaction of phagocyte membrane receptors with specific molecules, such as lipids or sugars, which form part of the cell wall of many microorganisms (Stuart *et al.*, 2005b; Stuart *et al.*, 2008). These receptors are known as pattern-recognition receptors (PRRs) because they recognize discrete conserved molecular patterns within microorganism molecules. In insects, several potential PRRs have been identified, and they can be grouped in various types: complement-like molecules, scavenger receptors, epidermal growth factor (EGF)-like repeat-containing receptors, peptidoglycan recognition proteins (PGRPs), integrins, and a highly variant receptor, Down syndrome cell-adhesion molecule (DSCAM) (Table 1).

Complement-like molecules

Thioester-containing proteins (TEPs) constitute an important group of proteins that includes the α2-macroglobulin family of protease inhibitors and the C3/C4 complement factors in vertebrates. The thioester active site typical of and the α2-macroglobulins and complement factors is present

Table 1 Insect phagocytic peceptors

Insect Receptors	Ligands	Mammalian counterpart	Opsonin	Insect	Refs
Complement-like molecules					
TEP VI	*Candida albicans*	Complement	Yes	*D. melanogaster* S2 cells	Stroschein-Stevenson *et al.*, 2006; Stroschein-Stevenson *et al*., 2009
TEP II	*E. coli*		Yes	*D. melanogaster* S2 cells	
TEP III	*S. aureus*		Yes	*D. melanogaster* S2 cells	
TEP1, TEP3	*E. coli, S. aureus*			*A. gambiae*	Moita *et al.*, 2005
Scavenger Receptors					
Croquemort	Apoptotic cells, *S. aureus*	CD36		*D. melanogaster*	Franc *et al.*, 1999; Stuart *et al.*, 2005a
Peste	*M. fortuitum*	SR-BI, SR-BII		*D. melanogaster*	Philips *et al.*, 2005
SR-CI	*E. coli, S. aureus*	?		*D. melanogaster*	Rämet *et al.*, 2001
EGF-like repeat-containing Receptors					
Eater	*E. coli, S. aureus S. marcescens*			*D. melanogaster*	Charroux *et al.*, 2009b; Defaye *et al.*, 2009; Kocks et al. 2005; Nehme *et al.*, 2011
Nimrod	*E. coli, S. aureus*			*D. melanogaster*	Kurucz *et al.*, 2007
Draper	Apoptotic cells, axon pruning, severed axons, *S. aureus*	CD91 (LRP)		*D. melanogaster*	Awasaki *et al.*, 2006; Freeman *et al.*, 2003; Hashimoto *et al.*, 2009; MacDonald *et al.*, 2006; Manaka *et al.*, 2004
Six-microns-under (SIMU)	Apoptotic cells, by glia in the nervous system			*D. melanogaster*	Kurant *et al.*, 2008
Peptidoglycan Recognition Proteins					
PGRP-SC1a	*S. aureus*	Mammalian PGRP*		*D. melanogaster*	Garver *et al.*, 2006
PGRP-LC	*E. coli*			*D. melanogaster*	Kurata, 2010; Rämet *et al.*, 2002
PGRP-LE	*L. monocitogenes*			*D. melanogaster*	Yano *et al.*, 2008
Integrins					
α integrins	*E. coli, Gram-positive bacteria*	Mammalian Integrins		Medfly (*C. capitata*)	Lamprou *et al.*, 2007
β integrins	*E. coli*			Medfly (*C. capitata*) *A. gambiae*	Mamali *et al.*, 2009; Moita *et al.*, 2006
DSCAM	*E. coli, S. aureus*	Immunoglobulin ?	Yes	*A. gambiae* *D. melanogaster* *D. melanogaster*	Dong *et al.*, 2006; Stuart *et al.*, 2007; Watson *et al.*, 2005

* Soluble molecules with no involvement in phagocytosis.
TEP, thioester-containing proteins; SR-CI, scavenger receptor class C 1; LRP, low-density lipoprotein receptor-related protein; PGRP. peptidoglycan recognition protein; DSCAM, Down syndrome cell-adhesion molecule.

in the TEP proteins. In insects, several TEPs are known in *D. melanogaster* (Lagueux *et al.*, 2000) and in *A. gambiae* (Christophides *et al.*, 2002). There are six TEP proteins (TEPI to VI) in the genome of *Drosophila*, including TEPV which is a putative pseudogene, and TEPVI (also named mcr: macroglobulin complement-related) in which the thioester site is mutated (Bou Aoun *et al.*, 2008). Some of the TEPs are upregulated after a bacterial infection (Lagueux *et al.*, 2000), with TEPIII being an

exception (Bou Aoun *et al.*, 2011). Their function was elucidated from an RNAi screen in *Drosophila* S2 cells, in which TEPVI was found to bind and increase phagocytosis of *C. albicans* (Stroschein-Stevenson *et al.*, 2006; Stroschein-Stevenson *et al.*, 2009). Similarly, TEPII binds *E. coli* and TEPIII binds *S. aureus*, and both increase phagocytosis (Stroschein-Stevenson *et al.*, 2006) (Table 1). In addition, TEPs also increase phagocytosis in *A. gambiae* (Moita *et al.*, 2005). Because these TEPs are soluble molecules that bind to microorganisms and induce phagocytosis by insect hemocytes, they behave like typical opsonins. Strengthen this idea is their structural relationship with mammalian complement components. In mammals, specific complement receptors bind the C3 complement factor deposited on microorganisms and promote phagocytosis (Jones *et al.*, 1999; Rosales, 2007). However, insect complement-like receptors specific for binding TEPs on microorganisms have not been described so far. Thus the mechanism by which some TEPs increase phagocytosis remains unclear. In addition, because TEPVI does not have the classical thioester motif, it is possible that some TEPs may have alternative functions, such as protease inhibitors of the α2-macroglobulin family.

Scavenger receptors

The scavenger receptors are unrelated multi-ligand receptors that share the capacity of binding to polyanionic ligands. For this reason they can bind multiple microorganisms and are thus important PRRs (Table 1).

Croquemort and its mammalian homolog CD36 are class B scavenger receptors. CD36 associates with integrins and mediates ingestion of apoptotic cells. Similarly, Croquemort is involved in phagocytosis of apoptotic cells during larval morphogenesis in *D. melanogaster* (Franc *et al.*, 1996; 1999) (Fig. 2). In addition, Croquemort has been reported to bind and induce phagocytosis of *S. aureus* in adult flies (Franc *et al.*, 1999), and this led to the characterization of CD36 as a phagocytic receptor for *S. aureus* (Stuart *et al.*, 2005a). Because CD36 functions together with phosphatidylserine receptors (Fadok *et al.*, 2000), it was proposed that Croquemort also required cooperation of phosphatidylserine receptor (PSR) for phagocytosis of apoptotic cells in *D. melanogaster* (Fadok *et al.*, 2001). However, PSR has been shown to be a nuclear protein and its role in phagocytosis is supported only by few PSR loss-of-function experiments (Wolf *et al.*, 2007). In mammals, other phosphatidylserine receptors have been described to be important for phagocytosis of apoptotic cells (Kobayashi *et al.*, 2007). Thus, whether Croquemort needs cooperation of phosphatidylserine recognition to promote ingestion of apoptotic cells remains an open question (Kinchen, 2010).

Peste is another class B scavenger receptor identified with an RNAi screen in *Drosophila* S2 cells. Peste can bind *M. fortuitum* (Philips *et al.*, 2005). This result has suggested that mammalian class B scavenger receptors may also be involved in recognition of mycobacteria. This idea is supported by experiments in which the mammalian class B scavenger receptors SR-BI and SR-BII conferred to non-phagocytic cells the capacity of ingesting *M. fortuitum* (Philips *et al.*, 2005).

SR-CI is another unrelated type of scavenger receptor. Scavenger receptor class C 1 (SR-CI) is expressed in *Drosophila* hemocytes during embryonic development, and it can bind *E. coli* (a Gram-negative bacteria) and *S. aureus* (a Gram-positive bacteria) (Rämet *et al.*, 2001). Its expression is also upregulated in larvae after a bacterial infection (Irving *et al.*, 2005). However, the role of SR-CI in phagocytosis is not clear since phagocytic defects due to mutations of this receptor are very weak, about 20 % of wildtype cells (Rämet *et al.*, 2001; Lazzaro *et al.*, 2006).

Epidermal growth factor (EGF)-like repeat-containing receptors

A new family of PRRs that contain EGF-like repeats to recognize different ligands is being documented in many species from *C. elegans*, insects, to mammals (Kocks *et al.*, 2005; Kurucz *et al.*, 2007).

Eater, a *D. melanogaster* protein, is a transmembrane molecule with 32 EGF-like repeats in its extracellular domain. Eater was the first EGF-like repeat receptor shown to be involved in bacterial recognition. It recognizes bacteria through its four N-terminal EGF-like repeats (Kocks *et al.*, 2005) and it is involved in phagocytosis (Chung *et al.*, 2011) (Table 1). Knockdown expression of Eater in *Drosophila* S2 cells resulted in reduced *E. coli* and *S. aureus* bacterial binding and ingestion. Also, hemocytes from Eater-deficient flies are impaired in phagocytosis of bacteria (Kocks *et al.*, 2005), and flies lacking the eater gene display more susceptibility to infection by ingested *Serratia marcescens* and to injected Gram-positive bacteria, such as *S. aureus* or *E. faecalis* (Kocks *et al.*, 2005; Charroux *et al.*, 2009b; Defaye *et al.*, 2009; Nehme *et al.*, 2011).

Nimrod is another *D. melanogaster* molecule similar to Eater. It is a transmembrane protein with ten EGF-like repeats in its extracellular domain. Silencing Nimrod expression in *Drosophila* S2 cells results in less phagocytosis of bacteria, while overexpression increases adhesion of these cells to tissue-culture plates (Kurucz *et al.*, 2007), suggesting that Nimrod may be both a phagocytic receptor and an adhesion molecule (Table 1).

Draper is yet another transmembrane protein with EGF-like repeats in its extracellular domain. It is expressed in glial cells and *D. melanogaster* hemocytes (Freeman *et al.*, 2003). It is important for phagocytosis of apoptotic cells in the central nervous system (Awasaki *et al.*, 2006; MacDonald *et al.*, 2006), by embryonic hemocytes, and by *Drosophila* S2 cells (Manaka *et al.*, 2004) (Table 1). Recently, a molecule expressed on apoptotic cells was identified as a ligand for Draper. This molecule, named Pretaporter, is an endoplasmic reticulum protein that relocates to the cell surface during apoptosis to serve as a ligand for Draper in the phagocytosis of apoptotic cells (Kuraishi *et al.*, 2009). Draper is also involved in phagocytosis of both *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) bacteria, and recently it was reported that

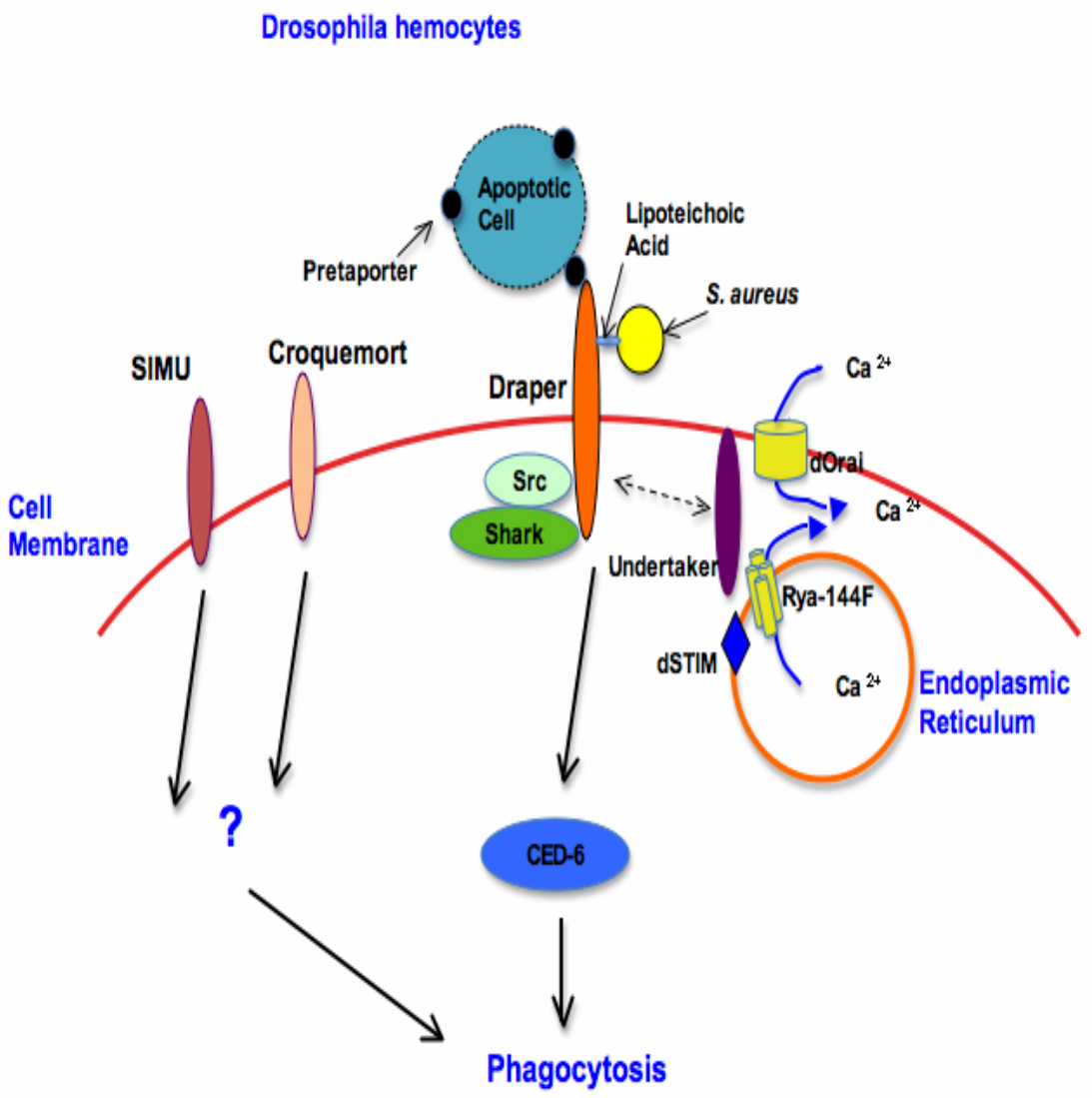

Fig. 2 Phagocytosis pathways in *Drosophila* hemocytes. Draper initiates phagocytosis of apoptotic cells or *S. aureus* bacteria by binding to Pretaporter, an endoplasmic reticulum protein that relocates to the cell surface of apoptotic cells, or to lipoteichoic acid on the surface of *S. aureus.* Draper binds in a Src kinase-dependent manner to shark, a non-receptor tyrosine kinase, similar to the mammalian Syk. Then, Draper is able to interact with CED-6 to promote phagocytosis. Also, Undertaker, a *Drosophila* Junctophilin protein, is required for Draper-mediated phagocytosis. Junctophilins couple Ca^{2+} channels at the plasma membrane to the Ryanodine receptors of the endoplasmic reticulum (ER). The Ryanodine receptor Rya-r44F, the ER Ca^{2+} sensor dSTIM, and the Ca^{2+}-release-activated Ca^{2+} channel dOrai are all in the same pathway promoting calcium homeostasis and phagocytosis. Croquemort and Six-microns-under (SIMU) are other phagocytic receptors in *Drosophila* hemocytes, but their signaling pathways are still unknown.

lipoteichoic acid serves as a ligand for Draper in the phagocytosis of *S. aureus* by *Drosophila* hemocytes (Hashimoto *et al.*, 2009). These reports confirm the idea that Draper is indeed a multi-ligand PRR.

Draper is a homolog of the *C. elegans* apoptotic receptor CED-1 (Gumienny *et al.*, 2001a, b), and similarly to this receptor, Draper also initiates phagocytosis by interacting with CED-6, an adapter protein containing a phosphotyrosine-binding domain, via an ITAM motif in its intracytoplasmic tail (Su *et al.*, 2002). This motif is also present in the apoptotic receptor CD91/low-density lipoprotein receptor-related protein (LRP) (Su *et al.*, 2002). Interestingly, in the mosquito *A. gambiae* a reverse genetics complementation screen (Moita *et al.*, 2005) designed to assign putative regulators of *E. coli* or *S. aureus* phagocytosis into separated functional groups clearly showed that CED-6 is functionally connected to the LRP for efficient phagocytosis of bacteria (Blandin *et al.*, 2007). Together these results suggest that Draper, CED-1, and CD91/LRP all activate a similar phagocytic machinery (see Fig. 2). Clearly, Draper binds apoptotic cells and *S. aureus* to initiate

phagocytosis. However, it is not clear that the signaling pathway shown in Fig. 2 is in fact the same one for both ligands. The model is a composite of the information available today.

Six-microns-under (SIMU) is another *Drosophila* phagocytosis receptor, which is expressed in highly phagocytic cell types during development and required for efficient apoptotic cell clearance by glia in the nervous system and by hemocytes (macrophages) elsewhere. SIMU belongs to a conserved family of proteins that includes CED-1 and Draper, and strongly binds to apoptotic cells, but does not require membrane anchoring, suggesting that it can function as a bridging molecule (Kurant et al. 2008). Phenotypic analysis revealed that SIMU acts upstream of Draper in the same pathway and affects the recognition and engulfment of apoptotic cells, while Draper affects their subsequent degradation (Kurant *et al.*, 2008; Kinchen, 2010) (see Fig. 2).

Peptidoglycan recognition proteins (PGRPs)

Peptidoglycan recognition proteins (PGRPs) are innate immunity proteins that are conserved from insects to mammals, recognize bacterial peptidoglycan, and function in antibacterial immunity and inflammation. PGRPs have at least one carboxy-terminal PGRP domain (approximately 165 amino acids long), which is homologous to bacteriophage and bacterial type 2 amidases. Mammals have four PGRPs (Dziarski *et al.*, 2006a, 2010). They are secreted proteins expressed in polymorphonuclear leukocytes (PGRP1), in liver (PGRP2), or on body surfaces and in secretions (PGRP3 and PGRP4). All PGRPs recognize bacterial peptidoglycan and three of them (PGRP1, PGRP3, and PGRP4 are directly bactericidal for both Gram-positive and Gram-negative bacteria (Dziarski *et al.*, 2010). Insects have up to 19 PGRPs, classified into short (S) and long (L) forms. The short forms are present in the hemolymph, cuticle, and fat-body cells, whereas the long forms are mainly expressed in hemocytes (Royet *et al.*, 2007; Charroux *et al.*, 2009a). The expression of insect PGRPs is often upregulated by exposure to bacteria. Insect PGRPs activate the Toll or Immune deficiency (Imd) signal transduction pathways (described later) or induce proteolytic cascades that generate antimicrobial products (Steiner, 2004; Dziarski *et al.*, 2006b). Several Drosophila PGRPs have lost this enzymatic activity and serve as microorganism sensors through peptidoglycan recognition. Other PGRP family members, such as PGRP-SC1 (Bischoff *et al.*, 2006) and PGRP-LB (Zaidman-Rémy *et al.*, 2006) have conserved the amidase function and are able to cleave peptidoglycan in vitro. However, the contribution of these amidase PGRPs to host defense in vivo remains unclear. In PGRP-SC1/2-depleted *Drosophila* larvae, an over-activation of the Imd signaling pathway was detected after bacterial challenge in the gut (Bischoff *et al.*, 2006).

In *Drosophila* at least three PGRPs have been shown to be important for phagocytosis of bacteria (Table 1). PGRP-SC1a is relevant for phagocytosis of *S. aureus* (Garver *et al.*, 2006), while the membrane-bound receptor PGRP-LC is involved in phagocytosis of *E. coli* (Gram-negative) but not Gram-positive bacteria (Rämet *et al.*, 2002). Interestingly, PGRP-SA was proposed to function in phagocytosis of *S. aureus* (Garver et al. 2006), but this effect was not found in another study (Nehme *et al.*, 2011). The *Drosophila* PGRP-LE functions synergistically with PGRP-LC in producing resistance to *E. coli* and *B. megaterium* infections (Takehana *et al.*, 2004). It is expressed in two forms, the full-length PGRP-LE acts as an intracellular receptor for monomeric peptidoglycan, whereas a version of PGRP-LE containing only the PGRP domain functions extracellularly to enhance PGRP-LC-mediated peptidoglycan recognition on the cell surface (Kaneko *et al.*, 2006). PGRP)-LE is also an important PRR against intracellular bacteria such as *Listeria monocytogenes* (Yano *et al.*, 2008). The PGRP-LE-mediated intracellular response against *L. monocytogenes* infection includes induction of autophagy (Kurata 2010) and induction of the gene Listericin in cooperation with the JAK-STAT (Janus kinase-signal transducers and activators of transcription) pathway (Goto *et al.*, 2010).

Integrins

Integrins are adhesion molecules found on the surface of virtually all cell types in mammals. They are responsible for binding to extracellular matrix proteins and to adhesion ligands on other cells (Shattil *et al.*, 2004; Arnaout *et al.*, 2007). Insect hemocytes aggregate in multiple layers during encapsulation and bind to microorganisms during phagocytosis. These functions can be mediated by integrins (Rosales, 2007) and indeed various integrins have been found in insect hemocytes (Table 1). β integrins are important during encapsulation of wasp eggs by *Drosophila* hemocytes (Irving *et al.*, 2005). α integrins are also relevant for encapsulation by *M. sexta* hemocytes (Levin *et al.*, 2005; Zhuang *et al.*, 2008). Various α and β integrins are required for microbial recognition by granulocytes and plasmatocytes of *P. includens* (Lavine *et al.*, 2003). Integrins are clearly important for phagocytosis of both *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) bacteria by medfly hemocytes (Foukas *et al.*, 1998; Lamprou *et al.*, 2007; Mamali *et al.*, 2009), and for phagocytosis of *E. coli* by *A. gambiae* hemocytes (Moita *et al.*, 2006).

Down syndrome cell-adhesion molecule (DSCAM)

Down syndrome cell-adhesion molecule (DSCAM) is an Ig superfamily receptor that is important for neural development and bacterial recognition in *D. melanogaster* (Table 1). DSCAM was first identified as a molecule involved in determining neuron connections during development (Schmucker *et al.*, 2000). To achieve this, DSCAM expresses many different isoforms derived from splice variants generated by combining variable and constant gene regions, similarly to the gene rearrangement that generates antigen receptor diversity in B and T lymphocytes in mammals. It is estimated that DSCAM could have more than 38,000 isoforms (Schmucker *et al.*, 2000). In immune tissues, hemocytes and fat-body cells, DSCAM could express more than 18,000 different

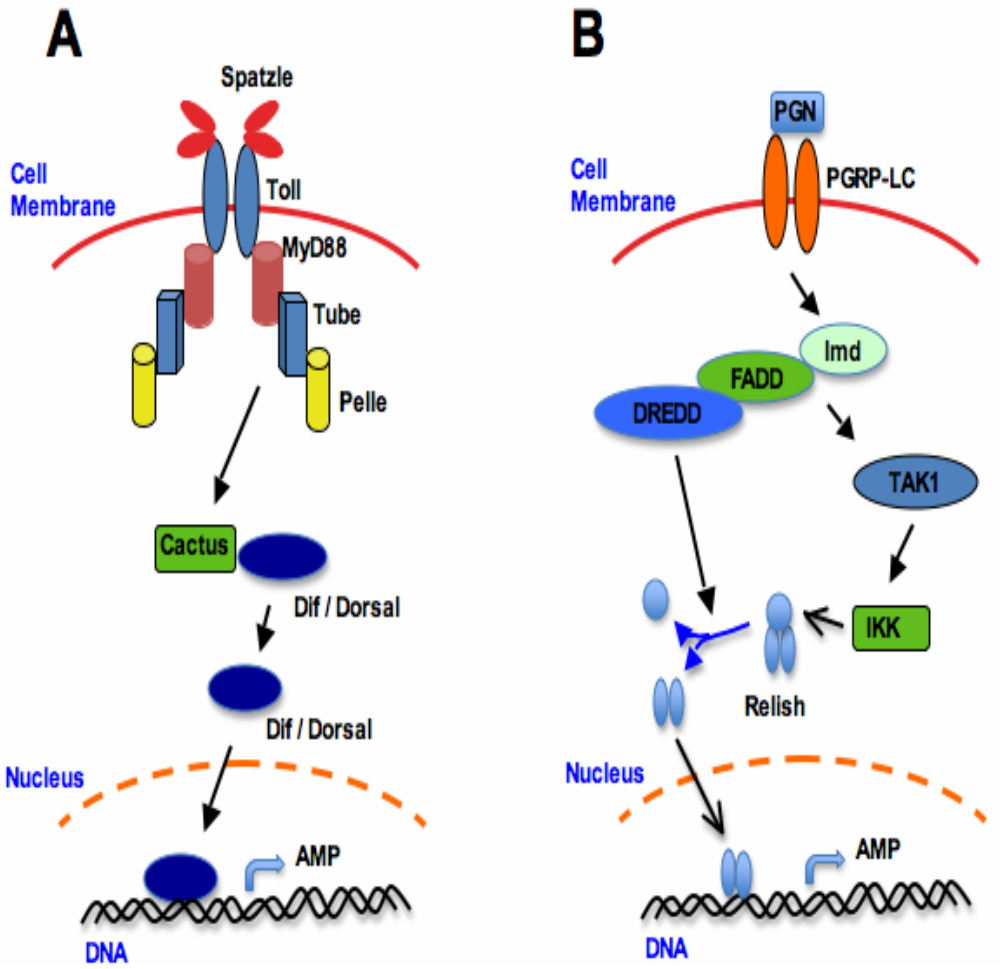

Fig. 3 Toll or Immune deficiency (Imd) signal transduction pathways. A) Activation of the transmembrane receptor Toll requires a proteolytically cleaved form of an extracellular cytokine-like polypeptide, Spätzle. After a complex of 4-Spätzle:2-Toll is formed the signaling pathway Myd88/Pelle/Tube leads to phosphorylation and degradation of Cactus (an IκB inhibitor) and translocation to the nucleus of the NF-κB transcription factors Dorsal and Dif. These factors in turn activate transcription of antimicrobial peptides (AMP). B) The Imd pathway is activated by binding of peptidoglycan (PGN) to the PGRP-LC receptor. This initiates signaling to the transcription factor Relish. Imd is connected to the caspase DREDD via the adaptor protein Fas-associated DD protein (FADD). DREDD is believed to have two functions: upstream, it activates the TAK1/IKKβ complex, which in turn phosphorylates Relish; downstream, DREDD cleaves phosphorylated Relish. The N-terminal NF-κB module of Relish then translocates into the nucleus where it activates transcription of antimicrobial peptides (AMP).

extracellular domains and also a similar number of soluble isoforms released to the hemolymph (Watson *et al.*, 2005). Therefore, DSCAM could produce sufficient numbers of microbial recognition receptors to provide a wide range of specificity (Neves *et al.*, 2004; Meijers *et al.*, 2007). Although, presently it is unclear how many DSCAM isoforms are produced by a single cell. DSCAM has been suggested to be important for phagocytosis of *E. coli* and *S. aureus* in *A. gambiae* (Dong *et al.*, 2006), and has also been found associated with phagosomes from *Drosophila* S2 cells (Stuart et al. 2007). In addition, there is an increase of secreted DSCAM isoforms after a bacterial challenge (Dong *et al.*, 2006). These characteristics have suggested that DSCAM might function similarly to antibody molecules in that they bind to microorganism and promote phagocytosis (Stuart *et al.*, 2008). However, it should be pointed out that data from *Drosophila* is only from cultured cells and there is not evidence for a role of DSCAM in phagocytosis in vivo. Also, the study in mosquitoes shows that silencing of DSCAM increases susceptibility to *E. coli* and *S. aureus* infections, but the connection with phagocytosis is weak. Thus, further studies are needed to confirm the role of DSCAM as an antibody-like molecule that promotes phagocytosis. In addition, the mechanism for DSCAM expression is different from the mechanisms of adaptive immunity, which are based on selection and clonal expansion of lymphocytes (a process not found in invertebrate hemocytes), producing a single type of immunoglobulin receptor on each cell (Blandin *et al.*, 2007).

Pallbearer

Pallbearer is an F box protein found in an in vivo screen for genes required for efficient phagocytosis of apoptotic cells by *Drosophila* S2 cells. F box proteins generally provide substrate specificity to Skp Cullin F box (SCF) complexes, acting as E3 ligases that target phosphorylated proteins to ubiquitylation and degradation via the 26S proteasome. Pallbearer functions in an SCF-dependent manner and its involvement in phagocytosis of apoptotic cells in vivo suggests a role for ubiquitylation and proteasomal degradation in this cellular process (Silva *et al.*, 2007).

Nonaspanins

Nonaspanins comprise an evolutionarily conserved family of proteins with an essentially unknown function. They are characterized by a large N-terminal extracellular domain and nine putative transmembrane domains. Three members in *Dictyostelium discoideum* (Phg1A, Phg1B and Phg1C) and *Drosophila melanogaster*, and four in mammals (TM9SF1-TM9SF4) have been found (Chluba-de Tapia *et al.*, 1997; Schimmoller *et al.*, 1998). Genetic studies in *Dictyostelium* demonstrated that Phg1A is required for cell adhesion and phagocytosis (Cornillon *et al.*, 2000; Benghezal *et al.*, 2003; Benghezal *et al.*, 2006). In *Drosophila*, Phg1A/TM9SF4-null mutant larval hemocytes were sensitive to pathogenic *E. coli* (Gram-negative), but not to *S. aureus* (Gram-positive) bacteria and TM9SF4-defective S2 cells showed reduced bacterial internalization (Bergeret *et al.*, 2008). These defects in phagocytosis were coupled to morphological and adhesion defects in mutant larval hemocytes, which had an abnormal actin cytoskeleton (Bergeret *et al.*, 2008).

Psidin

Psidin is a lysosomal protein required in hemocytes for both degradation of engulfed bacteria and activation of fat-body cells to produce antimicrobial-peptides (AMP). For a long time, the role of *Drosophila* phagocytes in the activation of the humoral immune response has not been clear. Previous studies to determine whether hemocytes play a role in activation of AMP production by the fat-body have relied on domino mutant larvae, in which proliferative tissues are disrupted and hemocyte numbers greatly reduced (Braun *et al.*, 1998). In these mutants, injection of *E. coli* results in normal activation of most AMPs, including Diptericin (Braun *et al.*, 1998), which suggested that activation of Imd- and Toll-pathways is independent of hemocytes. However, domino mutants failed to induce Diptericin during *Erwinia carotovora* (a Gram-negative) gut infection (Basset *et al.*, 2000), suggesting that hemocytes could deliver a signal from the gut to activate Imd signaling in the fat-body. Psidin was then identified in a screen for mutant larvae unable to induce Diptericin in response to injected *E. coli* (Wu *et al.*, 2001). More recently, *Drosophila* larvae Psidin mutants were not able to produce the AMP Defensin after *E. coli* (Gram-negative) or *M. luteus* and *B. subtilis* (Gram-positive) infections. This defect was not rescued by Psidin expression in the fat-body (Brennan *et al.*, 2007). In addition, mutant hemocytes presented impaired phagocytosis, since they could not degrade the ingested bacteria, and Psidin was found localized to lysosomes (Brennan *et al.*, 2007). Thus, the psidin gene acts in larval hemocytes, where it is required for the phagocytic degradation of internalized bacteria and for the induction of Defensin in the fat-body. These findings, whereby phagosome maturation is required for activation of humoral responses are reminiscent of the relationship in mammalian leukocytes, between endosome/phagosome function and immune activation. However, whereas the mechanisms for antigen processing and presentation in vertebrate adaptive immunity have been studied extensively (Houde *et al.*, 2003; Amigorena *et al.*, 2010), the connection between phagosome maturation and initiation of innate immunity are just beginning to be revealed. Future studies will help understanding how phagocytosis and destruction of microorganisms in phagolysosomes are coupled to activation of innate immune responses.

The receptors described above are all involved in insect (host)-microorganism (pathogen) interactions. Most of them have been shown to somehow participate in phagocytosis. However, some of these receptors can be classified as pure PRRs, that is receptors that have been selected during evolution for their ability to recognize conserved microbial structures that microorganisms cannot easily modify, as they are essential to their lifestyles. Among them, we have PGRPs and GNBPs. Some other receptors can be considered "true" phagocytic receptors, that is receptors that are required for fighting off specific pathogens. For example, TEPs and Draper. Several population genetic studies have been conducted with fruit flies, taking advantage of the complete genome sequencing of 12 lines of *Drosophila melanogaster* and one line of *D. simulans* (Jiggins *et al.*, 2003; Sackton *et al.*, 2007). These studies find that PGRP and Gram-negative binding protein (GNBP) genes are highly conserved. In contrast, *Drosophila* TEP genes evolve rapidly under positive selection (Jiggins *et al.*, 2006). Pattern recognition receptors that trigger humoral immunity are evolutionarily rather static, but receptors required for phagocytosis show considerable genomic rearrangement and adaptive sequence divergence (Lazzaro, 2008; Sackton *et al.*, 2010). Thus, we should be alert to make the distinction between regular PRRs that trigger *Drosophila* systemic immune responses and phagocytic receptors, which may not be adequately classified as regular PRRs.

Signaling pathways

Once a microorganism is detected by an immune cell, a series of signaling molecules are activated inside the cell to instruct it for different responses. These molecules follow particular signaling pathways that determine the final cellular response. In mammals the signaling pathways for phagocytosis are relatively well known, particularly for antibody and complement-mediated phagocytosis (Rosales, 2007). In insects, the signaling pathways

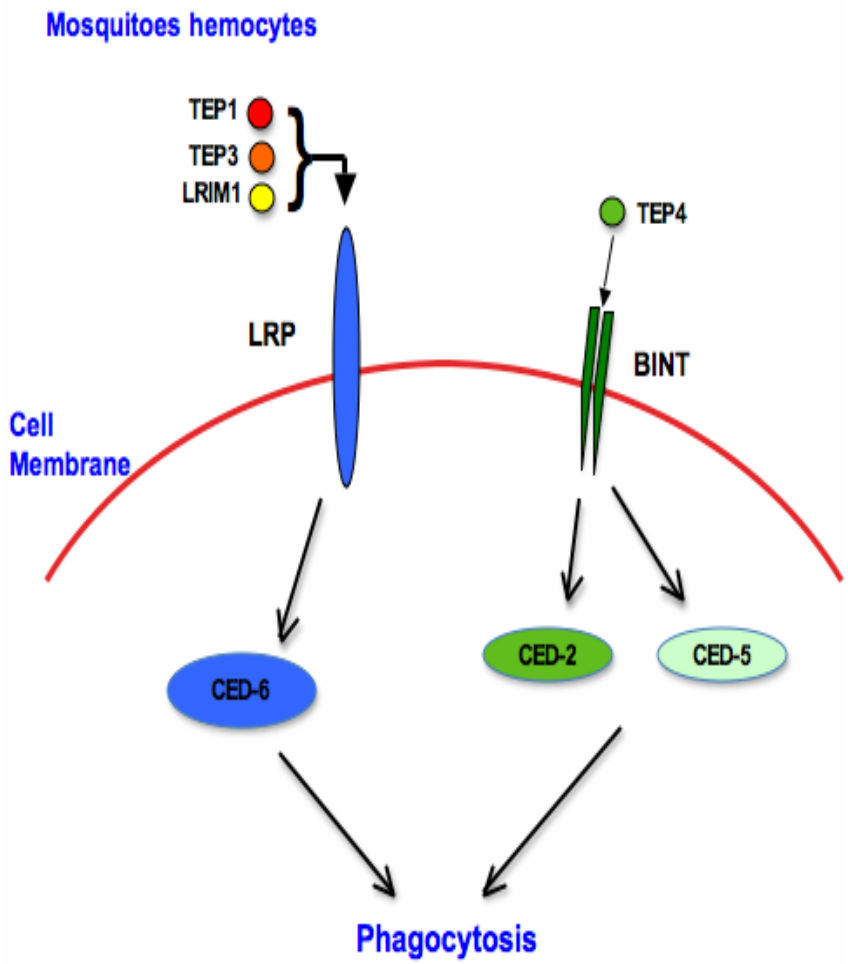

Fig. 4 Phagocytosis pathways in mosquitoes hemocytes. In the mosquito *A. gambiae* two major signaling pathways for phagocytosis of bacteria have been defined with a genetics screen. The intracellular molecules CED-2, CED-5, and CED-6 are the homologs of known components of the two pathways that mediate phagocytosis of apoptotic cells in *C. elegans*. Silencing CED-5 of CED-6 at the same time with identified regulators of phagocytosis permitted assignment of these regulators to one or the other pathway. One pathway, referred as CED-5, involves secreted putative opsonin TEP4, the integrin BINT, and the intracellular components CED-2 and CED-5. The other pathway, referred to as CED-6, involves the secreted proteins TEP1, TEP3, LRIM1, and the transmembrane receptor LPR (low-density lipoprotein receptor-related protein).

involved in humoral immune responses are relatively well described; but for cellular immune responses, the signaling pathways are only partially known. Although signaling pathways for humoral responses are not directly related to phagocytosis, I will briefly describe them first, for comparison reasons, followed by what is known about the signaling pathways for cellular (phagocytic) responses.

The humoral immune responses mainly involve the release of antimicrobial peptides by the fat-body, via the Toll (Valanne *et al.*, 2011) and the Immune deficiency (Imd) pathways (Kaneko *et al.*, 2005). Gram-positive bacteria and fungi predominantly induce the Toll signaling pathway, whereas Gram-negative bacteria activate the Imd pathway (Fig. 3).

The Toll signaling pathway

D. melanogaster Toll pathway was initially identified as a developmental pathway. It involves NF-κB signaling and is essential for embryonic development and immunity (Ashok, 2009; Valanne *et al.*, 2011). From there, the subsequent characterization of Toll-like receptors (TLRs) has reshaped our understanding of the mammalian immune system (Kawai *et al.*, 2011). Activation of the transmembrane receptor Toll requires a proteolytically cleaved form of an extracellular cytokine-like polypeptide, Spätzle (Shia et al. 2009), suggesting that Toll requires cooperation of other PRRs. This idea is supported by the fact that a mutation in a peptidoglycan recognition protein

(PGRP-SA) blocks Toll activation by Gram-positive bacteria (*M. luteu*s, *Streptococcus faecalis*, *B. thuringiensis*) and significantly decreases resistance to this type of infection (Michel *et al.*, 2001). Toll activation is not only mediated by PGRPs, but it requires GNBP1 (Gram-negative binding protein 1) for Gram-positive (*M. luteus or Enterococcus faecalis*) bacterial infections (Wang et al. 2006), and GNBP3 for fungal (*Beauveria bassiana*, *M. anisopliae*, *C. albicans, C. glabrata*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus*) infections (Gottar *et al.*, 2006; Mishima *et al.*, 2009). In addition, the Drosophila Persephone protease activates the Toll pathway when proteolytically matured by secreted fungal virulence factors. Thus, the detection of fungal infections in *Drosophila* relies both on the recognition of invariant microbial patterns and on monitoring the effects of virulence factors on the host (Gottar *et al.*, 2006). Toll activation initiates the signaling pathway Myd88/Pelle/Tube that leads to degradation of Cactus and translocation to the nucleus of the NF-κB transcription factors Dorsal and Dif (Imler *et al.*, 2002; Tauszig-Delamasure *et al.*, 2002; Valanne *et al.*, 2011) (Fig. 3A).

The Imd signaling pathway

The Imd pathway is activated by the PGRP-LC receptor (Gottar et al. 2002) and initiates signaling to the transcription factor Relish (Choe et al. 2002), via the pathway FADD/DREDD and the pathway TAK1/IKKb (Kaneko *et al.*, 2005; Aggarwal *et al.*, 2008; Silverman *et al.*, 2009) (Fig. 3B).

Phagocytosis signaling pathways

Arguable, the most relevant cellular immune response in insects is phagocytosis. Several signaling pathways have been described for this function in various insect species. In *D. melanogaster* hemocytes, an important phagocytic receptor is Draper (Table 1). This receptor mediates phagocytosis of apoptotic cells by activating Shark, a non-receptor tyrosine kinase, similar to the mammalian Syk. Shark binds to Draper via an ITAM motif present in the cytoplasmic tail of Draper. In addition, Draper/Shark interaction is dependent on phosphorylation of Draper by the kinase Src (Ziegenfuss *et al.*, 2008). Draper also interacts with CED-6 (Su *et al.*, 2002), an adapter protein containing a phosphotyrosine-binding domain, to initiate phagocytosis. Thus, Draper-mediated phagocytosis activates the Draper/Src/Syk/CED-6 pathway (Fullard *et al.*, 2009) (Fig. 2). In addition, it was recently found that Undertaker (UTA), a *Drosophila* Junctophilin protein, is also required for Draper-mediated phagocytosis. Junctophilins couple Ca^{2+} channels at the plasma membrane to those of the endoplasmic reticulum (ER), the Ryanodine receptors (Cuttell *et al.*, 2008). Draper, CED-6, UTA, the Ryanodine receptor Rya-r44F, the ER Ca^{2+} sensor dSTIM, and the Ca^{2+}-release-activated Ca^{2+} channel dOrai were placed in the same pathway promoting calcium homeostasis and phagocytosis (Cuttell *et al.*, 2008) (Fig. 2).

As mentioned before, in the mosquito *A. gambiae* two major signaling pathways for phagocytosis of bacteria have been defined with a genetics screen (Moita et al. 2005). One pathway, referred as CED-5, involves secreted putative opsonin TEP4, the integrin BINT, and the intracellular components CED-2 and CED-5 (Fig. 4). Another pathway, referred to as CED-6, involves the secreted proteins TEP1, TEP3, LRIM1, and the transmembrane receptor LPR (Blandin *et al.*, 2007) (Fig. 4).

In medfly hemocytes, phagocytosis of bacteria involves the pathway integrin/Src/FAK/MAP kinases, that ends in activation of an Elk-1-like protein (Mamali *et al.*, 2008a). Activated Elk-1-like is localized exclusively in the cell nucleus and it is associated with FAK (Mamali *et al.*, 2008b) (Fig. 5). MAP kinases can also be activated by other ligands such as LPS and latex beads (Lamprou *et al.*, 2005). The signaling pathways from these stimuli to MAP kinases are still not defined.

The signaling pathways for phagocytosis of bacteria by insect hemocytes are still incomplete. However, the pathways known resemble the pathways described in mammalian phagocytes. This suggests that many other signaling molecules described as relevant for phagocytosis in mammalian phagocytes will also be found to participate in phagocytosis by insect hemocytes. In support of this view, there are reports indicating that other signaling molecules are clearly involved in phagocytosis. It remains to place these signaling molecules within the corresponding signaling pathway. Some of these signaling molecules participating in phagocytosis by insect hemocytes are mentioned next.

Rho

Rho is a family of small GTPases, homologous to Ras, that includes Rho, Rac, and Cdc42. These GTPases are involved in regulating the actin cytoskeleton (Burridge *et al.*, 2004). In *Drosophila* Rac1 (Avet-Rochex *et al.*, 2007) and Rac2 (Williams *et al.*, 2006) are relevant for phagocytosis. In medfly hemocytes Rho GTPases participate in LPS and *E. coli* phagocytosis (Soldatos *et al.*, 2003).

FAK

Focal adhesion kinase (FAK) was first described as a kinase associated to focal adhesions, which are the cell adhesion structures where the actin cytoskeleton connects with the extracellular matrix via integrins. Upon integrin engagement, FAK is activated and functions as a docking molecule for Src family kinases and other signaling molecules. FAK plays a central role in various cell responses such as cell spreading and migration, cell proliferation and apoptosis (Schaller, 2010). An insect FAK homolog, DFAK56 has been found in the central nervous system, epidermis, nerve cord, and visceral mesoderm of *D. melanogaster* (Palmer *et al.*, 1999). FAK has been shown to be relevant for phagocytosis of *E. coli* by insect hemocytes (Metheniti *et al.*, 2001).

Syk

Spleen tyrosine kinase (Syk) is a non-receptor tyrosine kinase with a clear and essential role in mammalian Fc receptor and integrin signaling in phagocytes (Rosales, 2007). In these cells, Syk

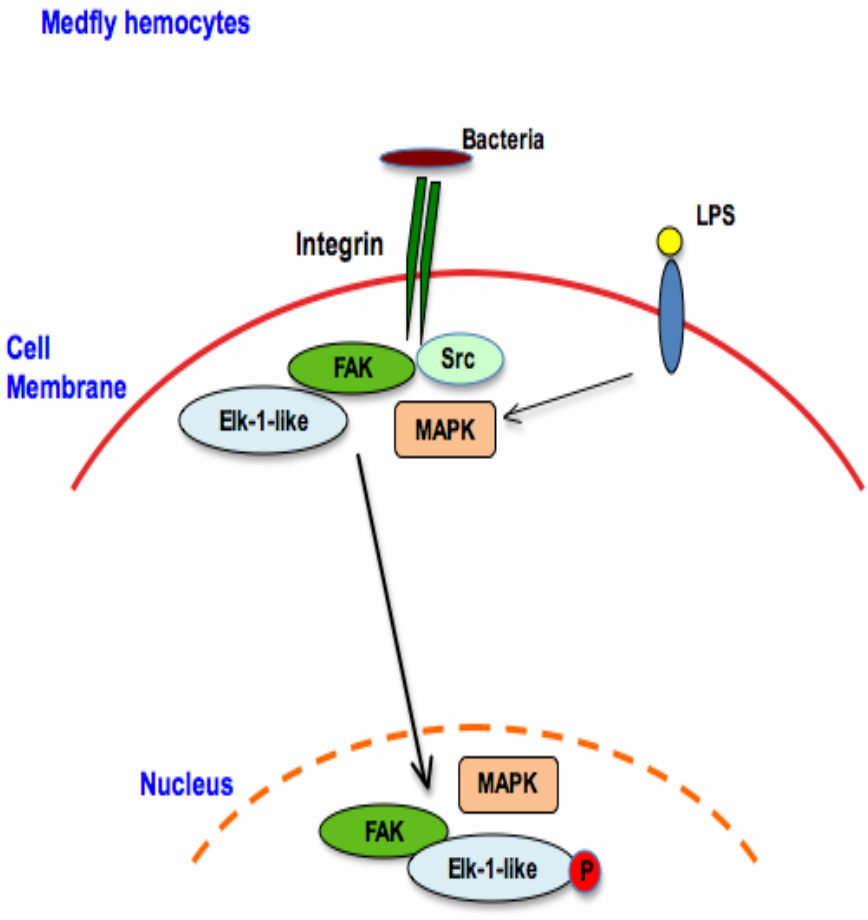

Fig. 5 Phagocytosis pathways in *medfly* hemocytes. In medfly hemocytes, phagocytosis of bacteria involves the pathway integrin/Src/FAK/MAP kinase, which ends in activation of an Elk-1-like protein. Upon integrin engagement, FAK is activated and functions as a docking molecule for Src family kinases and other signaling molecules such as MAP kinases. MAP kinases then phosphorylate Elk-1-like. Active, phosphorylated Elk-1-like is localized exclusively in the cell nucleus in association with FAK. MAP kinases can also be activated by other ligands such as LPS and latex beads, but the signaling pathways from these stimuli to MAP kinases are still not defined. Using pharmacological inhibitors for all three types of MAP kinases (the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), and the p38 mitogen activated protein) in medfly hemocytes greatly reduced bacterial ingestion, confirming the role for all MAP kinases in phagocytosis.

activates cytoskeleton rearrangements, gene expression, and phagocytosis. In insects, the homolog of Syk, Shark is important for Draper-dependent glial phagocytosis (Ziegenfuss *et al.*, 2008) (Fig. 3).

Src

Src is the prototype of the Src family of tyrosine kinases (Ingley, 2008). These kinases are involved in many cellular functions including cell proliferation, differentiation, and phagocytosis. Similarly, Src has been reported to participate in various *Drosophila* functions including maintenance of epithelial integrity (Langton *et al.*, 2007), fusome development and karyosome formation (Djagaeva *et al.*, 2005), and phagocytosis (Ziegenfuss *et al.*, 2008). Src also participates in phagocytosis by medfly hemocytes, in particular associated with FAK (Metheniti *et al.*, 2001; Soldatos *et al.*, 2003; Lamprou *et al.*, 2007).

MAP kinases

MAP kinases are a group of evolutionary conserved signaling kinases involved in many important cellular functions, such as cell proliferation, differentiation, development, apoptosis, inflammation, and phagocytosis. Three MAP kinase families are known: the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), and the p38 mitogen activated protein (p38). In *Drosophila*, Rolled, Basket, and Dp38 are the homologs of mammalian ERK, JNK, and p38, respectively (Han *et al.*, 1998; Lim *et al.*, 1999). They all have been implicated in phagocytosis by insect hemocytes. Inhibition of ERK in *M. sexta* hemocytes reduced phagocytosis of *E. coli* bacteria (de Winter et al. 2007). Also the use of pharmacological inhibitors for all three types of MAP kinases in medfly hemocytes greatly reduced

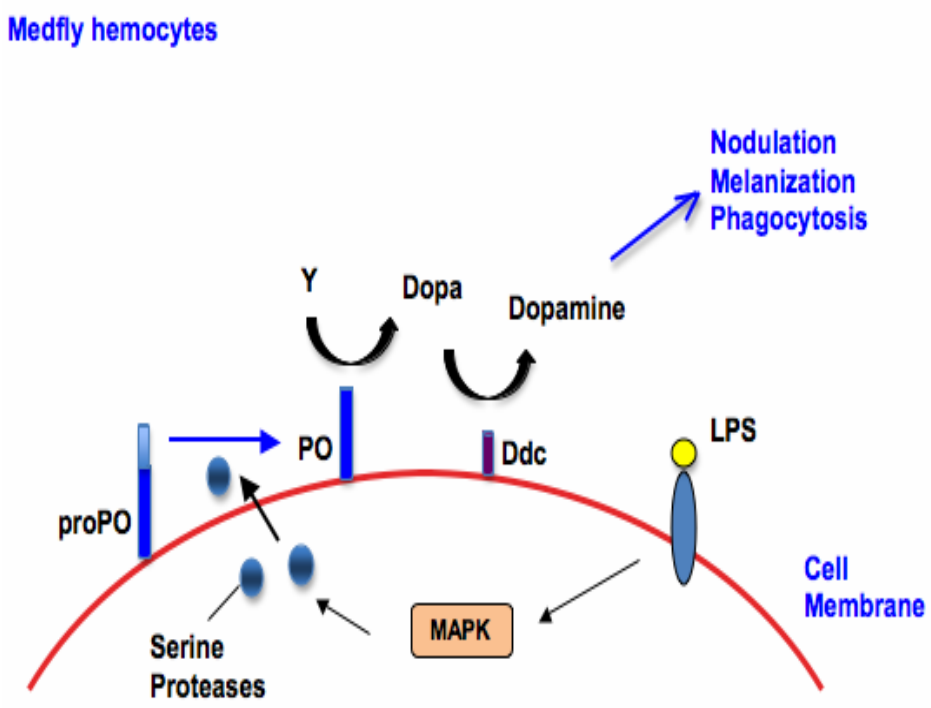

Fig. 6 proPO and Ddc pathways. After bacterial challenge, membrane-bound prophenoloxidase (proPO) is converted to active phenoloxidase (PO) through limited proteolysis by serine proteases. Active PO then catalyzes tyrosine into dopa. Another enzyme, dopa decarboxyilase (Ddc) is also expressed on the surface of these cells and it transforms dopa into dopamine. PO can also use dopa and dopamine as substrates to form quinones that are reactive intermediates for melanization, nodulation, and phagocytosis. MAP kinases activated by LPS or bacteria seem to be relevant for secretion of the serine proteases needed for initial proPO activation.

phagocytosis (Soldatos *et al.*, 2003; Lamprou *et al.*, 2005, 2007). Similarly, inhibition of a JNK-like protein in the mosquito *A. albopictus* cell line C6/36 resulted in reduced phagocytosis (Mizutani *et al.*, 2003).

PI-3K

Phosphoinositide 3-OH kinase (PI-3K) is a phospholipid kinase that provides cells with a survival signal that allows them to withstand apoptotic stimuli. PI-3K is also involved in many other important cellular functions including cell migration, lymphocyte activation and phagocytosis (Downward, 2004). In insects, PI-3K has been reported to participate in phagocytosis of bacteria in *M. sexta* (de Winter *et al.*, 2007) and endocytosis of LPS by medfly hemocytes (Soldatos *et al.*, 2003). Class I PI-3K (Rusten *et al.*, 2004; Berry *et al.*, 2007) and class III PI-3K (Juhász *et al.*, 2008) also seem to be involved in autophagy in *Drosophila*.

proPO

The prophenoloxidase (proPO) system is an important component of the innate immune system in insects. It is activated by bacteria infections and participates is several humoral and cellular responses including melanization, wound healing, encapsulation, nodulation, and phagocytosis (Cerenius *et al.*, 2008). After bacterial challenge, cell-free and membrane-bound proPO is converted to active PO (Fig. 6) through limited proteolysis by serine proteases (Cerenius *et al.*, 2004; Mavrouli *et al.*, 2005). Active PO then catalyzes the formation of quinones that are reactive intermediates for melanization and nodulation (Ling *et al.*, 2005; Jiang, 2008).

Ddc

Activation of proPO seems to be insufficient to induce ingestion of bacteria by medfly hemocytes (Lamprou *et al.*, 2007). However, another enzyme, dopa decarboxyilase (Ddc) is also expressed on the surface of these cells and it is reported to be important for nodulation, melanization, and phagocytosis, since treatment with small interfering RNA (siRNA) for Ddc markedly blocked *E. coli* phagocytosis (Sideri *et al.*, 2008) (Fig. 6). Also, a microarray analysis in Drosophila showed that Ddc expression levels increased after a bacterial infection (De Gregorio *et al.*, 2002). Ddc seems to be involved in wound healing, parasite defense, and behavior of insects (Hodgetts *et al.*, 2006).

Eicosanoids

Eicosanoids are active compounds biosynthesized by enzymatic oxygenation of arachidonic acid (AA) or other C20 polyunsaturated fatty acids. Phospholipase A2 (PLA2) is the enzyme

responsible for releasing AA from cellular phospholipids. Free AA can be metabolized by cyclooygenase (COX) to produce prostanglandins (PG), by lipooxygenase (LOX) to produce leukotrienes, lipoxins and other products, and by cytochrome P_{450}-epoxygenase to produce epoxyeicosatrienoic acids (Stanley *et al.*, 2009). PLA2 is expressed in fat-body cells and hemocytes and is increased after a bacterial infection (Tunaz *et al.*, 2003). Many reports show that eicosanoids are involved in several immune responses including phagocytosis (Figueiredo *et al.*, 2008; Castro *et al.*, 2009; Shrestha *et al.*, 2009), nodulation (Miller *et al.*, 1999; Zhao *et al.*, 2009), hemocyte spreading (Downer *et al.*, 1997), elongation (Miller, 2005), release of proPO (Shrestha *et al.*, 2008), and protection against nematode invasions (Hyrsl *et al.*, 2011).

Concluding Remarks

Phagocytosis is a well-established innate immune defense mechanism in higher organisms. It is fundamental for the host defense and also for stimulating the adaptive arm of the immune response. In insects, phagocytosis is also an innate immune response performed by hemocytes circulating in the hemolymph. Among the different types of hemocytes, plasmatocytes and granulocytes are consistently reported as phagocytic in many insect species. The cellular and molecular mechanisms for hemocyte phagocytosis are relatively well described for *Drosophila*, mosquitoes, and medfly. However, important variations in phagocytosis exist among different species. This underlines the fact that much new research is needed in more insect species to fully understand this cell process in insects. New techniques such as RNAi and flow cytometry, together with system-based approaches such as genomics and proteomics will certainly generate exciting advances in our understanding of phagocytosis. The signaling pathways for phagocytosis are only partially known. Several signaling molecules have been reported as relevant for phagocytosis, but we do not have information yet to place them in a particular signaling pathway. These gaps will certainly be filled as we continue studying how insects cope with the many pathogenic microorganisms they are exposed to.

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