Insect Science (2008) 15, 1—14, DOI 10.1111/j.1744-7917.2008.00183.x

Invited review

The insect cellular immune response

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Abstract The innate immune system of insects is divided into humoral defenses that include the production of soluble effector molecules and cellular defenses like phagocytosis and encapsulation that are mediated by hemocytes. This review summarizes current understanding of the cellular immune response. Insects produce several terminally differentiated types of hemocytes that are distinguished by morphology, molecular and antigenic markers, and function. The differentiated hemocytes that circulate in larval or nymphal stage insects arise from two sources: progenitor cells produced during embryogenesis and mesodermally derived hematopoietic organs. Regulation of hematopoiesis and hemocyte differentiation also involves several different signaling pathways. Phagocytosis and encapsulation require that hemocytes first recognize a given target as foreign followed by activation of downstream signaling and effector responses. A number of humoral and cellular receptors have been identified that recognize different microbes and multicellular parasites. In turn, activation of these receptors stimulates a number of signaling pathways that regulate different hemocyte functions. Recent studies also identify hemocytes as important sources of a number of humoral effector molecules required for killing different foreign invaders.

Key words antimicrobial peptides, clotting, cytokines, Diptera, *Drosophila*, encapsulation, hemocytes, immunity, insects, Lepidoptera, nodulation, phagocytosis

Introduction

Animals defend themselves against infectious organisms by two systems known as innate and acquired immunity. Innate immunity relies on germline encoded factors for recognition and killing of foreign invaders, whereas acquired immunity involves production of effector molecules that recognize specific antigens and development of an immunological memory (Fearon, 1997). Insects have a well developed innate response but are generally thought to lack an acquired immune system. The innate immune system of insects is subdivided into humoral and cellular defenses. Humoral defenses refer to soluble effector molecules such as antimicrobial peptides, complement-like proteins, and the enzymatic cascades that regulate melanin formation and clotting (Muta & Iwanaga, 1996; Cornelis & Soderhall, 2004; Blandin & Levashina, 2004; Theopold *et al*., 2004; Imler & Bulet, 2005). Cellular immunity in contrast refers to defense responses like phagocytosis and encapsulation that are mediated by blood cells (hemocytes) (Lackie, 1988; Strand & Pech, 1995; Gillespie *et al*., 1997; Irving *et al*., 2005). Dividing the insect immune system into humoral and cellular responses is more for convenience of discussion than functional since many humoral factors regulate hemocyte activity and hemocytes are important sources of many humoral defense molecules. We previously reviewed current knowledge of the insect cellular immune response in 2002 (Lavine & Strand, 2002). Since then, a number of studies have entered the literature that provide important new insights on hemocyte development and function in immunity. Most of this literature continues to focus on model species like *Drosophila melanogaster* and selected Lepidoptera. However, important data have also accrued in vector arthropods like mosquitoes. Here, I update this literature with emphasis on studies published since 2002.

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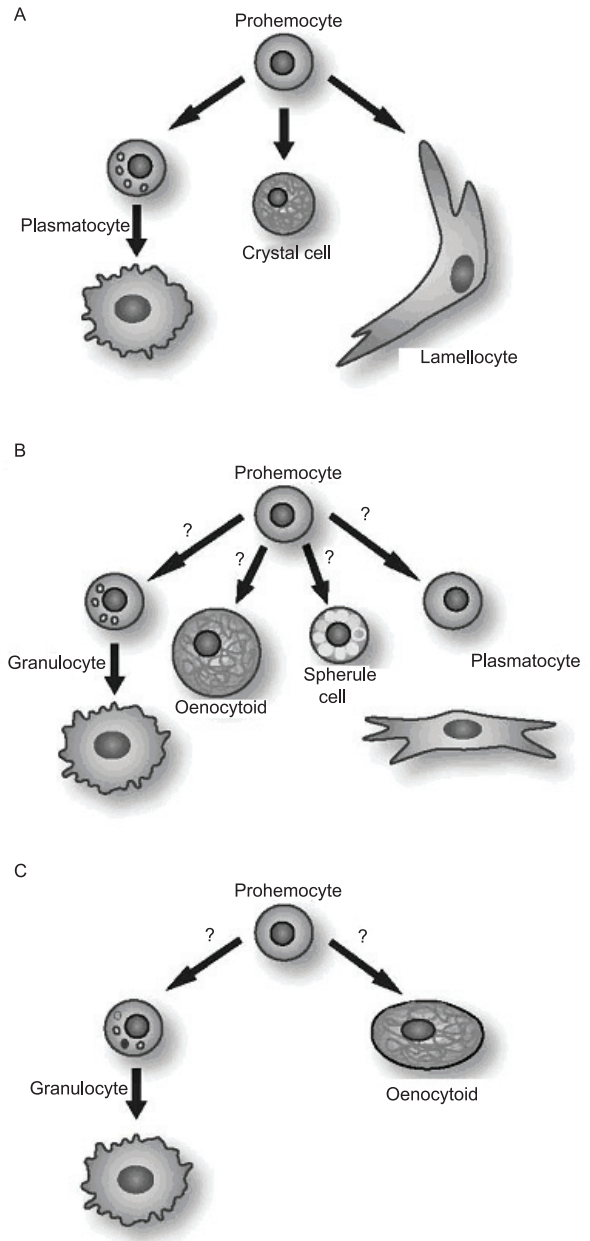
Hemocyte types

Insect hemocytes have historically been identified and classified using morphological, histochemical and functional characteristics (Gupta, 1985; Brehelin & Zachary, 1986). These characters have more recently been augmented by the use of antigenic and molecular markers (Willott *et al*., 1994; Gardiner & Strand, 1999; Lanot *et al*., 2001; Jung *et al*., 2005). Hemocyte-like cell lines, such as S2 cells from *Drosophila* and High Five cells from the lepidopteran *Trichoplusia ni* have also been used to characterize signaling pathways and other processes regulating hemocyte immune functions (Beck & Strand, 2003; Tauszig *et al*., 2000; Stuart & Ezekewitz, 2005; Cherry & Silverman, 2006) (see below).

The most detailed data on blood cell development in insects derive from *Drosophila* whose hemocytes are named differently from most other species. *Drosophila* larvae contain three terminally differentiated types of hemocytes in circulation named plasmatocytes, crystal cells, and lamellocytes (Lanot *et al*., 2001; Evans *et al*., 2003; Wertheim *et al*., 2005) (Fig. 1). Plasmatocytes represent 90%–95% of all mature hemocytes, are strongly adhesive *in vitro*, and function as professional phagocytes that engulf pathogens, dead cells and other entities. Molecular markers for plasmatocytes include the extracellular matrix protein peroxidasin and an uncharacterized surface marker called the P1 antigen (Nelson *et al*., 1994; Asha *et al*., 2003). Crystal cells are nonadhesive cells that comprise approximately 5% of the hemocyte population and express components of the phenoloxidase (PO) cascade such as prophenoloxidase 1 (PP01) the activation of which leads to the formation of melanin. Lamellocytes are essentially absent in healthy larvae but rapidly differentiate from prohemocytes when immune-challenged by intruders like parasitoid wasps and during metamorphosis (Lanot *et al*., 2001). Lamellocytes are large, flat, adhesive cells that are identified by reporters related to Jun kinase signaling and the L1 antigen (Lanot *et al*., 2001; Asha *et al*., 2003). The main function of lamellocytes is encapsulation of parasitoids and other large foreign targets. *Drosophila* also produces precursor prohemocytes which reside primarily in hematopoietic organs (see below) (Holz *et al*., 2003; Jung *et al*., 2005). However, a small number of prohemocytes are also observed in circulation (Lanot *et al*., 2001).

Differentiated hemocytes in other insects are usually named granulocytes (= granular cells), plasmatocytes, spherule cells, and oenocytoids. These names have been extensively used to name different hemocyte types in species from diverse orders including Lepidoptera, Diptera other than *Drosophila*, Orthoptera, Blattaria, Coleoptera, Hymenoptera, Hemiptera, and Collembola (see Jones, 1962; Lackie, 1988; Lavine & Strand, 2002; Ribeiro & Brehelin, 2006). In Lepidoptera, granulocytes are usually the most abundant hemocyte type (Fig. 1). These cells strongly adhere to foreign surfaces, spread symmetrically, and function as the professional phagocytes (Strand *et al*., 2006). Larger, granulocyte-like hemocytes called hyperphagocytic cells that differentiate following immune challenge have also been described from the hawkmoth, *Manduca sexta* (Dean *et al*., 2004). Plasmatocytes in contrast usually spread asymmetrically on foreign surfaces and are the main capsule-forming hemocytes (Strand *et al*., 2006). Non-adhesive hemocytes include oenocytoids that contain PO cascade components, and spherule cells that are potential sources of cuticular components (see Lavine & Strand, 2002). Some studies in Lepidoptera also report the presence of a small number of circulating hemocytes named prohemocytes that, similar to *Drosophila*, are considered progenitor cells (see below). Like *Drosophila*, several molecular and antigenic markers facilitate identification of these hemocyte types in model lepidopterans like *Manduca sexta* and *Pseudoplusia includens* (Mead *et al*., 1986; Willott *et al*., 1994; Jiang *et al*., 1997; Gardiner & Strand, 1999; Lavine & Strand, 2003; Beetz *et al*., 2004; Levin *et al*., 2005).

In the mosquito *Aedes aegypti*, Hillyer and Christensen (2002) classified circulating hemocytes into granulocytes, oenocytoids, adipohemocytes, and thrombocytoids on the basis of morphology, binding of selected lectins, and enzymatic activity. Other investigators, using only morphology, classified hemocytes from *Ae. aegypti* and *Culex quinquefasciatus* into plasmatocytes and oenocytoids (Andreadis & Hall, 1976) or recognized multiple cell types including prohemocytes (Foley, 1978; Kaaya & Ratcliffe, 1982). Castillo *et al*. (2006) used a combination of morphological, antigenic, and functional markers to conclude that *Anopheles gambiae* and *Ae. aegypti* produce three hemocyte types: granulocytes, oenocytoids, and prohemocytes (Fig. 1). Granulocytes are strongly adhesive, phagocytic, and the most abundant cell type in both species while oenocytoids and prohemocytes together comprise less than 10% of the total hemocyte population. Oenocytoids are the only cell type that constitutively exhibit PO activity, but granulocytes inducibly express PO activity following immune challenge. The uniform size, rounded morphology, large nuclear-to-cytoplasmic ratio, and lack of labeling by differentiation markers are consistent with prohemocytes being progenitor cells. However, it is possible mosquito prohemocytes could be a more specialized hemocyte type, such as a granulocyte precursor rather than a stem cell capable of differentiating into either granulocytes or oenocytoids. Granulocytes, oenocytoids, and prohemocytes are present in circulation in larvae, pupae and adult mosquitoes. In addition, no specialized capsule-forming



hemocytes are present in *An. gambiae* or *Ae. aegypti* (Castillo *et al*., 2006).

Much less is known about the hemocytes produced by other insects including numerous species of economic importance. Key challenges include the small size of many insects which makes collection and identification of hemocytes difficult due to the limited amount of hemolymph and cells in circulation. There also is clearly a need to develop a more uniform terminology for naming insect hemocytes in different species. For example, hemocytes named plasmatocytes, crystal cells, and lamellocytes in *Drosophila* are most similar morphologically and functionally to hemocytes named granulocytes, oenocytoids, and plasmatocytes respectively in Lepidoptera (see Fig. 1). Yet, using different names for these cells often leads to confusion; especially for individuals less familiar with the field or who have limited experience working with hemocytes from different insect groups (Lavine & Strand, 2002; Ribeiro & Brehelin, 2006).

Hematopoiesis

Circulating hemocytes arise during two stages of insect development (Jones, 1970; Akai & Sato, 1971; Feir, 1979; Ratcliffe *et al*., 1985; Traver & Zon, 2002; Holz *et al*., 2003). The first population of hemocytes is produced during embryogenesis from head or dorsal mesoderm while a second population is produced during the larval or nymphal stages from mesodermally derived hematopoietic organs. Current knowledge of how hematopoiesis is regulated derives primarily from the study of *Drosophila*. Key factors include the GATA homolog Serpent (Srp) which is required for normal hematopoiesis and *Pvf2* which affects hemocyte proliferation (Tepass *et al*., 1994; Munier *et al*., 2002). The genes *glial cell missing* (*gcm*) and *gcm2* function downstream of Srp and are required for

Fig. 1 Differentiated hemocyte types and proposed lineage relationships in selected insects. (A) *Drosophila* larvae contain three differentiated hemocyte types in circulation: phagocytic plasmatocytes; phenoloxidase (PO)-containing crystal cells; and capsule-forming lamellocytes. Plasmatocytes are rounded cells in circulation but readily bind and spread symmetrically on foreign surfaces. Each cell type derives from progenitor prohemocytes and proliferation of each cell type occurs from hemocytes already in circulation or in hematopoietic organs. (B) Larval stage lepidopterans like *Pseudoplusia includens*, *Manduca sexta*, and *Bombyx mori* contain four differentiated hemocyte types in circulation: phagocytic granulocytes, capsule-forming plasmatocytes, spherule cells, and PO-containing oenocytoids. Granulocytes and plasmatocytes are rounded cells in circulation but both bind to foreign surfaces with granulocytes spreading symmetrically and plasmatocytes spreading asymmetrically. Hematopoietic organs contain putative progenitor prohemocytes and plasmatocytes, whereas proliferation of other hemocyte types occurs primarily in circulation. Precise lineage relationships among lepidopteran hemocyte types remain unclear. (C) Larval and adult-stage mosquitoes like *Aedes aegypti* and *Anopheles gambiae* contain three hemocyte types in circulation: prohemocytes, phagocytic granulocytes, and PO-containing oenocytoids. Prohemocytes are putative progenitor cells but lineage relationships have not been experimentally defined. Whether circulating hemocytes in mosquitoes are of embryonic origin, derive from hematopoietic organs, or both is also unknown. See text for further details.

specification of plasmatocytes, whereas differentiation of crystal cells requires expression of the Runt-domain protein Lozenge (Lz) and Notch (Lebestky *et al*., 2000; Alfonso & Jones, 2002; Lebestky *et al*., 2003; Radtke *et al*., 2005). Alterations in Toll, Jak/Stat, and Ras-mitogen-activated protein kinase pathways cause significant hematopoietic defects in proliferation and differentiation (Dearolf, 1998; Sorrentino *et al*., 2004; Zettervall *et al*., 2004). Jak/Stat and Jun kinase signaling are also implicated in lamellocyte formation (Zettervall *et al*., 2004).

The hematopoietic organs of *Drosophila* are called lymph glands which form bilaterally along the anterior part of the dorsal vessel during embryogenesis (Jung *et al*., 2005). By the third instar each lymph gland consists of an anterior primary lobe and several posterior secondary lobes that are separated by pericardial cells. The primary lobe consists of three zones: (i) a posterior signaling center (PSC) that contains a unique population of putative pre-prohemocytes that do not express any maturation markers but do express the transcription factors Stat and Collier; (ii) a medullary zone that contains quiescent prohemocytes which also lack expression of maturation markers; and (iii) a cortical zone that contains plasmatocytes and crystal cells. Secondary lobes contain primarily prohemocytes (Crozatier *et al*., 2004; Jung *et al*., 2005). The majority of hemocytes that differentiate in the cortical region are released late in the third instar followed by degeneration of the lymph glands during metamorphosis (Lanot *et al*., 2001; Holz *et al*., 2003). Bromodeoxyuridine (Brdu) and transplantation studies further indicate that circulating plasmatocytes of embryonic origin also continue to proliferate during the larval stage (Holz *et al*., 2003). Taken together, these data indicate that the circulating hemocytes present in larval stage *Drosophila* arise from continued division of cells already in circulation and production and release of additional hemocytes from hematopoietic organs. Contributions from the hematopoietic organ also appear to be biased toward the final instar.

Maintenance of circulating hemocytes in larval Lepidoptera similarly involves proliferation of embryonically derived hemocytes already in circulation and the release of additional hemocytes from hematopoietic organs (Akai & Sato, 1971; Arnold & Hinks, 1976; Hinks & Arnold, 1977; Gardiner & Strand, 1999, 2000; Yamashita & Iwabuchi, 2001; Nardi, 2004). Lepidopteran hematopoietic organs are paired structures located in the meso- and metathorax (four organs total) in close proximity to the imaginal wing discs (Lavine & Strand, 2002). Antigenic markers and Brdu labeling studies with larval stage *Spodoptera frugiperda* and *Pseudoplusia includens* indicate that all hemocyte types in circulation proliferate with the possible exception of oenocytoids. Each hematopoietic organ in these species is singly lobed throughout larval development but similar to *Drosophila*, the number of hemocytes per organ increases greatly in the final instar to a maximum of 300 000 cells in *S. frugiperda* prior to the onset of metamorphosis. These hemocytes consist primarily of prohemocytes and plasmatocytes (Gardiner & Strand, 2000). Similar patterns are also reported for *M. sexta* and the silkmoth *Bombyx mori* (Nardi *et al*., 2003; Nakahara *et al*., 2003; Ling *et al*., 2005). Overall, these results suggest that the hematopoietic organs in Lepidoptera are an important source of plasmatocytes, particularly late in larval development, whereas circulating granulocytes, spherule cells and oenocytoids more likely derive from hemocytes already in circulation. The similarities between *Drosophila* and Lepidoptera suggest the possibility that a dual origin of circulating hemocytes from progenitor cells of embryonic origin and larval hematopoietic organs may be a conserved feature in insects generally. However, unlike *Drosophila*, very few signaling molecules or transcription factors have been identified as regulators of lepidopteran hematopoiesis (Nakahara *et al*., 2006).

Studies with different insect species indicate that the number of hemocytes in circulation can also rapidly change in response to stress, wounding or infection (Ratcliffe *et al*., 1985; Lackie, 1988). Some of these changes are due to differentiation events such as the rapid production of lamellocytes in *Drosophila* following parasitoid wasp attack (Sorrentino *et al*., 2002; Wertheim *et al*., 2005). In contrast, other studies make clear that many hemocytes are sessile yet can rapidly enter circulation and elevate the number of cells in circulation without any alteration in cell cycle times or turnover rates (Elrod-Erickson *et al*., 2000; Gardiner & Strand, 2000; Lanot *et al*., 2001; Moita *et al*., 2005; Castillo *et al*., 2006). The adhesion ligands, receptors, and downstream signaling factors regulating the entry of sessile hemocytes into circulation following immune challenge remain poorly understood. However, the GTPase Rac1 in combination with the Jun kinase Basket and actin stabilization were recently shown to be required for recruitment of sessile *Drosophila* hemocytes into circulation and encapsulation of parasitoids by lamellocytes (Williams *et al*., 2006). Reciprocally, release and activation of the cytokine plasmatocyte spreading peptide (PSP) from *P. includens* activates adhesion of plasmatocytes (Clark *et al*., 1997, 1998, 2004). Local activation of PSP causes plasmatocytes to aggregate and form capsules, whereas systemic activation causes plasmatocytes to drop out of circulation and become transiently sessile (Clark *et al*., 1997).

Hemocyte-mediated defense responses

Phagocytosis is a widely conserved defense response in

which binding of the target to its receptor induces the immune cell to form a phagosome. This results in engulfment of the target via actin polymerization-dependent mechanisms followed by maturation of the phagosome into a phagolysosome by a series of fission and fusion events with endosomes and lysosomes (Stuart & Ezekowitz, 2005). Hemocytes phagocytose a diversity of targets including bacteria, yeast, apoptotic bodies, and abiotic particles like synthetic beads and India ink particles (Lanot *et al*., 2001; Lavine & Strand, 2002).

Encapsulation refers to multiple hemocytes binding to larger invaders, like parasitoids and nematodes, that cannot be engulfed by a single cell. The binding of multiple hemocytes to aggregations of bacteria is also sometimes called nodulation (Ratcliffe & Gagen, 1976; Ratcliffe & Gagen, 1977). As previously noted, the main capsule-forming hemocytes in Lepidoptera are plasmatocytes but studies in several species indicate that granulocytes are also present. In some species, like *Manduca sexta*, the distribution of granulocytes and plasmatocytes in capsules appears random (Weigand *et al*., 2000) while in others it is highly organized with granulocytes being the first cells to bind to the target and plasmatocytes attaching thereafter. The latter case is well illustrated in *P. includens* where encapsulation of most foreign targets begins when a few granulocytes bind to the target (Pech & Strand, 1996, 2000). This is followed by the attachment of large numbers of plasmatocytes that adhere strongly to the target and one-another to form a multilayered sheath. Neither granulocytes nor plasmatocytes form capsules alone, but plasmatocytes readily encapsulate targets following granulocyte attachment, indicating that granulocytes produce factors that activate and recruit plasmatocytes. These factors include PSP (see above) as well as other unknown molecules. Capsule formation then ends when a monolayer of granulocytes attach and apoptose on the capsule periphery leaving an extracellular matrix-like layer (Grimstone *et al*., 1967; Pech & Strand, 1996; Liu *et al*., 1998). In effect, the periphery of the capsule likely assumes the characteristics of intact basement membrane which creates a self-surface to which plasmatocytes no longer bind. Lavine and Strand (2001) elaborated on these studies by finding that while encapsulation of most targets requires cooperation between granular cells and plasmatocytes, a few can be encapsulated by plasmatocytes alone if opsonized by unknown soluble receptors in plasma. In *Drosophila*, capsules are comprised predominantly of lamellocytes but whether other cell types, like plasmatocytes, participate in recognition of the target and recruitment of lamellocytes is unclear.

The capsules formed by insect hemocytes often melanize (Strand & Pech, 1995; Schmidt *et al*., 2001; Wertheim *et al*., 2005). As previously discussed, oenocytoids in Lepidoptera and crystal cells in *Drosophila* are sources of PO cascade components although plasma itself also contains these factors, making it unclear as to the origin of the melanin that forms around capsules. However, characterization of the PO cascade in *M. sexta* does indicate that pro-PO forms a complex with pro-PO activating proteases (PAPs), serine protease homologs (SPHs), and pattern recognition molecules like immunolectins that bind bacteria and nematodes (Yu *et al*., 2003) (see below). This finding suggests a mechanism for how melanin deposition can be restricted to the surface of a pathogen or capsule. It is also well known that melanin accumulates around a number of foreign targets in mosquitoes to form what are called melanotic capsules (Collins *et al*., 1986; Michel *et al*., 2005, 2006). No hemocytes appear to directly bind targets prior to melanotic encapsulation although hemocytes are potential sources of the PO cascade components required for melanin formation.

Coagulation of insect hemolymph occurs at sites of external wounding leading to the formation of a clot that traps micro-organisms and seals the wound (Theopold *et al*., 2004; Bidla *et al*., 2005). Microscopic studies in the lepidopteran *Galleria mellonella* and *Drosophila* reveal that soft clots initially consist of a fibrous matrix embedded with numerous hemocytes that predominantly appear to be granulocytes (*G. mellonella*) or plasmatocytes (*Drosophila*). This is followed by hardening of the clot due to cross-linking of proteins and melanization (Theopold *et al*., 2004; Scherfer *et al*., 2006). As with melanizing capsules, oenocytoids or crystal cells may also play an important role in clot melanization, although epidermal tissue and plasma are also sources of PO activity.

Regulators of pathogen recognition and hemocyte adhesion

Phagocytosis and encapsulation depends upon recognition of the target as foreign followed by activation of downstream signaling and effector responses. Opsonin-dependent pathways are regulated by humoral pattern recognition molecules that bind to the target followed by recognition and binding of the opsonized foreign target by surface receptors on hemocytes. Opsonin-independent pathways in contrast involve recognition and direct binding of the target by hemocyte surface receptors. In the case of microbial pathogens, several humoral pattern recognition receptors have been identified in *Drosophila*, lepidopterans, and mosquitoes that bind lipopolysaccharides, peptidoglycans and glucans associated with bacteria and/or fungi (Fig. 2). These include hemolin, LPS-binding protein, Gram-negative bacteria recognition protein (GNBPs), soluble pepti-

doglycan recognition proteins (PGRP-SA, and PGRP-SD), glucan recognition proteins (GRPs), soluble forms of Down's syndrome cell adhesion molecule (Dscam), and complement-like Tep proteins (Levashina *et al*., 2001; Irving *et al*., 2005; Moita *et al*., 2005; Wang *et al*., 2006; Dong *et al*., 2006; Terenius *et al*., 2007). Another subgroup of soluble PGRPs (PGRP-Sb1, Sc1a, 1b, and SC2) enzymatically degrade peptidoglycan. This appears to kill some bacteria (see Bangham *et al*., 2006) but other data suggest the possibility that the resulting peptidoglycan fragments act as signaling molecules that regulate hemocyte function (Garver *et al*., 2006). In the mosquito *An. gambiae*, the leucine-rich repeat protein LRIM1 is both an antagonist of *Plasmodium* (see below) and a possible opsonin of certain bacteria (Moita *et al*., 2005). Other humoral molecules implicated in recognition of foreign targets include a glutamine-rich protein purified from the beetle *Tenebrio molitor* (Cho *et al*., 1999) and immunolectins from *Manduca sexta* (Ling & Yu, 2006). Hemocytes themselves are a source for many of these factors, although other immune tissues, like the fat body, are also potential sources.

Cell surface receptors with roles in phagocytosis or encapsulation include the class B scavenger receptor (SR) (also called CD36 family members) Peste that binds certain intracellular bacteria, and the class C scavenger receptor dSR-CI and transmembrane protein Eater that bind several Gram-positive and -negative bacteria (Ramet *et al*., 2001; Kocks *et al*., 2005; Phillips *et al*., 2005) (Fig. 2). Membrane-bound PGRPs (PGRP-LCs) and transmembrane forms of Dscam are also implicated in phagocytosis of different bacteria in flies and mosquitoes (Ramet *et al*., 2002; Moita *et al*., 2005; Dong *et al*., 2006), while the class B scavenger receptor Croquemort in *Drosophila* and LDL receptor-related protein LRP1 in *An. gambiae* are involved in phagocytosis of apoptotic bodies (Franc *et al*., 1999; Moita *et al*., 2005). Scavenger receptors may also play an important role in the endocytic entry of dsRNA into insect cells (Saleh *et al*., 2006), whereas a third form of PGRP, designated PGRP-LE, was recently identified as an intracellular receptor with roles in recognition of pathogens after phagocytosis (Kaneko *et al*., 2006).

Integrins are dimeric transmembrane receptors composed of an α and β subunit. Insects encode multiple α and β subunits with βPS and αPS4 in *Drosophila* and related integrin subunits in lepidopterans and mosquitoes participating in both encapsulation and phagocytosis (Lavine & Strand, 2003; Levin *et al*., 2005; Irving *et al*., 2005; Wertheim *et al*., 2005; Moita *et al*., 2005) (Fig. 2). In *P. includens*, granulocytes are a source of PSP which activate plasmatocytes by binding to its cognate receptor (Clark *et al*., 2004). This in turn stimulates release of cytoplasmically stored adhesion molecules including integrins that regulate binding of plasmatocytes to the target and one-another during encapsulation (Strand & Clark, 1999; Lavine & Strand, 2003). RNAi experiments also implicate surface integrins in capsule formation by *M. sexta* (Levin *et al*., 2005) (Fig. 2). Downstream, integrin-mediated adhesion and phagocytosis in mammals requires tyrosine phosphorylation of other proteins including Cas, Fak, paxillan, and Pyk2 that localize to focal adhesions and link transmembrane integrins to the actin cytoskeleton (Gelman, 2003; Parsons, 2003). Less is known about these adapter molecules in adhesion and phagocytosis by insect cells although it is likely they play similar roles given that homologs of Fak (Fak56), Pyk2, and Paxillan (DpaxA) localize to focal adhesions (Pearson, 2003; Chen *et al*., 2005; Grabbe *et al*., 2004). RNA-actin-associated proteins like Arp 2/3 in phagocytosis (Pearson *et al*., 2003; Agaisse *et al*., 2005).

Other signaling pathways linked to recognition and binding of foreign targets by hemocytes include the Toll pathway which responds to Gram-positive bacteria, fungi, and certain viruses via activation of the Toll receptor and NF-κB transcription factors containing the Rel proteins Dif and Dorsal. Both PGRP-SA and soluble GNBPs are involved in activation of the Toll pathway (Imler & Zheng, 2005; Filipe *et al*., 2005) (Fig. 2). The Imd pathway responds to Gram-negative bacteria via binding by PGRP-LC and activation of NF-κBs containing Relish which results in induction of both novel effector genes and factors regulated by Toll signaling (Choe *et al*., 2002; Ramet *et al*., 2002; Imler & Zheng, 2005) (Fig. 2). Tep proteins play a role in activation of JAK/STAT signaling (Agaisse & Perrimon, 2004), while JNK signaling is associated with adhesion and phagocytosis via regulation of the cytoskeleton (Boutros *et al*., 2002). Genome-wide studies confirm significant expression levels of several components of the Toll, Imd, JAK-STAT, and JNK pathways as well as the PO cascade in hemocytes following immune challenge by microbes or parasitoids (Irving *et al*., 2005; Wertheim *et al*., 2005). Recent results also indicate that hemocytes orient to wound sites via chemotaxis in the *Drosophila* embryo. The identity of these chemotactic factors is unknown but the ability of hemocytes to respond to them involves both phosphoinositol 3 kinase (PI3K) and Rac signaling activity (Stramer *et al*., 2005; Wood *et al*., 2006). Lastly, insect pro-POs lack signal peptides which has led to the suggestion that the release of these enzymes by oenocytoids (Lepidoptera) and crystal cells (*Drosophila*), depends upon cell lysis (see Cornelis & Soderhall, 2004; Nappi & Christensen, 2005). Precisely how recognition of a foreign target stimulates cell lysis and PO release is unclear but alterations in the function of the GTPase Rho A appears to block the response in *Drosophila* crystal cells, possibly by interfering with essential rearrangements of the cytoskeleton (see Bangham *et al*., 2006).

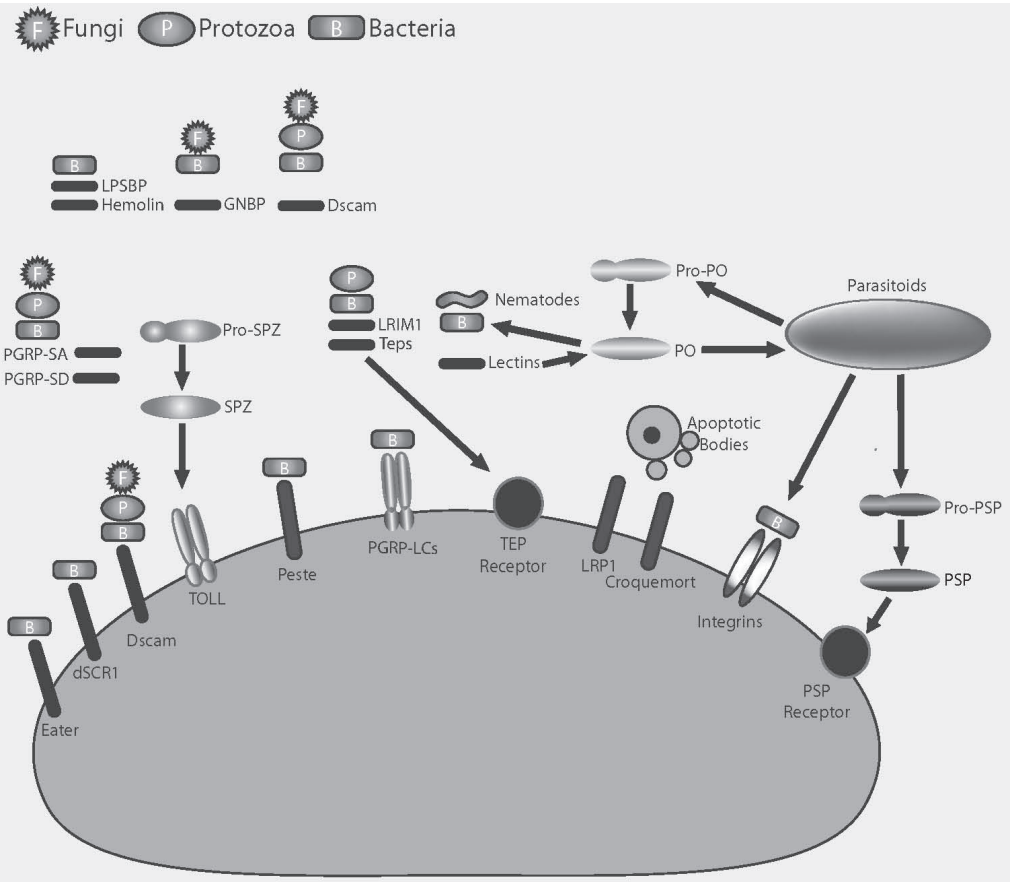


Fig. 2 Recognition of foreign targets by insect hemocytes involves multiple receptors. In mosquitoes and *Drosophila*, soluble and cellular forms of Dscam and Tep proteins bind and mediate phagocytosis of several different microbes. Mosquito Tep and LRIM1 proteins are also involved in killing *Plasmodium* and possibly other protozoans. Other soluble receptors including LPSBP, GNBP, and PGRP have been identified from several insects and bind different microbes. In *Drosophila*, binding of microbial ligands by PGRP-SA and -SD also stimulates proteolytic processing of pro-spaetzle (Pro-SPZ) to form the Toll ligand spaetzle (SPZ). Another soluble receptor, hemolin, is known only from Lepidoptera, whereas immunolectins bind bacteria and nematodes and also form complexes with phenoloxidase (PO) that potentially facilitate localized deposition of melanin. In *Drosophila*, several cellular receptors including Eater, Peste, dSCRI, and PGRP-LC bind micro-organisms. Another CD36 homolog, Croquemort, mediates uptake of apoptotic bodies while LRP1 has a similar function in mosquitoes. Dimeric integrins identified in *Drosophila*, mosquitoes, and Lepidoptera mediate phagocytosis of certain bacteria and binding of capsule-forming hemocytes to parasitoids. Parasitoids and other encapsulation targets also induce proteolytic processing in Lepidoptera of pro-PSP to bioactive PSP which stimulates hemocyte adhesion after binding its cognate receptor.

Hemocytic-mediated effector responses

In addition to recognition molecules, hemocytes produce a number of extra- and intracellular effector molecules that are implicated in killing foreign invaders. Among the most important extracellular effector molecules are antimicrobial peptides (AMPs) regulated by the Toll and Imd pathways (Imler & Bulet, 2005). Although the fat body is a primary site of antimicrobial peptide expression, studies in diverse insects reveal that many AMPs are also expressed in hemocytes (Lowenberger, 2001; Hoffmann & Reichhart, 2002; Boman, 2003; Lackie, 1988; Yamano *et al*., 1994; Dimopoulos *et al*., 2000; Bartholomay *et al*., 2004; Irving *et al*., 2005). Lavine *et al*. (2005) further determined that AMP expression varies among hemocyte types in Lepidoptera with granulocytes and plasmatocytes expressing multiple AMPs, and spherule cells and oenocytoids expressing only lysozyme.

A major bottleneck following *Plasmodium* infection in mosquitoes occurs when ookinetes transition to oocysts during passage through the midgut. The two most apparent events associated with parasite mortality are ookinete lysis and melanization (Whitten *et al*., 2006). Although incom-

pletely understood, three molecules with recognition functions, TEP1, LRIM1 and APL1, also have killing activity. In addition, hemocytes appear to be the primary source of these effector molecules (Blandin *et al*., 2004; Osta *et al*., 2004; Riehle *et al*., 2006). As previously discussed, oenocytoids and granulocytes are also sources of PO cascade components in *An. gambiae* (Castillo *et al*., 2006). POs are expressed as an inactive zymogens (pro-POs) in all insects and are converted to active PO by serine proteases called pro-PO activating proteinases (PAPs). Knockdown of the serpin SRPN2, a PAP inhibitor, in *An. gambiae* increases melanization and reduces survival of *Plasmodium berghei* but has no apparent effect on survival of *P. falciparum* (Michel *et al*., 2006). This suggests the quinones and reactive intermediates produced following activation of the PO cascade are important in killing some parasites following melanotic encapsulation, but not others.

Genome-wide analyses in *Drosophila* similarly indicate that several genes encoding Teps, pro-POs and other PO cascade components are expressed in hemocytes (Irving *et al*., 2005; Wertheim *et al*., 2005). Similar to the effects of SRPN2, loss of function mutants for another putative PAP inhibitor, Spn27A, in *Drosophila* also results in increased melanization. Whether this effect increases resistance to infection is unclear but loss of Spn27A does increase susceptibility to wounding, suggesting that melanization may be more important for clotting than direct killing of pathogens (Ramet *et al*., 2001; De Gregorio *et al*., 2002). On the other hand, studies in Lepidoptera suggest melanin or other factors produced following activation of the PO cascade have anti-viral activity (Trudeau *et al*., 2001; Popham *et al*., 2004).

Intracellular killing of phagocytosed pathogens in mammalian macrophages occurs in the phagolysosome. The hydrolytic environment of the phagolysosome limits replication of many micro-organisms, while release of reactive oxygen intermediates, proteases and other factors including PGRPs and AMPs directly kill pathogens (Selsted & Ouellette, 1995; Reeves *et al*., 2002; Tydell *et al*., 2006). Few functional studies have been conducted on the phagosomes and phagolysosomes in insect hemocytes. However, like mammals, insect-produced phagosomes contain hundreds of proteins that include similar effector molecules (Stuart & Ezekowitz, 2005; Stuart *et al*., 2007). Parasitoid wasps and other large multicellular organisms also usually die after encapsulation. Several factors including asphyxiation, products associated with activation of the PO cascade, and AMPs have been suggested as killing agents (Salt, 1970; Nappi & Christensen, 2005). Similar to the insect phagocytosis literature though, little experimental data exists on the precise mechanisms underlying the death of encapsulated targets.

The least understood defense responses in insects currently is anti-viral immunity. The mammalian immune system recognizes viral pathogens at the cell surface or in endosomal vesicles through pattern recognition receptors such as Toll-like receptors. Helicase proteins present in the cytoplasm also function as pattern-recognition receptors that recognize intracellular dsRNA intermediates associated with replication of single-stranded RNA viruses and DNA virus transcription and induce the interferon pathway (see Li *et al*., 2004; Cherry & Silverman, 2006). dsRNA also induces the RNAi pathway which directly degrades viral RNA. Cytoplasmic helicase proteins have not been identified in insects but RNAi as an intracellular viral defense mechanism has been demonstrated in mosquitoes and *Drosophila* (Keene *et al*., 2004; Wang *et al*., 2006; Galiana-Arnoux *et al*., 2006). Infection by some viruses activate the Toll and Imd pathways while others stimulates Jak-STAT signaling (Dostert *et al*., 2005; Zambon *et al*., 2006). Susceptibility to infection and/or small decreases in viral replication are also detected, suggesting these pathways regulate production of antiviral factors. The identity of these factors and their modes of action remain to be characterized. Recognition of viruses and intracellular anti-viral defense mechanisms could obviously involve a variety of cell types in addition to hemocytes. However, I would speculate that hemocytes will likely be found to play important roles in anti-viral defense given that many insect viruses disseminate through the hemocoel during the course of a systemic infection.

Concluding remarks

While humoral defense responses are still overall better understood than cellular defenses, the gap is rapidly closing as more studies on insect hemocytes enter the literature. Genetic and RNAi screens in model species like *Drosophila* clearly offer a powerful approach for identifying molecules required for regulating cellular defense responses. Such studies must still be complemented by bioassay-driven, biochemical approaches given the important role proteolytic cascades and other post-translational processing events play in regulating insect immune defenses. Studies on pathogens that have co-evolved with particular insect species will also be essential in understanding the role of cellular and other defense mechanisms in natural infections.

Acknowledgments

I thank J. A. Johnson for assistance with the figures. Some work discussed here was funded by grants from the Na-

tional Institutes of Health (AI38917 and AI063605) and US Department of Agriculture National Research Initiative (2005-05382) to MRS.

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Accepted March 15, 2007