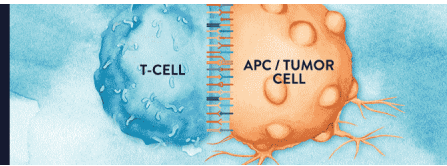


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BRIEF REVIEWS

***Drosophila* Hemopoiesis and Cellular Immunity**Michael J. Williams¹

In Drosophila melanogaster larvae, three classes of circulating cellular immune surveillance cells (hemocytes) can be identified: plasmacytes, crystal cells, and lamellocytes. Plasmacytes are professional phagocytes most similar to the mammalian monocyte/macrophage lineage and make up ~95% of circulating hemocytes. The other ~5% of circulating hemocytes consists of crystal cells, which secrete components necessary for the melanization of invading organisms, as well as for wound repair. A third cell type known as lamellocytes are rarely seen in healthy larvae and are involved in the encapsulation of invading pathogens. There are no obvious mammalian counterparts for crystal cells or lamellocytes, and there is no equivalent to the lymphoid lineage in insects. In this review, I will discuss what is currently known about Drosophila hemopoiesis and the cellular immune response and where possible compare it to vertebrate mechanisms. The Journal of Immunology, 2007, 178: 4711–4715.

All multicellular organisms are continually exposed to microorganisms and thus have evolved effective immune defense mechanisms to eliminate pathogens. The mammalian immune response is comprised of two major components: innate and adaptive. Insects also have a very effective immune response that until recently was thought to rely solely on the phylogenetically more ancient innate response. Recent evidence may point to insect having a primitive form of adaptive immunity (1–3). Although research on many different insects has contributed to our current knowledge of the mechanisms underlying the innate immune response, the fruit fly *Drosophila melanogaster* has been a very useful model toward a better understanding of how this response is regulated, especially humoral immunity (4, 5).

There are two main components to the *Drosophila* innate immune response, the humoral and cellular systems, both of which are activated upon immune challenge. The humoral response is mainly induced in an abundant tissue known as the fat body. The fat body is thought of as the insect equivalent to the liver. When activated, the fat body can produce and secrete into the hemolymph large amounts of antimicrobial peptides. The

cellular response involves phagocytosis, nodule formation, and encapsulation of pathogens. Over the past few decades, our understanding of how the humoral response is regulated has grown considerably (4–6). Yet, the processes that govern the cellular response are unclear, and only recently has it been studied at the molecular level. This review will focus on recent discoveries concerning the cellular response and on the cell lineages that are engaged in these processes.

The mammalian hemopoietic cellular immune response consists of the lymphoid and myeloid lineages. The lymphoid lineage is the main component of the adaptive immune response, possessing a unique ability to achieve somatic gene rearrangement, which enables it to produce a myriad of Abs and receptors. The mammalian innate cellular immune response comes from both the lymphoid and myeloid lineages; among these neutrophils, macrophages, and dendritic cells are professional phagocytes. Interestingly, the innate immune response, by producing costimulatory molecules and immunomodulatory cytokines, has a regulatory role in adaptive immunity (7). In *Drosophila*, there is no equivalent of a lymphoid lineage, and the cellular immune response consists of circulating immune surveillance cells (hemocytes). Hemocytes play a key role in immune surveillance and are active against pathogens and parasites. When an invading organism is recognized as foreign, circulating hemocytes should remove it, either by phagocytosis and/or encapsulation. When the morphology of the hemocytes is compared, three basic types of cells can be identified. The most abundant cells are the plasmacytes, which resemble the mammalian monocyte/macrophage lineage. These are small cells involved in phagocytosis and encapsulation, as well as producing antimicrobial peptides. In addition to plasmacytes, *Drosophila* has two additional circulating immune surveillance cells that have no clear counterpart in mammals. The largest and least abundant cells are the lamellocytes. Lamellocytes are involved in the encapsulation of invading pathogens too large to undergo phagocytosis. Crystal cells secrete components of the phenol oxidase cascade and are involved in the melanization of invading organisms and in wound repair (8). Many of the hemopoietic factors have been conserved across taxonomic groups ranging from flies to humans, and aspects of hemopoiesis and hemocyte function appear to be conserved. Comparative studies using *Drosophila* as a

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² Abbreviations used in this paper: Srp, Serpent; Dscam, Down syndrome cell adhesion molecule; EGF, epidermal growth factor; Gcm, Glial cell missing; Gcm2, Glial cell missing-2; Hop, Hopscotch; Kn, Knot; Lz, Lozenge; lz, lozenge; PGRP-LC, peptidoglycan recognition protein LC; TEP, thio-ester containing complement-related protein; tsp68c, tetraspanin68c; ush, u-shaped; VEGF, vascular endothelial growth factor.

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model should enhance our understanding of blood cell development, wound repair, and the innate immune response.

Hemopoiesis and cell differentiation

Over the past few years, it has become evident that *Drosophila melanogaster* and vertebrates share several important hemopoietic factors in common. In particular, the transcription factors of the GATA, RUNX, and Friend-of-GATA families have been shown to regulate several steps in both vertebrate and *Drosophila* hemopoiesis (9). Although the *Drosophila* genome encodes five GATA factors (including Serpent (Srp)),² it is Srp that is expressed before all differentiation markers in embryonic prohemocytes (10). During embryogenesis, the plasmatocytes and crystal cells both arise from this early population of prohemocytes (11) (Fig. 1A). Srp is involved in this differentiation, and its expression is necessary for cell maintenance during development (12, 13). The *Drosophila* genome encodes three RUNX transcription factors, of which Lozenge (Lz) is involved in hemopoiesis. Initially, it was observed that in a *lozenge* (*lz*) loss-of-function mutant no crystal cells are formed (11). Later, it was discovered that Lz doesn't do this alone but forms a complex with Srp and together they induce crystal cell formation (13) (Fig. 1A). Plasmatocyte differentiation requires two related transcription factors: Glial cells missing (*Gcm*) and Glial cells missing-2 (*Gcm2*) (14–16). In the absence of both these transcription factors, plasmatocytes fail to differentiate normally, and their number is reduced severely, whereas crystal cell formation appears unaffected (16). Previously, it was thought that during their differentiation crystal cells must express *lz* but not *gcm/gcm2* and plasmatocytes express *gcm/gcm2* but not *lz* (11). More recently, Bataille et al. (17) showed that this is not exactly the case. Their work determined that, during the early stages of development, *gcm* is expressed in all prohemocytes but is then down-regulated in the anterior-most cells of the hemopoietic progenitors (Fig. 1A). These cells where *gcm* is down-regulated then begin to express *lz*. There is a significant increase in the number of crystal cells in *gcm* and *gcm2* double mutants. This shows that, at least in a subset of prohemocytes, *gcm/gcm2* expression is necessary to inhibit crystal cell development. The authors go on to show that *gcm/gcm2* inhibition of crystal cell formation is a two-step process. Initially, *gcm/gcm2* regulate the number of cells that express *lz*, then subsequently *gcm/gcm2* interfere with *lz* maintenance. If *gcm/gcm2* are removed, then *lz* is maintained in a subset of cells, and they differentiate into crystal cells instead of plasmatocytes. This article shows that prohemocytes are bipotent progenitors. The Friend-of-GATA homolog *u-shaped* (*ush*) antagonizes crystal cell development (18) (Fig. 1A). In *Drosophila*, *ush* is highly expressed in hemocyte precursors and throughout the development of the plasmatocyte lineage. *ush* is also expressed in crystal cell precursors but down-regulated as these cells develop (18).

As in embryos, several signaling pathways associated with hemopoiesis in *Drosophila* larvae are also involved in regulating mammalian hemopoiesis. In mammals, as well as *Drosophila*, overactivation of the Ras/Raf pathway leads to uncontrolled proliferation (19). Two studies showed that expression of constitutively active Ras causes overproliferation of larval hemocytes (20, 21). Furthermore, Zettervall et al. (21) went on to show that overexpression of the epidermal growth factor (EGF) receptor, known to signal upstream of Ras, can also induce hemocyte overproliferation. They also showed that two ETS tran-

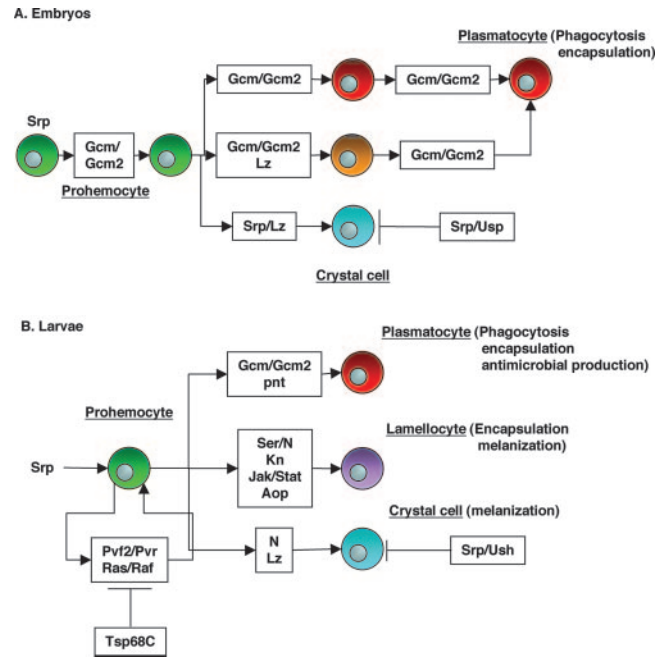


FIGURE 1. Hemopoietic development in *Drosophila* embryonic and larval stages. *A*, In embryo expression of the GATA transcription factor, Srp is required for the formation of prohemocytes. In midstage embryos, expression of the transcription factors Gcm and Gcm2 is initiated in all prohemocytes. In some hemocytes, *Gcm/gcm2* expression is down-regulated, leading to the expression of the RUNT family transcription factor Lz. Continued expression of Lz induces a subset of the prohemocytes to become crystal cells. Continued expression of *Gcm/gcm2* is necessary for the formation of embryonic plasmatocytes. *B*, In larvae, Srp is also required for the formation of prohemocytes. Hemocyte proliferation is under the control of the PDGF VEGF receptor Pvr and the Ras/Raf pathway. Proliferation is inhibited by the tetraspanin Tsp68C. Similar to embryos, expression of *Gcm/gcm2* is required for the formation of larval plasmatocytes. The Ets transcription factor Pointed (Pnt) may also be involved in plasmatocyte formation. The Notch and JAK/STAT pathways, along with the transcription factor Kn, are involved in the formation of the larval-specific cell type known as lamellocytes. The inhibitor Ets transcription factor Anterior open (Aop) may also be involved in lamellocyte formation. Although similar to embryos, Lz is necessary for crystal cell formation. Unlike embryos, Notch is also involved in the formation of larval crystal cells. The Friends-of-GATA transcription factor U-Shaped (Ush) is an inhibitor of crystal cell formation.

scription factors (Pointed and Anterior open), known to be downstream of the Ras/Raf/MAPK pathway, are involved in hemocyte specification. Overexpression of the Pointed-P1 transcript induced a significant increase in plasmatocyte numbers, whereas overexpression of a constitutive active form of the inhibitor Anterior open induces lamellocyte formation. Interestingly, in mice, knocking down tetraspanins has been shown to induce lymphocyte proliferation (22). Sinenko et al. (23) showed that in *Drosophila* loss of at least one tetraspanin, *tetraspanin68c* (*tsp68c*), was sufficient to induce overproliferation of larval hemocytes. Also, overexpression of *Drosophila* Tsp68C was able to inhibit Ras-induced hemocyte proliferation (Fig. 1B).

In mammals, the JAK/Stat pathway is involved in both hemopoiesis and immunity. In *Drosophila*, it has been shown that hyperactivation of the JAK homolog Hopscotch (Hop) induces plasmatocyte proliferation and the differentiation of lamellocytes (21, 24, 25) (Fig. 1B). Yet, in *hop* loss-of-function larvae, the numbers of circulating hemocytes are comparable to wild-type larvae (26, 27). Interestingly, Hop is required for the larvae

to mount an effective immune response to parasitization by parasitoid wasps (27). This may be due to an inability of a hemopoietic organ, known as the lymph gland, to be activated. All of this suggests that the JAK/STAT pathway may be more involved in regulating the cellular immune response than in the regulation of hemopoiesis.

The *Drosophila* receptor tyrosine kinase Pvr shares homology with the vertebrate platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) receptors. During embryogenesis Pvr controls the migration and survival of hemocytes (28–31). In larvae the Pvr pathway may also be involved in hemocyte proliferation (Fig. 1B). Overexpression of Pvr in hemocytes or misexpression of the ligand Pvf2 causes a significant increase in either circulating hemocyte numbers or expansion of the lymph gland (21, 32). Zettervall et al. (21) showed that expressing a dominant-negative Pvr construct was sufficient to decrease the number of circulating hemocytes.

Overactivation of the *Drosophila* Toll pathway, which has been shown to be involved in both *Drosophila* and vertebrate immunity upstream of NF κ B/I κ B signaling pathway, also induces hypertrophy of the lymph gland and hemopoietic defects that include overproliferation and aberrant differentiation (21, 33). More recently, Matova and Anderson (34) showed that the REL transcription factors Dif and Dorsal, known to signal downstream of the Toll receptor, are essential for proper circulating hemocyte numbers, as well as for proper plasmatocyte function. Larvae that are *Dif dorsal* double mutants have three times fewer hemocytes than wild-type larvae. This is partially due to an increase in apoptosis. Furthermore, overexpression of Dif or Dorsal in hemocytes induces proliferation (34). Dif and Dorsal are also necessary for proper hemocyte function because loss of both REL factors in hemocytes inhibits the clearance of bacteria from the hemolymph.

Similar to vertebrates Notch signaling is required during *Drosophila* embryogenesis for development of all cardiogenic mesoderm, including the lymph gland (35). In larvae Notch signaling is necessary in a compartment of the lymph gland, known as the posterior signaling center, for the determination of the larval crystal cells in the lymph gland (36, 37). In the posterior signaling center Serrate (Ser) signals through Notch to induce a subpopulation of prohemocytes to commit to crystal cell lineage by inducing the expression of Lz (Fig. 1B). Recently, the early B cell factor homolog Collier (also known as Knot (Kn)), was shown to be expressed in the posterior signaling center before Ser expression, and is necessary to control Ser expression in these cells (38). When Collier expression is removed from the lymph gland, the mutant larvae fail to produce lamellocytes upon parasitization. This indicates a role for collier in lamellocyte specification (Fig. 1B).

Wound healing

After the embryonic hemocytes differentiate in the head mesoderm, the plasmatocytes begin to migrate out into the rest of the embryo. Embryonic plasmatocytes migrate along predetermined routes until the whole embryo is populated by stage 17 (39). For the plasmatocytes to migrate out of the head region, they require the activity of the *Drosophila* PDGF/VEGF receptor, Pvr and three ligands Pvf1, Pvf2, and Pvf3 (29). When the Pvr receptor or all three ligands are removed, the plasmatocytes fail to migrate out of the head region (29, 31). The ligands Pvf1, Pvf2, and Pvf3 are redundant for hemocyte migration out of the

head region. Furthermore, ectopic expression of one of the Pvr ligands, Pvr2, induces a chemotactic response in embryonic plasmatocytes (29). More recently, it was shown that the small GTPases Rac1, Rac2, and Cdc42 are also required for proper plasmatocyte migration during embryogenesis (40). The Rac GTPases are known to signal downstream of Pvr during border cell migration in oogenesis (41), and it would be interesting to know if they are also required downstream of Pvr during embryonic plasmatocyte migration.

In mammals, macrophages migrate to wound sites to remove cell and matrix debris, and it was believed that the Rho family GTPases Rac1 and Rac2 were necessary for their migration. Yet recently, it was shown that, although they are necessary for proper macrophage morphology, they are dispensable for migration (42). In *Drosophila* embryos, this is not the case. Stramer et al. (43) showed that wounding of a *Drosophila* embryo induced hemocytes to migrate to the wound location and that this migration required the activities of Rac1, Rac2, and Rho GTPase. Once there, similar to mammalian macrophages, the embryonic hemocytes engulfed cellular debris and apoptotic corpses. Removal of Rac1 and Rac2 reduced the number of hemocyte lamellipodia induced by wounding, and very few hemocytes were recruited to the wounds. This suggests that the Rac GTPases are necessary for the migration of embryonic plasmatocytes to wounds. Embryos mutant for Rho1 also failed to migrate to laser-induced wounds. Hemocytes lacking Rho1 activity sense the wound and try to migrate to it but are held back because they are unable to retract their trailing edge. A similar defect has been described in human monocytes (44). In monocytes, the small GTPase Cdc42 is required to recognize and respond to chemotactic signals (45–47). When Cdc42 signaling was blocked in embryonic plasmatocytes, they were still able to migrate to the wound, but the motility of the plasmatocytes was affected. Similar to what has been described for both yeast and mammalian cells, hemocytes lacking Cdc42 were not able to maintain their cell polarity (43, 48). These results show that, similar to mammalian leukocytes, *Drosophila* plasmatocytes require the Rho family GTPases for proper migration to wound sites.

Wood et al. (49) demonstrated that embryonic hemocytes respond to developmental and wound signals via different signaling pathways. Their experiments show that embryonic hemocytes follow Pvf2 and Pvf3 expression to migrate along the CNS, and the embryonic hemocytes follow Pvf2 for migration along the dorsal vessel. Next, they looked at PI3K signaling in hemocytes. In mammalian neutrophils, PI3K signaling is important for migration toward a chemoattractant signals (50). Using both a dominant-negative form of PI3K and a specific PI3K chemical inhibitor, they show that PI3K is dispensable for developmental migration along the CNS or dorsal vessel but necessary for hemocyte migration toward a wound. They also show that Pvr signaling is not involved in chemotaxis toward a wound. In mammals, PI3K can induce Rac leading to lamellipodia formation and is proposed to feedback to activate PI3K. It would be interesting to see whether a similar mechanism is used in *Drosophila* hemocytes.

Phagocytosis

Phagocytosis is an evolutionarily conserved process essential for the removal of invading pathogens and apoptotic bodies. It is

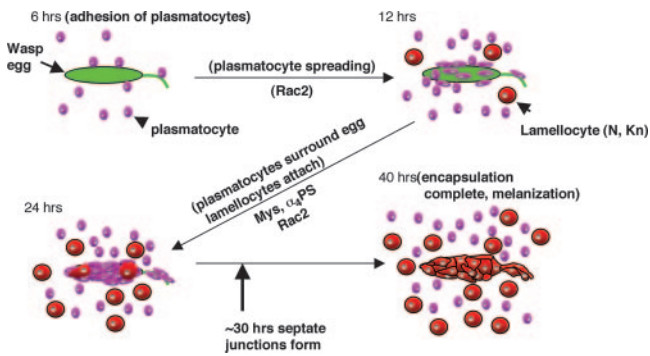


FIGURE 2. Diagram of the encapsulation of a parasitoid wasp egg. By 6 h postparasitization, plasmatocytes have started to attach to the wasp egg. Once attached, the plasmatocytes begin to spread around the egg, requiring the small GTPase Rac2. By 12 h postparasitization, lamellocytes begin to appear; this requires the Notch (N) signaling pathway and the transcription factor Collier (also known as Kn). By 24 h postparasitization, the plasmatocytes have completely surrounded the wasp egg, and lamellocytes are beginning to adhere. The β_2 -integrin Myospheroid (Mys) and α -integrin α_4 PS are required for lamellocyte adherence to the wasp egg. Rac2 is required for the lamellocytes to maintain a spread conformation once attached to the wasp egg. After the plasmatocytes surround the egg, they form septate junctions. Finally, the lamellocytes completely surround the egg and the cellular capsule melanizes.

important for both the innate and adaptive mammalian immune responses. During development, it is necessary for proper tissue remodeling (51). In *Drosophila*, the plasmatocytes are the professional phagocytes of the immune response. As mentioned earlier, they are most similar to mammalian macrophages. In order for phagocytosis to occur, receptors on the surface of the engulfing cell must first recognize an invading pathogen or apoptotic body. Previous studies in insects have identified a number of conserved proteins, including complement-like factors, pathogen-recognition receptors, and cytoskeletal proteins necessary for proper phagocytosis (52–56). In mammals and insects, activation of surface receptors induces intracellular signaling pathways leading to the cytoplasmic remodeling required for internalization, phagosome maturation, and particle dissolution.

In *Drosophila*, only a few genes have been implicated in the phagocytosis of invading microbes. One of the first to be identified was the scavenger receptor dSR-CI as a pattern recognition receptor that binds to bacteria (56). This receptor is responsible for only a small amount of the total binding of bacteria by a phagocytically active *Drosophila* cell line. To look for additional genes involved in phagocytosis R met et al. (57) conducted a RNA interference-based screen in the *Drosophila* immune-reactive S2 cell line and reported 34 genes possibly involved in the phagocytosis of bacteria. Among these genes is the peptidoglycan recognition protein LC (PGRP-LC), which is involved in the engulfment of Gram-negative, but not Gram-positive, bacteria. Similarly, the mosquito PGRP-LC was also found to be involved in the phagocytosis of bacteria (54). However, the loss of PGRP-LC only reduced the phagocytic capability of the S2 cells to 65% of normal, suggesting that other receptors must be involved in the recognition and engulfment of invading microbes. To look for more genes involved in phagocytosis, Kocks et al. (53) used RNAi to target the Srp gene, a transcription factor that is required for phagocytosis and hemocyte differentiation (57). Using this method, they were able to identify 46 genes that required Srp for their expression in

a *Drosophila* cell line. Knocking-down each of these transcripts revealed one gene, which the authors called *eater*, having a clear role in the phagocytosis of both *Escherichia coli* and *Staphylococcus aureus*. Loss of the *eater* transcript in S2 cells decreased phagocytic activity by 70%. *eater* is predicted to encode a transmembrane receptor having 32 EGF-like repeats in its extracellular domain. It is the first EGF-like repeat receptor shown to be involved in microbial recognition. Similar to scavenger receptors, Eater appears to recognize a broad range of microbial pathogens. In *Drosophila* larvae, Eater is expressed in plasmatocytes and loss of Eater reduced the phagocytosis of several bacterial species by up to 80%.

One of the most exciting recent finds is that in *Drosophila* the Ig superfamily receptor Down syndrome cell adhesion molecule (Dscam) binds to bacteria and is required for efficient phagocytosis by plasmatocytes (1). Through alternative splicing, Dscam has the potential to produce ~38,000 isoforms with distinct extracellular domains. Watson et al. (1) went on to show that only ~18,000 isoforms are expressed in fat body cells and hemocytes, and only a subset of the potential isoforms bind to *E. coli*. This leads to the possibility that Dscam is sufficient to provide a wide range of pathogen recognition receptors in *Drosophila*. Dong et al. (2) reported the mosquito Dscam gene, *AgDscam*, also has a complex genome organization that can produce >31,000 potential alternative splice forms. Furthermore, by microarray analysis, Dong et al. (2) show that *AgDscam* responds to infection by producing pathogen-specific splice forms. Lowering *AgDscam* expression in mosquitoes lowers their resistance to infections with bacteria, as well as the malaria parasite *Plasmodium*. It would be interesting to know how the production of the pathogen specific isoforms is regulated.

The *Drosophila* genome encodes six thio-ester containing complement-related (TEP) proteins. Recently, the TEP proteins have been linked to phagocytosis in *Drosophila* (58). Previously, Moita et al. (54) showed that three mosquito TEP proteins (TEP1, TEP3, and TEP4) are required for efficient phagocytosis of bacteria. Similar to the mammalian complement factors, *A. gambiae* TEP1 opsonizes bacteria via a thio-ester bond (59).

Encapsulation

In *Drosophila*, when an invading organism is recognized as foreign, circulating hemocytes should remove it, either by phagocytosis and/or encapsulation. This reaction can be observed when parasitoid wasps lay their eggs in the hemocoel of *Drosophila* second instar larvae. The invasion elicits a strong cellular response, with the release of plasmatocytes from the lymph gland (60) and possibly also the sessile population found throughout the larvae (21). Furthermore, it causes the differentiation of numerous lamellocytes (8, 61, 62). Once the wasp egg is recognized, capsule formation ensues. This requires circulating plasmatocytes to change from a nonadhesive to adhesive state enabling them to bind to the invader. First, the plasmatocytes attach and spread around the chorion of the wasp egg. After the cells have spread, they form septate junctions, thus separating the egg from the hemocoel (63, 64). The last phases of capsule formation include lamellocyte adherence and melanization due to crystal cell degranulation (63) (Fig. 2). From these encapsulation events, it is obvious that adhesion and cell shape change is an essential part of the cellular response against parasitoid wasp eggs. The challenge is to understand the spatial

and temporal regulation of the signaling events involved in this cellular response. One family of proteins known to be centrally involved in the regulation of cell migration and cell shape change is the Rho GTPases (Rho, Rac, and Cdc42). Recently, two articles have been published showing that both *Drosophila* Rac GTPase (Rac1 and Rac2) are necessary for proper encapsulation eggs from the parasitoid wasp *Leptopilina bouvardi* (64, 65). In Rac2 mutants, the plasmatocytes and lamellocytes recognize and attach to the wasp egg but fail to spread around it. This lack of spreading means septate junctions fail to form, and the capsule fails to melanize. Williams et al. (65) showed that Rac1 and the *Drosophila* JNK (Basket) are also required for proper encapsulation, but the reason is still unknown. Interestingly, it has also been published that the *Drosophila* β_2 -integrin (known as Myospheroid) is necessary for proper encapsulation of the wasp egg (66). In *myospheroid* mutants, lamellocytes still form but fail to attach to the wasp egg. In many cell types, it has been shown that Rho family GTPases signal downstream of integrins, and it would be interesting to see if the same is true during the hemocyte response to parasitoid wasp eggs.

The whole process of parasite encapsulation raises several interesting questions. First, what is the signal produced by plasmatocytes once they have recognized an invader that they cannot engulf. This signal is perceived by prohemocytes in the lymph glands, which then differentiate and are released into circulation. Wertheim et al. (67) performed a microarray to look at genes induced after larvae were parasitized. They found that both the JAK/STAT and Toll pathways responded to parasitization. This corresponds with work showing that both the *Drosophila* JAK homolog Hop and the Toll pathway are required for a proper encapsulation response (27). It could be that these pathways are required for prohemocytes to react to some signal from activated circulating hemocytes. Another unanswered question is how the hemocytes recognize the wasp egg?

Concluding remarks

The field of *Drosophila* humoral immunity has witnessed significant development over the last decade, expanding our general understanding of innate immunity. Until recently, *Drosophila* has not been a leading model in the analysis of hemopoiesis and innate cellular immunity. Yet, a number of recent studies have demonstrated that the *Drosophila* cellular immune response can help in our understanding of the mechanisms involved in hemopoiesis and immune activation. It may also prove to be a valuable tool in understanding the molecular mechanisms underlying the various functions of myeloid-type blood cells.

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Disclosures

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