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Review

Signaling pathways implicated in the cellular innate immune responses of *Drosophila*

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Abstract

The phylogenetically conserved innate immune systems of insects and other invertebrates employ blood cells (hemocytes) that are functionally reminiscent of vertebrate macrophages, attesting to the importance of phagocytosis and other cell-mediated responses in eliminating various pathogens. Receptor-ligand binding activates signaling cascades that promote collaborative cellular interactions and the production of pathogen-specific cytotoxic responses. Numerous comparative genetic and molecular studies have shown the cytotoxic effector responses made by cells of the innate immune system to be evolutionarily conserved. Comparative analyses of genomic sequences provide convincing evidence that many of the biochemical processes manifested by immune-activated hemocytes are similar to those made by activated vertebrate macrophages. Included in this genomic repertoire are enzymes associated with reactive intermediates of oxygen and nitrogen, cellular redox homeostasis, and apoptosis, the synthesis of extracellular matrix, cell adhesion and pattern recognition molecules. Surprisingly, little is known of the types of cytotoxic molecules produced by invertebrate hemocytes, and the signaling and transcriptional events associated with their collaborative interactions when engaging pathogens and parasites. This review examines certain aspects of the blood cell-mediated defense responses of *Drosophila*, and some of the signaling pathways that have been implicated in hemocyte activation, differentiation, and the regulation of hematopoiesis.

Key words: Melanotic encapsulation; phagocytosis; apoptosis; hematopoiesis; immune signaling pathways; *Drosophila*

Introduction

Eukaryotic cells possess intrinsic mechanisms for detecting perturbations resulting from aberrant development of rogue cells and pathogen challenge. The origin and molecular bases for non-self recognition, the biochemical reactions the destroy non-self, and the cell signaling pathways mediating these responses are regulated by complex homeostatic mechanisms (Nappi and Ottaviani, 2000). To contend with the immensenumber of foreign molecules likely to be encountered, vertebrate species employ both innate and adaptive immune mechanisms (Gillespie ***et al***., 1997; Medzhitov and Janeway, 1997, 2000; Janeway and Medzhitov, 2002). Innate immunity is a conserved mechanism of defense, involving common cell signaling pathways, transcriptional elements, and cytotoxic effector responses (Beutler, 2004). Depending on the

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species, the innate immune effector responses generated in response to pathogens include phagocytosis, opsonization, activation of complement and coagulation cascades, activation of pro-inflammatory signaling cascades, and apoptosis. In addition, the innate immune system functions in activating and modulating the adaptive immune response through the induction of co-stimulatory molecules and cytokines (Gillespie *et al*., 1997; Medzhitov and Janeway, 1997, 2000; Hoffmann *et al*., 1999; Medzhitov, 2001; Janeway and Medzhitov, 2002; Bodian *et al*., 2004; Ottaviani *et al*., 2004). Non-self recognition by the adaptive immune system is mediated by B and T lymphocytes, and relies on somatic gene rearrangements to perpetuate a highly diverse repertoire of receptors that contribute to immune specificity and memory. Engagement of these antigen-specific receptors by their cognate antigens activates signaling cascades that promote the clonal expansion of lymphocytes, their differentiation and collaborative interactions with other immune cells, and the production of pathogen-specific cytotoxic responses. Adaptive immunity is an efficient system of defense that generates virtually limitless somatic gene rearrangements that produce antigen-specific receptors, but it has a limitation in that clonal expansion and differentiation of lymphocytes are delayed responses, typically manifested 4-7 days after encountering pathogens (Janeway and Medzhitov, 2002). Consequently, the ability of vertebrates to detect and efficiently eliminate pathogens is dependant on the collaborative interactions of the two non-self discriminatory systems, the adaptive immune system manifesting both specificity and memory components, and the innate immune system, which, because of its more rapid engagement of pathogens, is generally considered to be the first line of defense (Kimbrell and Beutler, 2001).

Insect innate immunity

Insects possess diverse and effective immune mechanisms for combating prokaryotic and eukaryotic infections. First-line defenses include cells and/or cell products associated with the external cuticle, gut and tracheal lining. Organisms that breach these barriers and invade the host hemocoel encounter constitutive and inducible defenses that include phagocytosis and encapsulation by macrophage-like blood cells or hemocytes, reactive intermediates of oxygen and nitrogen, activation of various proteolytic cascades that lead to blood coagulation, melanization, the synthesis of antimicrobial peptides by the fat body (a structure analogous to the mammalian liver), and the release of stress-responsive proteins and substances believed to function in opsonization and iron sequestration (Gillespie *et al*., 1997; Hoffmann *et al*., 1999; Medzhitov and Janeway, 2000; Nappi and Ottaviani, 2000; Hoffmann and Reichhart, 2002; Janeway and Medzhitov, 2002; Hoffmann, 2003). The extent to which the above substances cause or contribute to the destruction of pathogens and parasites remains largely undetermined.

Inexplicably, much of the current literature dealing with innate immunity in insects fails to acknowledge the role played by the blood cells. Although invertebrate hemocytes are considered to be functionally similar to vertebrate macrophages, little has been done to document, as integral components of the insect innate immune system, such well known macrophage-derived cytotoxic molecules (e.g. reactive intermediates of oxygen and nitrogen, perforin, granzymes). Apart from studies of the factors regulating antimicrobial gene expression, little is known about the signaling pathways that control the more immediate and ubiquitous responses made by the blood cells, namely phagocytosis and encapsulation. An essential component of these cellular responses is melanization, which is manifested at the site of cuticular penetration, and on the surfaces of foreign organisms soon after they enter the host hemocoel and encounter hemocytes. The pigment is derived from the oxidation of monophenols (e.g., tyrosine) and diphenols (e.g., L-DOPA, dopamine) and the ensuing polymerization of their respective orthoquinones, a cascade of reactions initiated by the rate-limiting enzyme tyrosinase (i.e. phenoloxidase, PO), and involving the participation of at least three additional enzymes; phenylalanine hydroxylase (PAH), dopa decarboxylase (DDC), and dopachrome conversion enzyme (Fig. 1) (Sugumaran, 2002). There is currently a paucity of experimental evidence to accurately define the role of melanin and its precursors in insect innate immunity, and essentially nothing is known of the molecular mechanisms initiating the hemocyte-mediated melanogenic responses accompanying non-self recognition. What is known is that PO-mediated melanization responses must be localized, target-specific and very tightly regulated processes in order to prevent fatal systemic activation in the open circulatory system of an insect.

Detection of non-self: involvement of pattern recognition

Given the limitations of just an innate immune system, insects nevertheless effectively discriminate

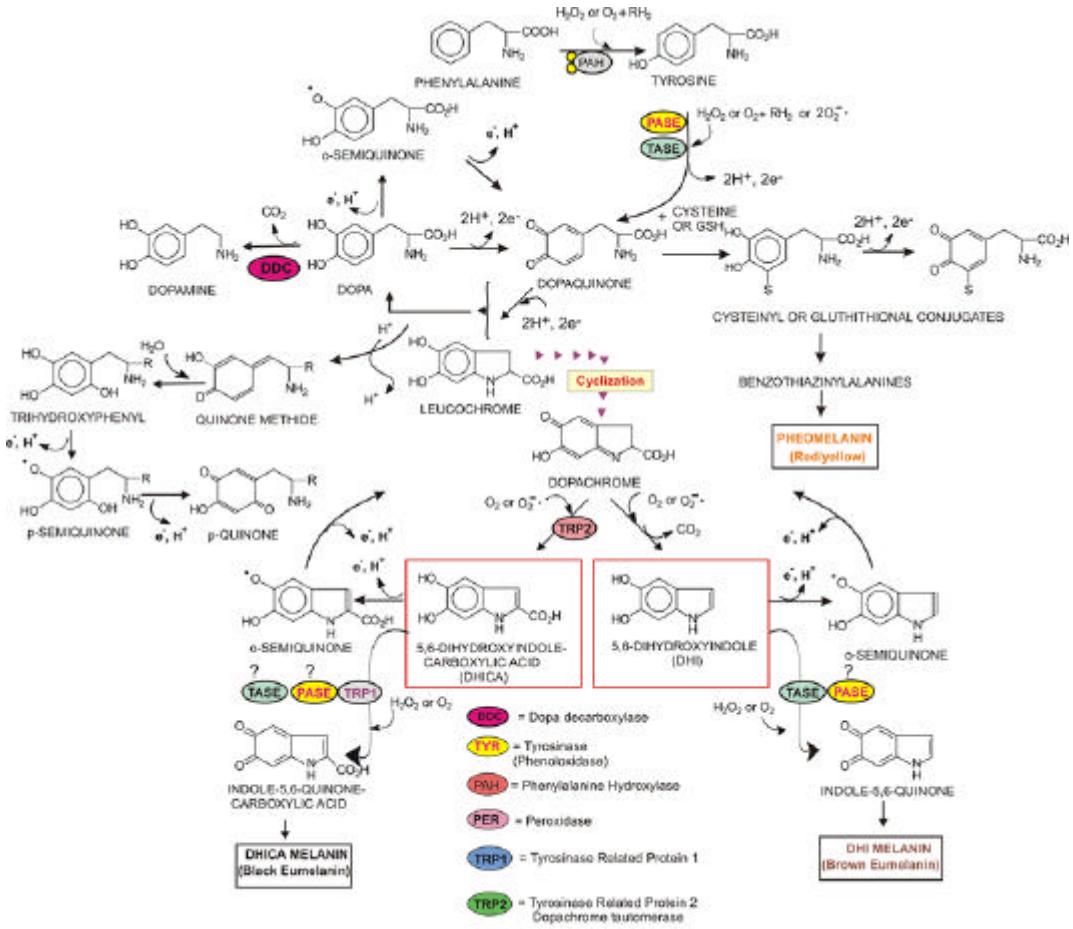

Fig. 1. Summary of mammalian melanogenesis. Tyrosinase (TYR), tyrosinase-related protein 1 (TRP1) and tyrosinase-related proteins 2 (TRP2), are membrane-bound melanosomal proteins that are thought to interact as a multi-enzyme complex in regulating melanogenesis. TYR catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation DOPA to DOPAquinone, and the oxidation 5,6-dihydroxyindole (DHI) to indole-5, 6-quinone. TRP2 functions as a DOPAchrome tautomerase, producing 5,6-dihydroxyindole-2-carboxylic acid (DHICA) from DOPAchrome, preventing the latter from undergoing a spontaneous decarboxylation to DHI. TRP1 is believed to function in the melanogenic pathway as a DHICA oxidase, promoting the oxidation and polymerization of DHICA monomers into eumelanin. The substrate for Tyr, tyrosinase, is derived from phenylalanine in a reaction catalyzed by phenylalanine hydroxylase (PAH). Some of the oxidations catalyzed by TYR may also be catalyzed by peroxidase (PER), but the involvement of PER has not been established for many systems. The critical decarboxylation of DOPA to dopamine results from the action of an aromatic amino acid decarboxylase termed DOPA decarboxylase (DDC). It remains to be established if this enzyme can also facilitate the conversion of DOPAchrome to DHI. DHICA melanin has not been reported in insects.

between self-molecules and the varied types of pathogens they encounter. Much of what little is known about non-self recognition in insects is based on responses made following their subjection to injections of microorganisms or microbial products. Of concern is the fact that the injection methods bypass the normal routes of entry into the host hemocoel, and the large amount of exogenous material introduced is overwhelming and completely unnatural.

Pathogen recognition by the mammalian innate immune system is mediated by macrophages, polymorphonuclear leukocytes, dendritic cells and natural killer cells, and is dependant on the presence of a finite number of germ-line encoded pattern recognition receptors (PRRs). The latter recognize and bind to pathogen-associated molecular patterns (PAMPs), which are conserved motifs unique to microorganisms and essential for their survival (Medzhitov and Janeway, 1997). PAMPs that serve as ligands include bacteria-derived lipoteichoic acid, peptidoglycan (PGN), and lipopolysaccharide (LPS), and β-glucan of fungi. LPS is the principal glycolipid component of the outer membrane of Gram-negative bacteria. PGN, which is a polymer of β(1-4)-linked N-acetylglucosamine and N-acetylmuramic acid, is abundant in the cell wall of Gram-positive bacteria, and forms a thin layer beneath the LPS-containing outer membrane in Gram-negative bacteria. PGRPs, which recognize PGN and Gram-positive bacteria, are highly conserved from insects and mammals (Dziarski, 2004).

Since PAMPs are invariant between microorganisms of a given class, only a limited number of germ line-encoded pattern recognition receptors are required to combat microbial infections. Implicated as mediators of pathogen recognition by cells of the mammalian innate immune system are Toll-like receptors (TLRs), some of which are ligand-specific (e.g., TLR3, 5, and 9), while others respond to many structurally unrelated molecules derived from different groups of pathogens (e.g., TLR4 recognizes both viral components and Gram-negative LPS). In addition, some pathogens are recognized by more than TLR (e.g., both TLR2 and TLR4 recognize gram-positive organism-derived pathogen-associated molecular patterns (Medzhitov, 2001). The signaling domain of each TLR is the most conserved part of the receptor and is referred to as TIR (Toll/IL-1R), which denotes TLR, interleukin-1 receptor (IL-1R).

It is widely acknowledged that PRRs may be secreted molecules or components of the cytosol or plasma membrane, but is uncertain if these molecules are directly involved in non-self recognition, or if they represent downstream components activated by separate recognition signals. If insect PRRs indeed bind to unique PAMPs, one can only speculate about the common molecular properties that must be manifested by fungi, Gram-positive bacteria and some Gram-negative bacteria, in order for these morphologically and physiologically diverse pathogens to separately activate the insect Toll pathway, but not the Imd pathway, which is said to be reactive against certain other Gram-negative bacteria and not fungi.

Some of the unresolved issues about insect antimicrobial immunity concern the degree of specificity of the Toll and Imd pathways in differentially discriminating among the varied pathogens. The *Drosophila* Toll receptor cannot be considered a pattern recognition receptor, since its ligand is an endogenously-derived cytokine-like molecule, Spaetzle, and not a pathogen-associated molecular molecule. Pathogen recognition occurs upstream of Toll, and may involve a serine protease inhibitor encoded by the *necrotic* gene, as a loss-of-function mutation activates Toll (Levashina *et al*., 1999). Fungal-dependent Toll activation is known to require the gene *persephone*, which encodes a serine protease. However, over expression of the gene in non-immune challenged flies also leads to Toll activation and antimicrobial gene expression (Ligoxygakis *et al*., 2002). The Imd pathway is believed to be activated by Gram-negative binding protein (GNBP).

Insect PGRPs have been grouped into two classes; short PGRPs (PGRP-S), which are small extracellular proteins (19-20 kDa), and long PGRPs (PGRP-L), which have longer transcripts and are either intracellular or membrane-spanning proteins. PGRP-S are constitutively synthesized or induced primarily in cells of the fat body, epidermis, gut, and to a lesser degree in the hemocytes. PGRP-L is mainly expressed in hemocytes. When activated by PGN or Gram-positive bacteria, PGRP-SA activates proteases that cleave Spaetzle, which then activates Toll. Activation of Toll initiates a signal transduction cascade that results in the activation of Rel family transcription factors (similar to Nuclear Factor kappa B, NF-*K*B), which translocate into the nucleus, bind to the *k*B sites, and activate genes of the innate immune system. The Toll pathway also is activated during fungal infection through a Gram-negative binding protein-3 (GNBP). PGRP-S binding to PGN and Gram-positive bacteria activates the prophenoloxidase cascade leading to melanization reactions. Prophenoloxidase-mediated melanization reactions also are elicited by fungi, LPS, and during cuticular injury. Gram-negative bacteria and some Gram-positive bacilli activate, via PGRP-L, the

Imd pathway that leads to expression of other innate immune genes. Components of the Imd signal transduction cascade resemble those of the mammalian TNF-á receptor-inducer pathway.

Some PGRPs are not very specific, reacting with both Gram-positive and Gram-negative bacteria, as well as with fungi and LPS. Functions uniquely manifested by insect PGRPs include activation of the Toll and Imd pathways, activation of prophenoloxidase-mediated melanization, and phagocytosis (Michel *et al*., 2001). In *Drosophila*, three PGRPs have been identified as being involved in immune related processes, a soluble molecule (PGRP-SA), and transmembrane components (PGRP-LC and PGRP-LE). PGRP-SA lacks protease activity, but its activation results in the processing of the Toll ligand Spaetzle, suggesting PGRP-SA functions upstream of the serine protease cascade that activates Toll. PGRP-LC lacks recognizable signaling motifs, but may activate a proteolytic signaling cascade to generate a Toll ligand. *Drosophila* PGRP-LC also may play a role in phagocytosis (Ramet *et al*., 2002b) and activation of the Imd pathway (Dziarski, 2004). Drosophila PGRP-LE also functions in activation of the Imd pathway, as well as the prophenoloxidase cascade. A list of some mammalian and insect PGRPs is given in Table 1.

Blood cell-mediated responses in *Drosophila*

Phagocytosis

Phagocytosis is very likely a highly conserved process among eukaryotic organisms. The process is initiated upon contact with, and recognition of, non-self, altered or rouge cells, or apoptotic cells. Responses that rapidly ensue following the tethering of non-self determinants to specific cell surface receptors include cytoskeleton modifications, vesicle trafficking, internalization, and destruction of the engulfed the target within phagosomes (Ramet *et al*., 2002b). In *Drosophila*, several membrane receptors on certain hemocytes (plasmatocytes) have been implicated in phagocytosis. During embryonic development phagocytosis of apoptotic cells is dependent on the *croquemort* gene (Franc *et al*., 1996, 1999), which encodes a mammalian CD36 homologue. The latter is a class B scavenger receptor that participates with other elements in engulfing apoptotic cells (Fadok *et al*., 1998a). The *Drosophila* scavenger receptor, dSR-CI, apparently mediates binding to both Gram-negative and Gram-positive bacteria, (Pearson *et al*., 1995), and at least one GNBP has been shown to bind to LPS and â-1,3-glucan (Kim *et al*., 2000; Ramet *et al*., 2001).

It has been proposed that *Drosophila* thioester-containing proteins (TEPs) function either as opsonins to promote phagocytosis, in a manner similar to mammalian complement factor C3, or as protease inhibitors, in an $á_2$-macroglobulin-like manner (Lagueux *et al*., 2000). TEPs have been shown to alter *in vitro* phagocytic activity. In a blood cell line derived from the mosquito *Anopheles gambiae*, phagocytosis of Gram-negative bacteria is strongly reduced when transcription of the TEP gene *atep1* is impaired (Levashina *et al.,* 2001).

Melanotic encapsulation

The cellular innate immune response of *Drosophila melanogaster* larvae against pathogens too large to be phagocytosed involves the proliferation and precocious differentiation of hemocytes, and their rapid employment in forming multicellular, melanotic capsules around avirulent parasites (Fig. 2) (Carton and Nappi, 1997, 2001; Vass and Nappi, 2000, 2001).

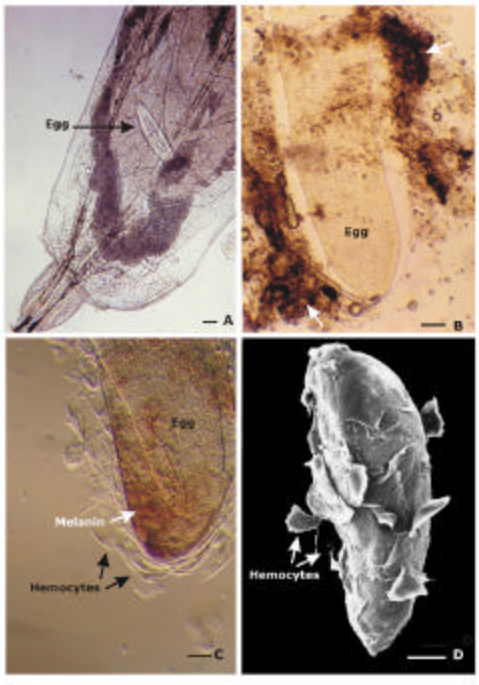

Fig. 2. (A) Egg of *Leptopilina* in the body cavity of a *Drosophila* larva soon after infection (Bar = 80 µm). (B and C) Light micrographs of the early stages of hemocyte-mediated melanotic encapsulation of *Leptopilina* (Bars = 15 µm). Scanning electron micrograph of a fully-formed hemocyte capsule surrounding the parasitoid egg (Bar = 50 µm)

Table 1

	PRGP PROTEINS				
	Fruit fly	**Mosquito**	**Silkworm**	**Human**	**Mouse**
EFFECTOR FUNCTIONS					
Antibacterial				PGRP-S	PGRP-S
Amidase Activity	PGRP-LB PGRP-SB1 PGRP-SB2 PGRP-SC1a PGRP-SC1b PGRP-SC2	PGRP-LB PGRP-S2 PGRP-S3	BTL-LP1	PGRP-L	PGRP-L
Toll Pathway Activation	PGRP-SA				
Imd Pathway Activation	PGRP-LC PGRP-LE				
PPO Activation	PGRP-LE		PGRP-S		
Phagocytosis	PGRP-LC				
To be Determined	PGRP-LAa1 PGRP-Lab PGRP-Lac PGRP-LD PGRP-LF PGRP-SD	PGRP-LA1 PGRP-LA2 PGRP-LC PGRP-S1	BTL-LP2	PGRP-Iα PGRP-Iβ	

Fruit fly, *Drosophila melaogaster;* Mosquito, *Anopheles gambiae;* Silkworm, *Bombyx mori;* Human, *Homo sapiens*; Mouse, *Mus musculus.*

There is good evidence that hemocyte proliferation and differentiation in response to infection occurs principally, if not completely, within the larval hematopoietic lymph glands (Lanot *et al.*, 2001; Sorrentino *et al.*, 2002). Virtually identical reactions occur during the formation of melanotic tumors (Nappi *et al.*, 1984, 2004). These heritable, benign or neoplastic growths appear at various times during larval development, either circulating in the hemocoel or attached to the viscera (Stark, 1918, 1919; Hartung, 1950; Harshbarger and Taylor, 1968; Ghelelovitch, 1968; Gateff, 1978, 1994; Sparrow, 1978; Gateff *et al.*, 1996; Woodhouse *et* al., 1998). The pigmented masses are generally comprised of aggregations of adhering blood cells, or various endogenous tissues encapsulated by these cells. The encapsulated tissues are either aberrant structures perceived as non-self, or normal tissues infiltrated and encapsulated by hemocytes undergoing aberrant proliferation and neoplastic differentiation.

Hemocytes and transcriptional regulation of hematopoiesis

There is currently little information regarding the nature and mode of action of the signaling molecules involved in regulating the cascade of hemocyte-mediated encapsulation responses in insects, or of the specificity of the responses (Lavine and Strand, 2002). Studies of hematopoiesis in *Drosophila* provide a basis for better understanding of the complex interactions of signaling molecules. Although the origins and fates of the various hemocyte types in *Drosophila* are not completely understood, embryonic hemocytes, which originate exclusively from head mesoderm, constitute a homogenous population with respect to their potential for phagocytosis (Tepass *et al.*, 1994). During larval

development, hematopoiesis is known to occur in the lymph glands situated along the anterior portion of the dorsal blood vessel (Stark and Marshall, 1930; Shatoury, 1955; Shatoury and Waddington, 1957; Gateff, 1994; Lanot *et al.*, 2001; Evans and Banerjee, 2003). Progenitor cells in the lymph glands give rise to three distinct hemocyte lineages: plasmatocytes, crystal cells and lamellocytes. Plasmatocytes comprise 95% of the blood cells in circulation during larval development. These cells appear to be functionally similar to mammalian monocytes and macrophages, possessing surface receptors that include a Scavenger Receptor, a CD36 homologue, and a peptidoglycan recognition protein (Fadok *et al.*, 1998b; Aderem and Underhill, 1999; Meister and Lagueux, 2003). Crystal cells, which represent approximately 5% of the circulating hemocytes, possess prophenoloxidase-containing cytoplasmic inclusions that, when released into the surrounding hemolymph, initiate melanogenesis, a response that accompanies the cellular encapsulation response. Lamellocytes are cells that normally appear in circulation in low numbers just before pupation (< 1%), but they undergo massive and precocious differentiation in response to infection, and then are released into the hemolymph to combat parasites and pathogens. In response to immune challenge by avirulent strains of the endoparasitic wasp *Leptopilina boulardi*, the total number of circulating hemocytes increases (2-3 fold increase) as does the percentage of lamellocytes (20-40%), but crystal cells are virtually absent (Nappi and Walker, 1959; Streams, 1969; Nappi, 1987).Virulent strains of the wasp circumvent melanotic encapsulation by introducing immune suppressive factors (ISF) that interfere with the blood cell-mediated response (Fig. 3). The lymph glands are the sole source of the additional cells recruited to assist those already in circulation at the time of infection in forming melanotic capsules. Plasmatocytes and lamellocytes comprise the cellular components of the capsules, while crystal cells presumably lyse and/or release the crystalline inclusions in close proximity to the developing capsule, thereby initiating the phenoloxidase-mediated melanization reaction. The cytotoxic molecules generated by these collective blood cell responses have never been unequivocally determined experimentally, but reportedly include reactive intermediates of oxygen, nitrogen and melanin (Nappi *et al.*, 1995, 2000; Nappi and Vass, 1998, 2001; Carton and Nappi, 2001).

Hematopoiesis is under the control of a number of transcription factors and signaling pathways, including GATA factors, JAK/STAT or Notch pathways (Rehorn *et al.*,1996; Qiu *et al.*, 1998; Lebestky *et al.*, 2000, 2003; Duvic *et al.*, 2002; Remillieux-Leschelle *et al.*, 2002; Evans and Banerjee, 2003). The precocious appearance of lamellocytes in the lymph glands of immune and auto-immune reactive larvae, and subsequently in the circulation, suggests the hematopoietic tissues detect stimuli originating from blood cell encounters with non-self entities within the hemocoel, and respond by releasing activated cells specifically programmed to eliminate pathogens or aberrant tissues (Fig. 4).

Several transcription factors and signaling pathways govern hemopoietic lineages (Rehorn *et al.,* 1996; Lebestky *et al.*, 2000, 2003; Waltzer *et al.*, 2003). As *Drosophila* hemocyte stem cells begin to differentiate in the lymph glands, they express the GATA factor Serpent (Srp), and later both Srp and the Friend-of-GATA homologue, U-shaped (Ush). Two zinc-finger-containing transactivators, termed glial cells missing (Gcm), cooperatively direct plasmatocyte differentiation (Fig. 5). Plasmatocyte precursors express the transcription factors Gcm and Gcm2, while maintaining Srp and Ush expressions. In crystal cell precursors, Srp and Ush are down-regulated and their expressions eventually lost, while the Serrate/Notch (S/N) pathway and the transcription factor Lozenge (Lz) become active. A small fraction of Lz cells turn off Lz expression and manifest Gcm expression. These Lz-silent Gcm-expressing cells may be precursors of a plasmatocyte lineage that gives rise to lamellocytes (Evans and Banerjee, 2003). Srp has been shown to be required for humoral immunity by regulating the genes in the larval fat body (Petersen *et al.*, 1999; Tingvall *et al.*, 2001). Regulation of the lamellocyte pathway has not yet been established, but involvement of JAK/STAT signaling has been proposed (Luo and Dearolf, 2001).

Innate immune signaling pathways

The binding of cell surface receptors to their cognate ligands activates signaling cascades that promote collaborative cellular interactions and the production of pathogen-specific cytotoxic responses by the innate immune systems, which recent comparative genetic and molecular studies have shown to be evolutionarily conserved. The basic components of cell signaling cascades include receptor-ligand interactions, receptor activation and signal initiation, recruitment of cytoplasmic adaptor molecules, activating/inactivating enzyme complexes, modulators of transcription factors, and translational and post-translational modifiers (Fig. 6). Especially intriguing is the fact that the varied and complex homeostatic mechanisms associated with non-self recognition and

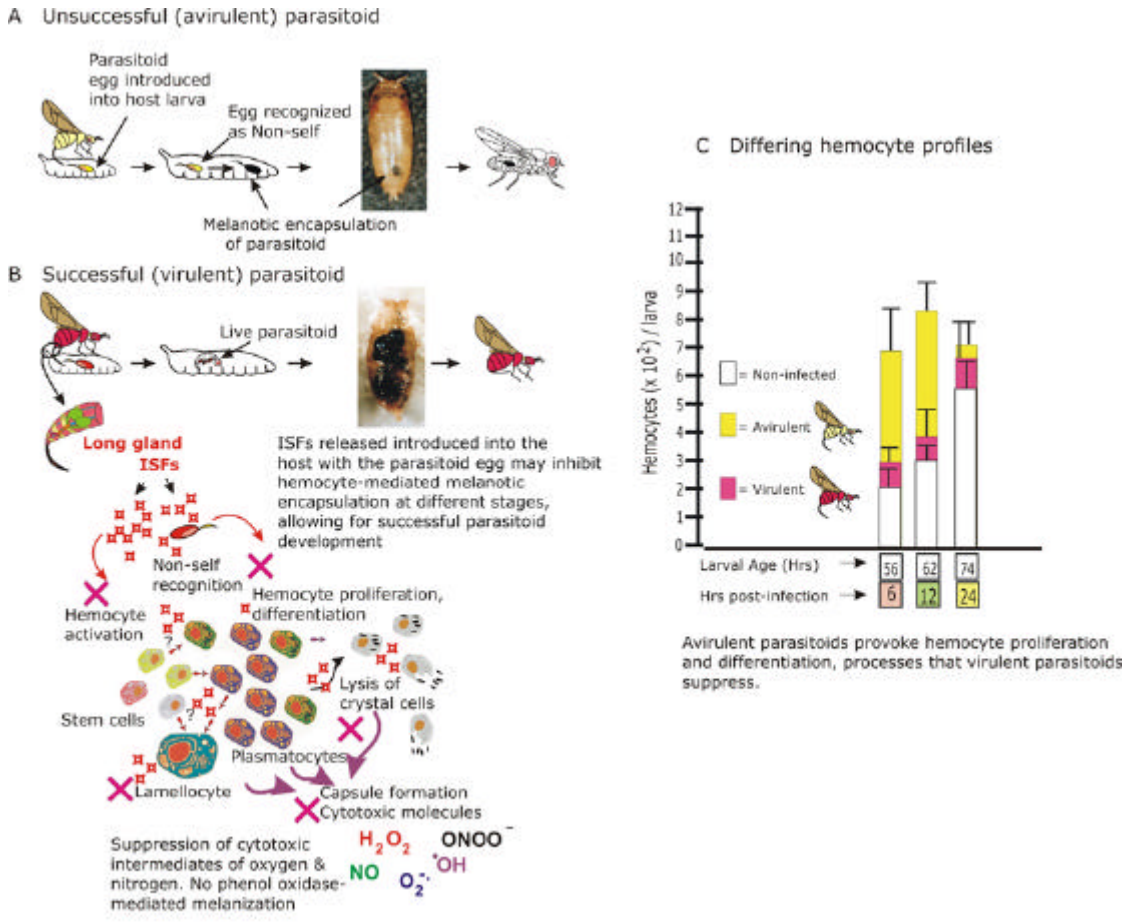

Fig. 3. (A and B) Diagrams illustrating the fates of different strains of the endoparasitic wasp *L. boulardi* in a given host strain of *D. melanogaster*. Virulent wasp strains inject into the host immune suppressive factors (ISF) derived from the long gland that block melanotic encapsulation. ISF from different strains and species of *Leptopilina* appear to target different elements in the cascade of host responses, from initial recognition processes to the synthesis of cytotoxic effector molecules. (C) Comparative blood cell analyses indicate that some virulent strains can block the hemocyte-mediated response, which against avirulent wasps involves and increase in the number of circulating hemocytes, and a precocious mass differentiation of plasmatocytes to capsule-forming lamellocytes (Nappi *et al*., 2004).

the initiation of cytotoxic responses are executed with relatively few signaling systems, frequently using a common transcription factor, namely NF-*K*B, as an integral component in executing the varied immune effector responses. NF-*K*B has been identified in virtually all cell types, and the presence of its responsive sites in the promoters and enhancers of genes that encode for cytokines, acute phase proteins, and cell adhesion molecules, implicates the transcription factor as an indispensable component in the coordination of immune responses.

Much of what is known about immune responsive signaling pathways in insects stems from studies of the synthesis of antimicrobial peptides by the fat body, the functional equivalent of the mammalian liver. The major regulators of immune peptide gene expression in insects are Rel/ NF-*K*B-like transcription factors, the activities of which are believed to be modulated by signaling pathways that are similar to the mammalian IL1-R/TLR pathways. Against microbial agents two intracellular signaling pathways target distinct Rel/ NF-*K*B-like transcriptional elements that mediate immune gene expression. The Toll pathway has been shown to regulate host responses primarily against Gram-positive bacteria and fungi, whereas resistance against Gram-negative bacteria is

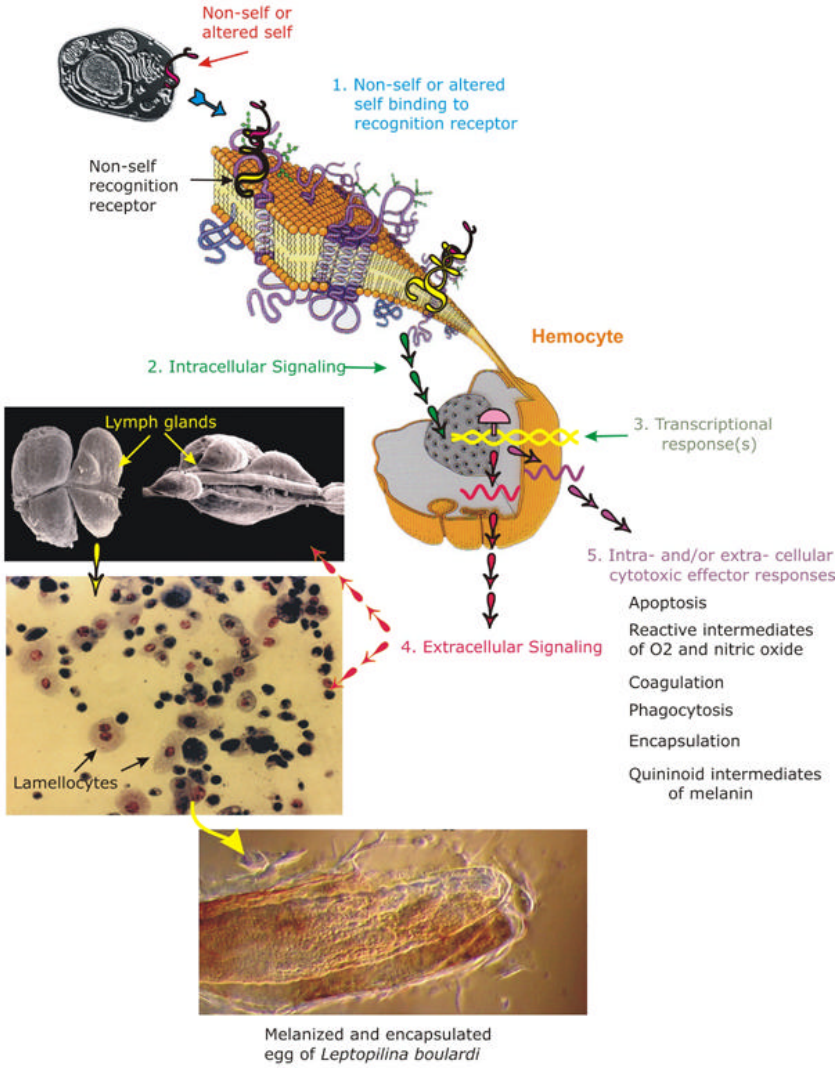

Fig. 4. An overview of the signaling events believed to be involved in the hemocyte-mediated melanotic encapsulation of response of *Drosophila* against *Leptopilina*. Studies showing blood cell poliferation and differentiation within the hematopoietic lymph glands upon infection (Sorrentino ***et al.***, 2002), and the ensuing increase in the number of cells in circulation, implicate the involvement of extracellular signals, perhaps originating from pathogen engagement with circulating cells or with substances within the hemolymph.

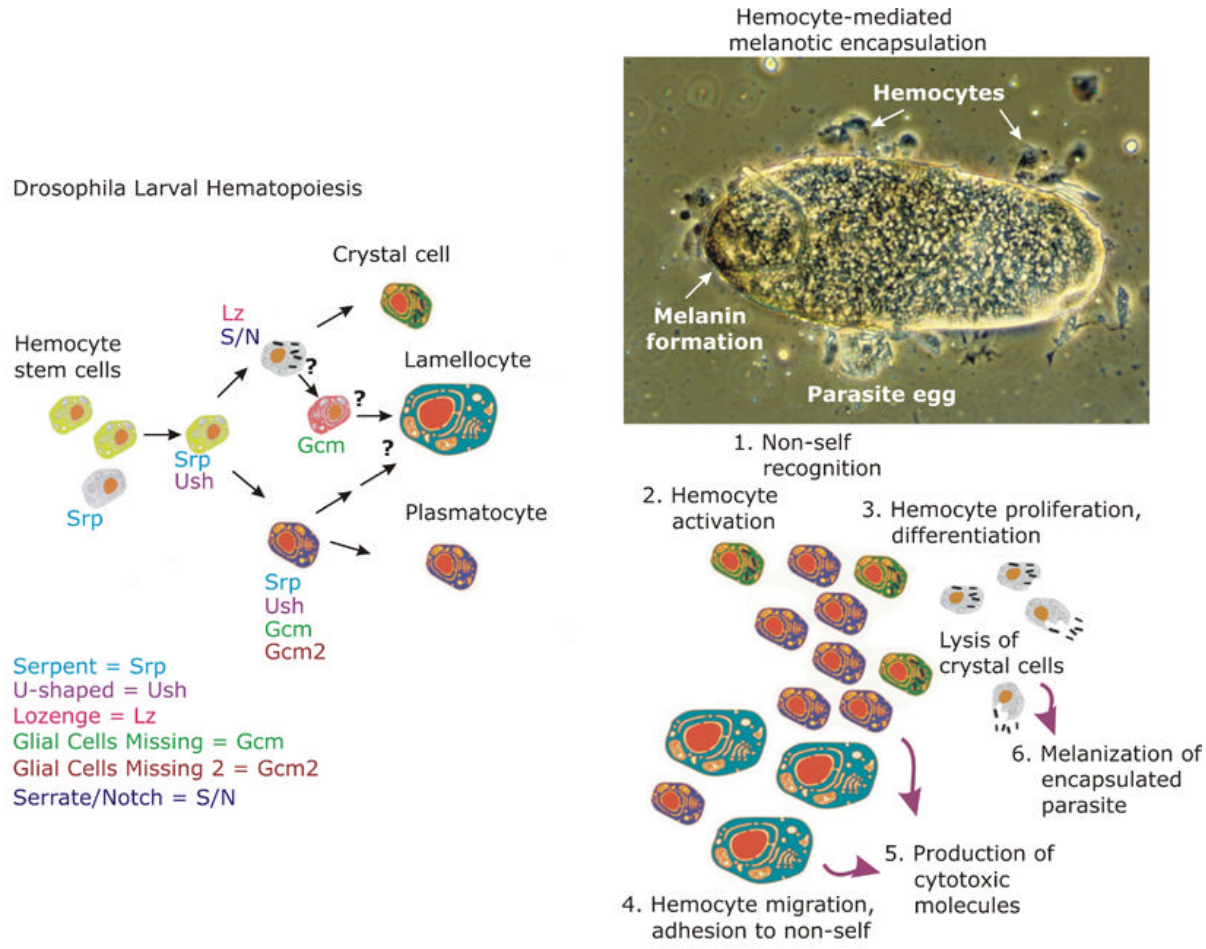

Fig. 5. Transcriptional control of *Drosophila* larval hematopoiesis. PVR, the *Drosophila* homologue of PDGFR/VEGFR, and one of its three putative ligands, PVF2, control proliferation of progenitor hemocytes, the process appearing to be regulated, at least partially, by the Raf/MAPK, JAK/STAT, and Toll pathways. Early hemocyte identity is specified by the GATA factor Serpent (Srp). Two Zinc-finger transactivators, Gcm and Gcm2, and U-shaped are additional factors required for plasmatocyte development. The fate of crystal cells is programmed by Serrate/Notch (S/N) and Lozenge (Lz) factors. The Friend-of-GATA homologue U-shaped (Ush) inhibits crystal cell development. Transcription factors that determine lamellocyte development have not yet been identified.

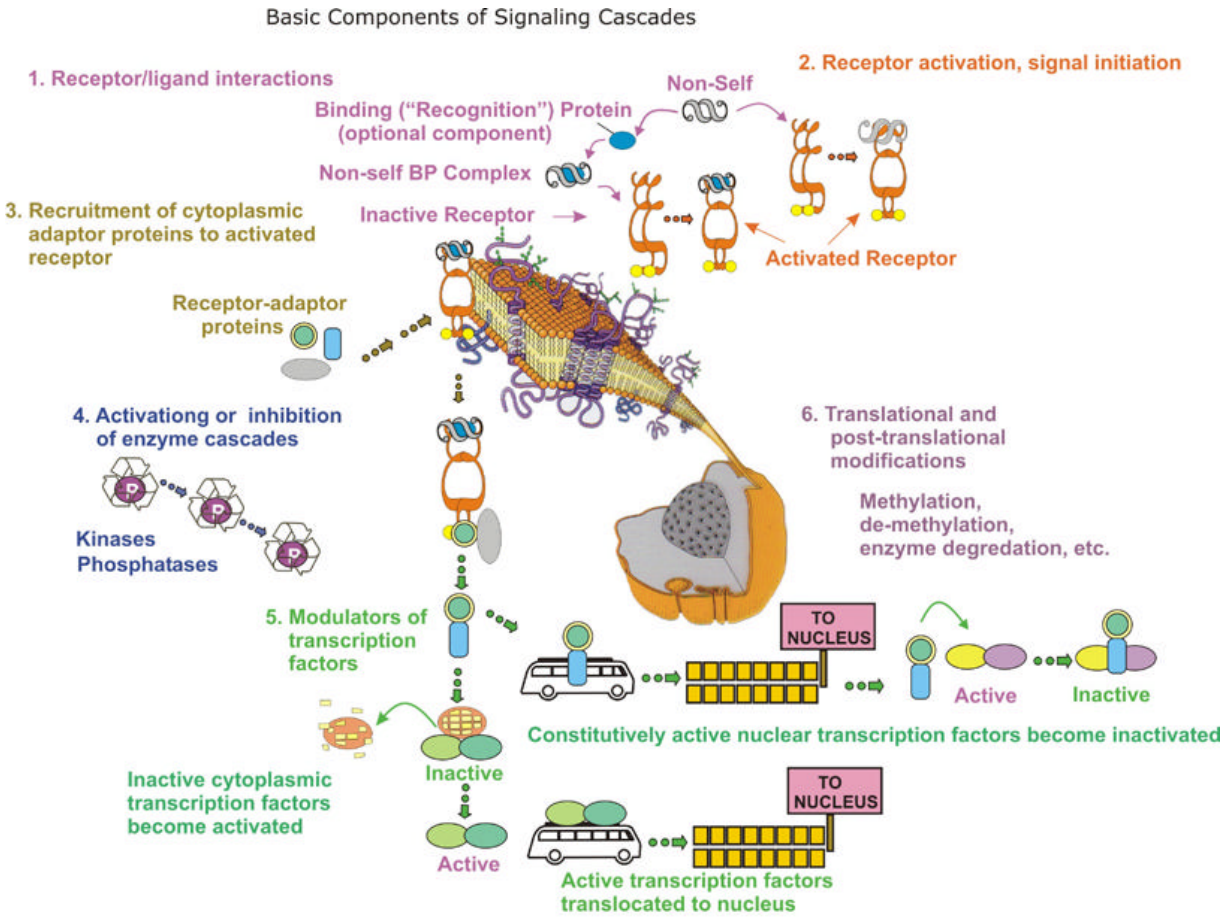

Fig. 6. Outline of some of the essential components of signaling pathways leading to innate immune responses. Hemocyte non-self recognition may involve plasma membrane receptors functioning independently, or in cooperative interactions with hemolymph binding ('recognition') proteins. Some pathways render constitutively active transcription factors inactive, while others promote the nuclear translocation of activated factors.

mediated principally by the Imd pathway (Lemaitre, 1999). However, pathogen specificity of these two pathways is uncertain. Although activated through significantly different mechanisms, these intracellular signaling pathways regulating antimicrobial gene expression in insects and mammals employ certain common components, suggesting an evolutionary link between these two groups (Hoffmann and Reichhart, 2002; Tzou *et al.*, 2002; Hoffmann, 2003).

At least three additional, distinct and evolutionarily conserved signaling pathways have been shown to be immune responsive in insects: c-Jun N-terminal kinase (JNK), the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway, and the Ras/Raf/mitogen activated protein kinase (MAPK) pathway. Although numerous components of these pathways have been shown to be involved in immune gene expression, less is known about the mechanisms that either repress these genes in the absence of infection or those that down regulate the genes following infection for restorative homeostasis. An outline of the interactions of these pathways with the Toll and Imd pathways is given in Fig. 7.

Toll and Imd pathways

The Toll pathway, which is responsive to infections by fungi and both Gram-positive and Gram–negative

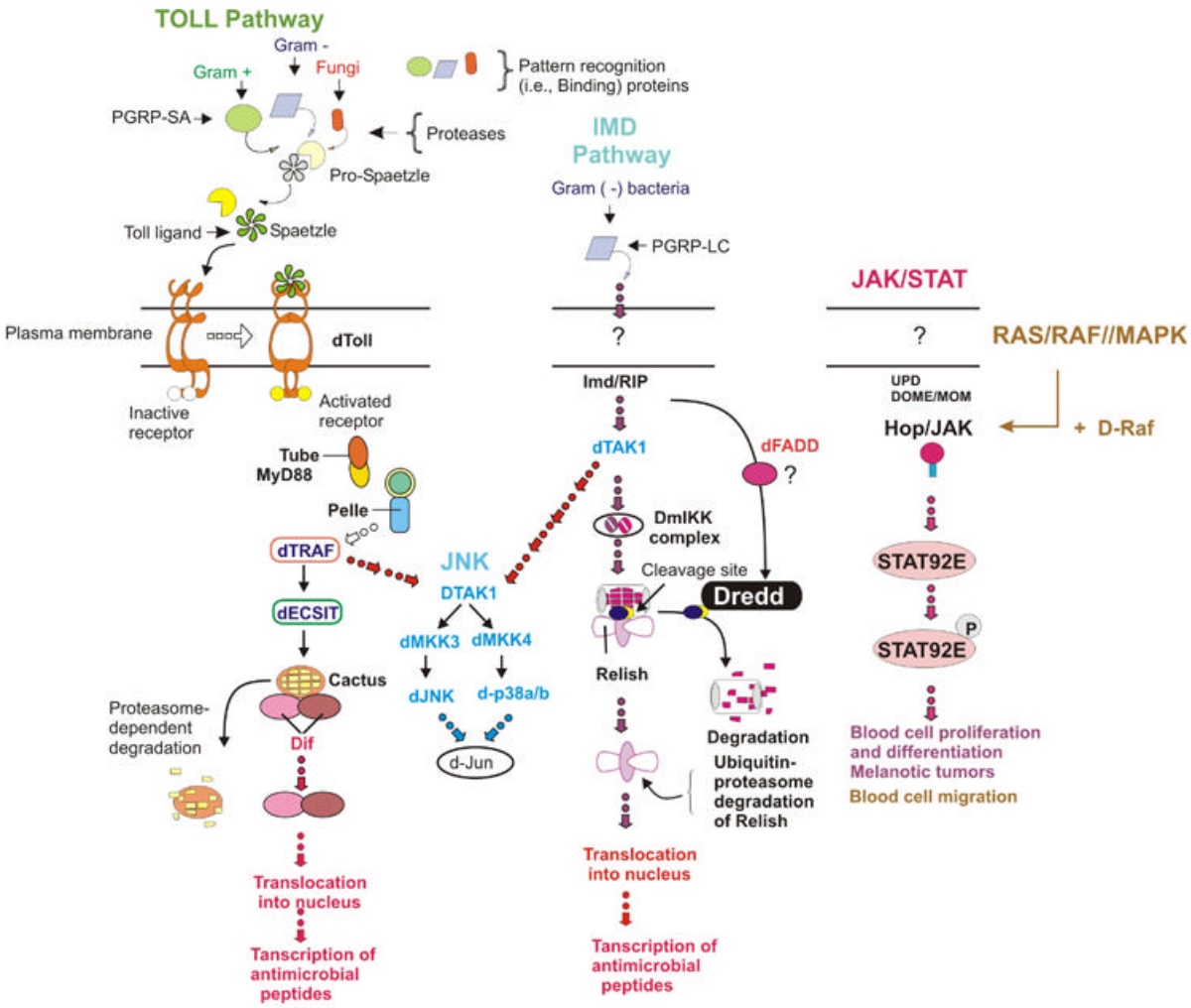

Fig. 7. Overview of the signaling pathways known to be involved in *Drosophila* humoral and cell-mediate innate immunity.

bacteria, leads to activation of two *Drosophila* NF-*K*B homologs, Dorsal and/or Dif (Dorsal-like immunity factor) (Bulet *et al*., 1999). The Imd pathway is responsive to Gram-negative bacteria or LPS treatment, and employs the *Drosophila* NF-*K*B homolog Relish (Lemaitre *et al.*, 1995; Lemaitre, 1999). Activation of the Toll signaling pathway is initiated with the cleavage of an extracellular protein termed pro-Spaetzle, which generates the active, cytokine-like polypeptide Spaetzle (Fig. 7), the ligand for the Toll transmembrane receptor. This cleavage is induced by a proteolytic cascade that is activated early during infection. Components of the intracellular region of the Toll receptor are similar to the intracellular region of the mammalian IL-1R, commonly referred to as the TIR domain (Imler and Hoffmann, 2000; Imler and Zheng, 2004). The cytoplasmic TIR domain of Toll interacts with three components to form a receptor-adaptor complex. Each component contains a death domain. Two of these molecules are adaptor proteins, the *Drosophila* homologue of MyD88, and Tube. The third component of the complex is Pelle, which possesses a serine-threonine kinase domain, and is homologous to mammalian interleukin-1 receptor-associated kinases (IRAKs). Toll receptor binding by Spaetzle engages the receptor-adaptor complex, which in turn activates a signaling cascade

that causes the degradation of the Inhibitory (I)-*K*B homolog, Cactus, and the ensuing nuclear translocation of the Rel proteins Dorsal and/or Dif. The dissociation of NF-*K*B homologs involves a signal-dependent phosphorylation of Cactus, followed by its proteasome-mediated degradation. The identity of the Cactus kinase is unknown. Toll activates Dif in adults, and Dorsal and/or Dif in larvae. Dorsal is also required during early embryogenesis for the Toll-dependent patterning of the dorsoventral axis.

It remains to be determined how bacterial infections cause the cleavage of pro-Spaetzle to Spaetzle, and the ensuing activation of Toll. There is some indication that activation of the Toll pathway may involve PGRPs or GNBPs, as mutants of two genes that encode some of these proteins, s*emmelweis* and *osiris*, manifest altered Toll signaling and immune responsiveness to infection by Gram-positive bacteria (Michel *et al.,* 2001; Hoffmann, 2003).

Several elements comprise the Imd pathway: dRIP, a death domain of the Imd protein that is similar to mammalian Receptor Interacting Protein, or RIP; Relish, a Rel protein related to mammalian P105 and P100 NF-*K*B precursors; Dredd, an apical caspase related to caspase-8 of mammals; dmIKK (a *Drosophila* inhibitor kappa-B kinase complex comprised of two elements, dmIKKâ and dmIKKã); dFADD, an adaptor protein that acts between Imd and Dredd; and dTAK1), a *Drosophila* homologue of TAK1 believed to be involved in the regulation of apoptosis and JNK signaling. dTAK1 is a MAPKKK that functions upstream of the dmIKK complex and downstream of Imd. The target of the Imd pathway is Relish, which, when cleaved, translocates into the nucleus. Like the mammalian NF-KB proteins, p100 and p105, Relish is composed of a DNA binding Rel-homology domain and an inhibitory ankyrin-repeat domain. The cleavage of Relish is dependent on both Dredd and the DmIKK complex. The peptidoglycan recognition protein PGRP-LC acts upstream of Imd, and probably functions as a receptor for the Imd pathway. The ligand for Imd is not known (Fig. 7).

JAK/STAT pathway

The Janus kinase (JAK)/signal transducers and activators of transcription (STATs) cascade (JAK/STAT) is an evolutionarily conserved, intracellular signaling pathway that plays a central role during hematopoeisis, cell movement, cell fate determination, and regulation of the cellular immune response (Zeidler *et al*., 2000; Hou *et* al., 2002). The outcome of JAK/STAT signaling in development depends on its collaborative interations with other signals and/or pathways, such as Wnt, the Ras/Raf/MAPK cassette, and those associated with cell cycle regulation. During cell migration, the JAK/STAT signaling pathway may connect to elements that regulate cytoskeletal modifications (Dearolf, 1999).

In vertebrates, the JAK/STAT pathway is activated by a large number of cytokines and growth factors. Cytokine receptors constitutively associate with JAKs. Cytokine binding induces conformational changes in its receptor, which leads to activation of the associated JAKs. Activated JAKs autophosphorylate and/or transphosphorylate, and then phosphorylate the cytokine receptors. The receptor tyrosine motifs phosphorylated by JAKs serve as docking sites for the SH2 domains of STATs. Upon binding, the STATs are activated by tyrosine phosphorylation and detach from the receptor. Subsequently they dimerize, translocate to the nucleus, bind to specific DNA elements in the promoter regions of genes, and activate transcription (Shuai, 2000; Decker *et al*., 2002; Hou *et al*., 2002; O'Shea *et al*., 2002; Shuai and Liu, 2003).

It is presently difficult to understand how can the same JAK/STAT pathway be used to produce completely different developmental outcomes in different tissues. Characterization of the genes that are regulated by JAK/STAT, as well as the identification of the interacting, combinatorial signaling pathways, are promising avenues toward understand how this pathway can be involved in such a wide array of functions (Dearolf, 1999; Zeidler *et al*., 2000; Shuai and Liu, 2003).

In addition to the JAKs and STATs, other regulators of the signaling pathway include PIAS, a protein inhibitor of activated STAT, SOCS, a suppressor of cytokine signaling, and STAM, a signal transducing adaptor molecule. PIAS proteins negatively regulate the JAK/STAT pathway by binding to, and inhibiting, STAT activity (Hari *et al*., 2001; Karsten *et al*., 2002).

In *Drosophila,* the JAK/STAT pathway homologue is comprised of four main components, Unpaired (UPD), which is a secreted glycoprotein that associates with the extracellular matrix, the receptor Domeless/Master of marelle (DOME/MOM), the JAK, Hopscotch (HOP), and the STAT, STAT92E (Agaisse and Perrimon, 2004) (Fig. 7). In addition to these four components, three classes of proteins that modulate JAK/STAT signal transduction in mammals have homologs in *Drosophila*; DPIAS, DSOCS, and STAM. DPIAS negatively regulates the HOP/STAT92E pathway and is required for blood cell and eye development (Betz *et al*., 2001). DSOCS suppress the activity of HOP/ STAT92E. DSTAM

protein lacks the ITAM domain that is used by the mammalian STAMs to bind JAKs (Dearolf, 1999).

There is some evidence that the *Drosophila* JAK/STAT signaling pathway controls blood cell proliferation. Two dominant temperature-sensitive mutations that hyperactivate HOP (hopTum-l and hopT42) lead to hemocyte-mediated melanotic tumor formation (Corwin and Hanratty, 1976; Hanratty and Dearolf, 1993; Harrison *et al*., 1995; Luo *et al*., 1995; Luo and Dearolf, 2001). The blood cell phenotype associated with these mutations indicates that hyperactivation of JAK/STAT activity enhances cell proliferation via the Ras/Raf/MAPK pathway. Interestingly, the differentiation of hemocytes and their aggregation to form melanotic tumors can be suppressed by a reduction in STAT92E activity (Dearolf, 1998). In addition, DPIAS activity is known to enhance melanotic tumor formation (Betz *et al*., 2001). Blood cell proliferation and differentiation may occur either in the hematopoietic lymph glands, or in circulation. Similar hemocytic responses have been observed in response to immune challenge (Walker, 1959; Nappi and Streams, 1969; Nappi, 1984, 1987; Nappi and Silvers, 1984; Nappi *et al.*, 1984; Rizki and Rizki, 1982, 1994; Nappi and Carton, 1986). The succession of cellular events initiated in parasitized larvae may be initiated by signals originating from circulating hemocytes upon contact with foreign surfaces in the hemocoel, and the transmission of this information to induce the hemocyte differentiation in the lymph gland (Lanot *et al*., 2001; Nappi *et al*., 2004). The JAK/STAT-dependent differentiation of blood cells in the lymph glands in response to either the development of aberrent tissues or to parasitization implicates signaling molecules, but the nature of these substances remain to be determined.

JNK and ERK

Various studies of intracellular signals that regulate cell growth, differentiation, and stress responses have demonstrated that many kinds of signals culminate in the activation of mitogen-activated protein kinases (MAPKs). These enzymes are activated through phosphorylation by MAPK kinases (MAPKKs), and mediate various signaling inputs into transcription factors. Three subgroups of the MAPK superfamily have been identified: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) or stress-activated protein kinase, and p38 (or Mpk2). The ERK cascade plays a central role in the transduction of mitogenic signals, employing a specific MAPK/ERK kinase (MEK). Both JNK and p38 are activated in response to various stresses and inflammatory cytokines.

Homologues of all three subgroups of vertebrate MAPKs have been identified in *Drosophila melanogaster*: DSOR1 (Downstream suppressor of Raf-1) is the *Drosophila* homolog of MEK. It has been shown to repress the apoptotic protein Hid (Head involution defective), and thus to function as a survival factor. DJNK, a homologue of JNK, is a mediator of cell morphogenesis and cell polarity signaling, as well as a stress-signaling transducer. D-p38a and D-p38b are *Drosophila* homologs of p38 (Hou *et al*., 2002). They have been reported to modulate signal transduction from a transforming growth factor, inhibit the production of antimicrobial peptides, and to transduce stress signals.

The triggering of the JNK in response to microbial challenge appears to represent a branch of the Imd pathway. Induction of some genes by Imd and TAK requires JNK, while others are stimulated by the activation of a branch that uses IKK and Relish (Fig. 7). Interestingly, the transcriptional program activated by JNK signaling comprises genes that regulate cytoskeletal function, many of which are associated with wound healing processes (Ramet *et al*., 2002a). Indeed, correlative evidence is mounting implicating JNK-mediated signaling as a conserved pathway in which the cytoskeletal modifications it initiates function in both in wound repair and innate immunity (Takatsu *et al*., 2000; Mihaly *et al*., 2001; Park *et al*., 2004).

JNK activity is modulated by ceramide levels. Ceramide may be produced from sphingolipids via action of serine palmitoyltransferase (SPT) and ceramide synthase, or by the sphingomyelin hydrolytic pathway. SPT catalyzes the first step in the biosynthesis of sphingolipids, i.e., the condensation of serine and palmitoyl-CoA to yield 3-ketosphinganine. 3-Ketosphinganine is metabolically converted to ceramide and various other sphingolipids. Ceramide is also produced through the sphingomyelin pathway, which is initiated by hydrolysis of sphingomyelin. In dipterans, sphingomyelin is not present. However, an alternative sphingolipid species, ceramide phosphoethanolamine, is present and is presumed to be hydrolyzed to ceramide. A decrease in the rate of de novo sphingolipid synthesis via SPT removes repression of the DJNK cascade and elicits apoptosis. Ksr, a caspase activated protein kinase (CAPK) is known to be activated by ceramide (Fig. 13) The *Drosophila* death domain protein Rpr (Reaper) elicits apoptosis by causing an elevation in ceramide level (Abrams, 1999; Kuranaga *et al*., 2002; Chai *et al*., 2003).

Signaling pathways associated with hematopoiesis

Little is presently know of the signal transduction pathways that influence hemocyte progenitor cells to proliferate and differentiate precociously, and to attack and destroy pathogens and abnormally developing endogenous tissues. Implicated as mediators of the activity the transcriptional regulators governing hematopoiesis in *Drosophila* are the JAK/STAT (HOP/STAT92E) and Toll/Cactus (NF-*K*B) signal transduction pathways (Qiu *et al.*, 1998; Luo and Dearolf, 2001). The *Drosophila* Ras/Raf/MAP kinase pathway, with the homologue D-Raf, acting downstream of HOP, plays a role in melanotic tumor formation (Luo and Dearolf, 2001), influences the migration of blood cells from the lymph gland into the hemolymph, and contributes to the survival of circulating blood cells. Interestingly, the Ras/MAP kinase signal is not required for blood cell proliferation and differentiation in the lymph gland (Luo and Dearolf, 2001). Also, over expression of the HOP/STAT92E pathway leads to the formation of neoplastic, melanotic tumors, a process that involves the massive proliferation and precocious differentiation of blood cells or hemocytes. The blood cell changes may occur in the larval hematopoietic organ (lymph glands), and/or in circulation. These pathways mediate hyper-proliferation phenotypes that resemble leukemia in mammals (Dearolf, 1998; Harrison *et al.*, 1995; Luo *et al.*, 1995). Lamellocyte differentiation and aggregation, but not the proliferation of hemocyte progenitor cells, can be suppressed by a reduction in STAT92E activity (Dearolf, 1998). The *Drosophila* Ras/Raf/MAP kinase pathway also participates in hematopoiesis controling the migration of blood cells from the lymph gland into the hemolymph, but apparently is not required for blood cell proliferation and differentiation (Luo and Dearolf, 2001). In vertebrates, interferons intergrate early innate immune responses by inducing immediate transcriptional responses through the JAK/STAT pathway (Decker *et al.*, 2002).

Additional evidence indicating the involvement of the JAK/STAT signaling pathway in insect defense responses is its activation in response to septic injury. In the mosquito *Anopheles gambiae*, STAT activation has been observed in response to bacterial challenge, suggesting this cascade controls other genes encoding humoral factors (Barillas-Mury *et al.*, 1999). Also, the gene encoding the complement-like protein TEP1 appears to be a target for the JAK/STAT pathway in *Drosophila* (Lagueux *et al.*, 2000). Thus, understanding the activation and function of the JAK/ STAT and JNK pathways during pathogenesis promises to reveal important insights into innate immunity.

The Ets family of transcription factors regulates cell growth and differentiation, and the activities of many of its signaling intermediates are modulated through phosphorylation by the evolutionarily conserved Ras/mitogen-activated protein kinase (MAPK) cascade. In *Drosophila melanogaster*, cellular proliferative and differentiative responses are regulated by various homeostatic controls, including the Ets-domain transcription activator POINTED (PNT) and the transcriptional repressor YAN, both of which respond to receptor tyrosine kinase (RTK) signaling through GTPase Ras and the mitogen-activated protein kinase (MAPK) cascade. In unstimulated cells lacking appropriate RTK signaling, the constitutively active transcriptional repressor YAN is bound to DNA preventing transcription by the transcriptional activator POINTED2 (PNT). Upon appropriate RTK signaling, phosphorylated MAPK enters the nucleus and phosphorylates both YAN and PNT. This promotes the dissociation and nuclear export of YAN, and causes the transcriptional activator PNT to bind to the target genes freed from YAN suppression (Fig. 8). The RTK-initiated MAPK-mediated phosphorylation of YAN is facilitated by MAE (Modulator of the Activity of ETS), which plays a pivotal role, along with CRM1, in mediating the nuclear export of YAN. MAPK-phosphorylated PNT releases MAE, which forms a complex with MAPK-phosphorylated YAN. The YAN-MAE complex then associates with CRM1, which causes the release of MAE and the ensuing CRM1-mediated export of YAN from the nucleus (Baker *et al.*, 2001). Thus, in response to appropriate signaling by RTK, MAE modulates the levels of both the transcriptional activator PNT and the transcriptional repressor YAN, thereby fine-tuning the proliferative and differentiative responses. Misregulation of RTK signaling and/or activation of the Ets-domain transcription factors PNT and YAN cause neoplastic transformation in the larval blood cells leading to their infiltration and encapsulation of normal endogenous tissues. Neoplastic transformation in the larval blood cells results from augmented and sustained activation of the JAK/STAT (HOP/STAT92E) signaling pathway, as well as from mutations involving nucleosome remodeling factors (Badenhorst *et al.*, 2002)

Cytokines and hematopoiesis

The *Drosophila* homologue of the mammalian platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF) receptor, and the

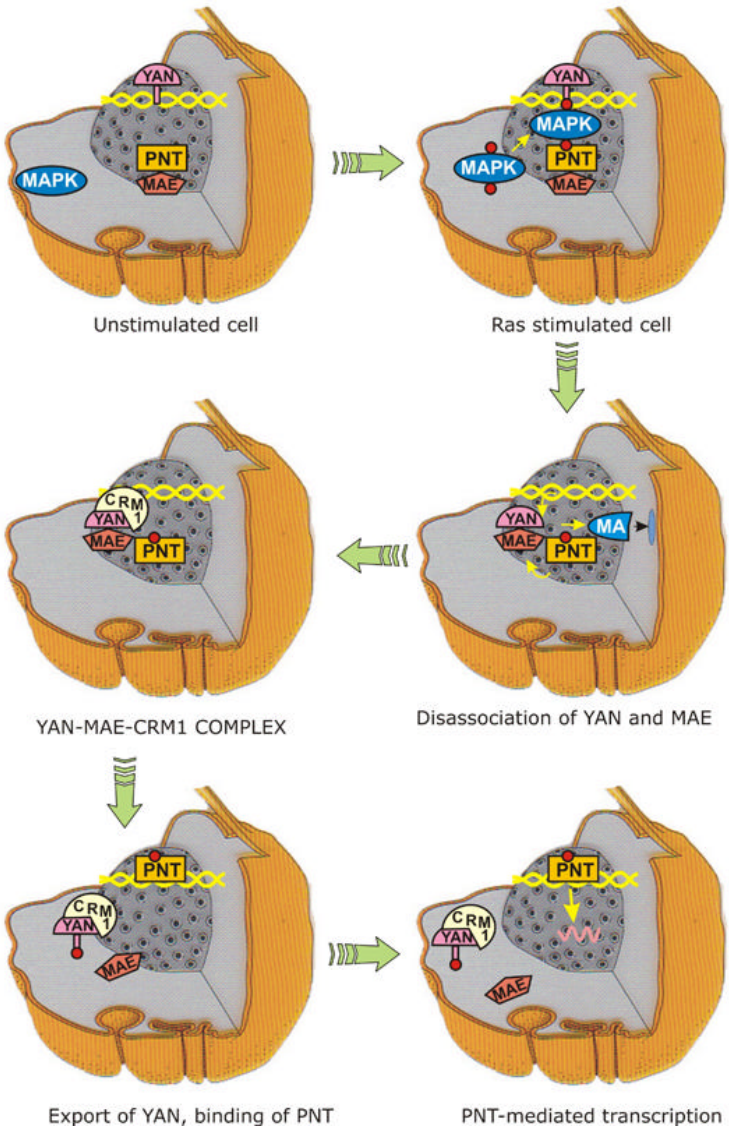

Fig. 8. The conserved Ras/MAPK cascade is an integral part of the processes of cell division, differentiation, movement and death. In *Drosophila*, MAE (modulator of the activity of Ets) is a signaling intermediate linking MAPK signaling to the nuclear export of the constitutively active transcriptional repressor Yan. This allows the transcriptional activator Pointed (PTS) to bind to DNA leading to transcription.

ligand designated PVF2, are believed to participate in the control of hemocyte proliferation (Heino *et al*., 2001; Munier *et al*., 2002). The *Drosophila* proteins, which appear to behave similarly to the active forms of mammalian PDGFs and VEGFs in transducing signals from specific cell surface receptors, have been shown to function in embryonic blood cell migration (Cho *et al*., 2002). Implicated in the PDGF/VEGF-mediated control of hemocyte proliferation and migration is Ras and the Raf/mitogen-activated protein kinase (MAPK) pathway (Asha *et al*., 2003), the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway, and the Toll pathway (Qiu *et al*., 1998).

Lipids and immune signaling

Unfortunately, little attention has been given to the role of lipids in insect immune signaling. This is

surprising, given that a diversity of eukaryotic signaling systems employ lipid and lipophilic molecules. Steroids, which typically act through nuclear receptors to directly affect transcription, can also signal through membrane receptors that activate receptor tyrosine kinases (RTK) and G-protein-coupled receptors (GPCR), which in turn activate second messengers and Protein kinase C (PKC). Depending on the specific pathway, PKC can activate either Ras-Mitogen-Activated Protein Kinase (Ras-MAPK) cascades, or second messenger systems (e.g., IP_3/Ca^{2+}/cAMP). PKCs and MAPKs have their cellular effects either by activating other protein kinases, or by transcriptional regulation via molecules like ERKs (Wheeler and Nijhout, 2004)

Prostaglandins belong to a class of lipids called eicosanoids, which are derived from arachidonic acid. In vertebrates, prostaglandins serve as critical mediators of inflammation. In insects and many other invertebrates, prostaglandins appear to be mainly involved in regulating the cellular immunological defense mechanisms and oviposition (Stanley-Samuelson, 1987; Stanley-Samuelson *et al.*, 1997; Stanley *et al*., 1998, 1999). Prostaglandins act primarily by binding to G-protein-coupled cell surface receptors and exert their effect through Ca^{2+} and cAMP second messenger signaling (Fig. 9).

Phospholipase A_2 (PLA_2) is responsible for releasing arachidonic acid from cellular phospholipids, a reaction that is envisioned to be the first step in the biosynthesis of eicosanoids. Eicosanoids have been shown to mediate cellular immune reactions against bacterial infections (Stanley and Howard, 1998; Miller *et al.*, 1999; Stanley *et al*., 1999; Tunaz *et al.*, 2003). PLA_2 occurs in the fat body and hemocytes, and is elevated in response to bacterial challenge (Tunaz *et al*., 2003). Specific inhibitors of either PLA_2, cyclooxygenase or lipoxygenase, significantly inhibit the induction of the immune genes in *Bombyx mori* (Morishima *et al*., 1997). Inhibition of PLA_2 signaling abrogated significantly the cellular melanotic encapsulation response of *D. melanogaster* larvae against the endoparasitic wasp *L. boulardi* (Carton *et al.*, 2002). Moreover, recent studies have demonstrated a relationship between *Drosophila* PLA_2 and lipopolysaccharide-activation of the Imd pathway in insect immunity (Yajima *et al*., 2003) (Fig. 10).

Apoptosis as an immune strategy

Normally, nonmalignant cells have a genetically determined lifespan regulated by temporally integrating homeostatic mechanisms that mediate both life- and death-promoting processes. Two main cell death pathways are available to terminal cells, apoptosis and oncosis, both of which progress into irreversible necrosis, the end state of cellular toxicity. Apoptosis is a series of processes by which aged, aberrant, or infected cells are systematically dismantled and packaged into small “apoptotic bodies” that are removed by phagocytosis. Typically, cell death by apoptosis is executed without accompanying inflammatory responses (Fadok *et al*., 1998b). The morphological changes that characterize apoptosis include cell shrinkage, phosphatidylserine externalization on the plasma membrane, DNA cleavage into base pair fragments, maintenance of ATP levels, blebbing of the plasma membrane, nuclear pyknosis, dissolution of the nucleolus, and extensive cytoplasmic vacuolation. Oncosis is characterized by cell or organelle swelling, and marked reduction of ATP levels (Majno and Joris, 1995; Levin *et al*., 1999).

The switch from cell viability to apoptosis-mediated death frequently involves a family of aspartic acid-specific proteases known as caspases that coordinate as well as execute cell death process. The substrate specificity and mechanism of activation of caspases provide a tight control on the activities of these enzymes, minimizing unregulated inadvertent, and potentially injurious responses (Waterhouse and Trapani, 2002). Caspases involved in apoptotic signaling typically function in a hierarchical manner, with initiator or apical caspases activating downstream effector or executioner caspases (Boatright and Salvesen, 2003; Salvesen and Abrams, 2004). Cells that do not execute the apoptotic death program either remain immortal, or they undergo oncosis, ultimately exhibiting necrotic morphology.

Humans express four initiator caspases (caspases 2, 8, 9, 10), three effector caspases (caspases 3, 6, 7), three cytokine activator caspases (caspases 1, 4, 7), and one (caspase 14) involved in keratinocyte differentiation. Initiator caspases contain caspase recruitment domains (CARD) or death effector domains (DED) that preceding their catalytic domains. Initiator caspases are the first components to initiate proteolytic activity and commit cells to death. Recruitment and activation of initiator caspases is achieved by adapter molecules that bind to death receptors via CARDs or DEDs. Executioner caspases 3, 6, and 7 are typically downstream proteins that lack the domains associated with initiator caspases (Salvesen and Abrams, 2004). It should be noted that the designation of caspases as initiator or executioner based on their substrates and domain profiles is not

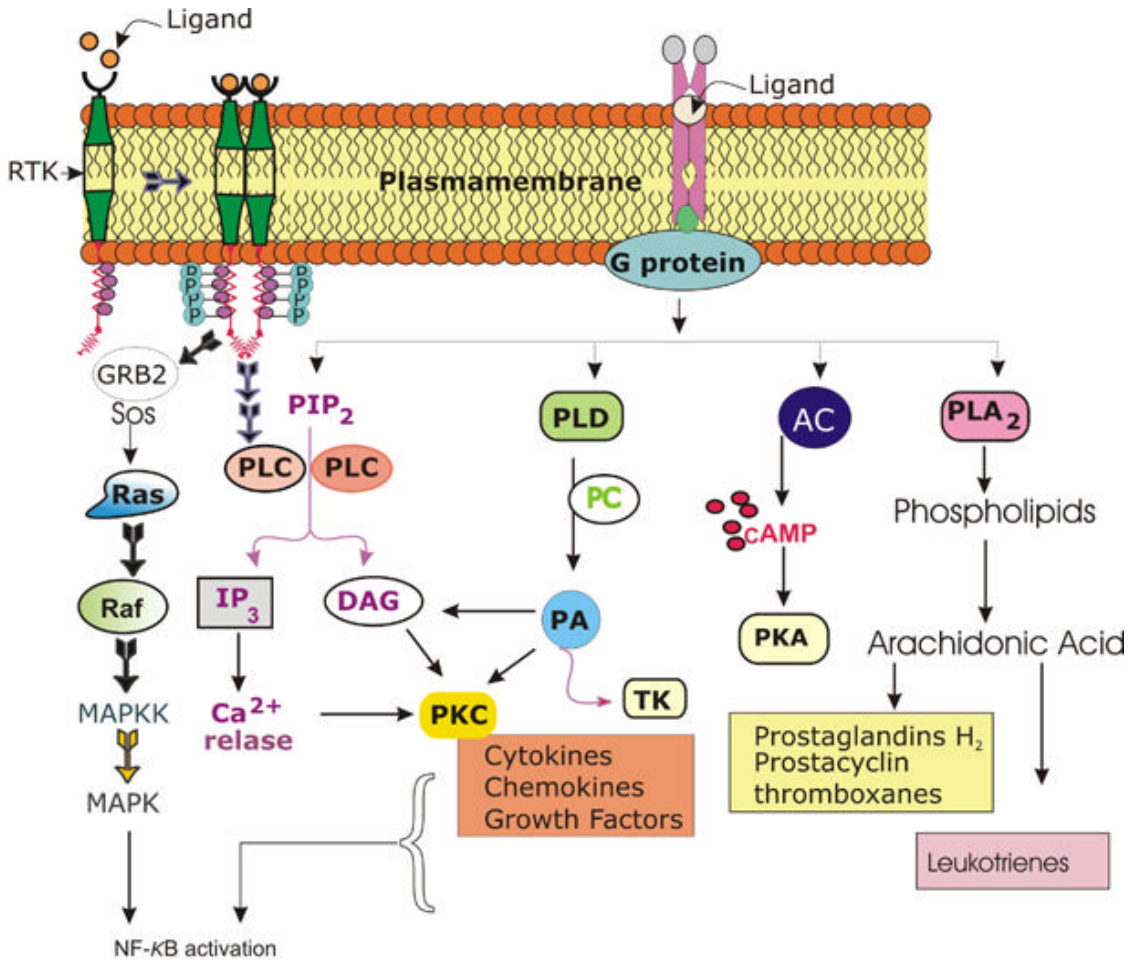

Fig. 9. Ligand binding induces conformational changes in receptors, which then initiate signal propagation. Receptor tyrosine kinase receptors (RTK) can initiate intracellular signals via the Ras/MAPK cascade, and by activating a specific protein kinase C (PCK). Upon ligand binding, tyrosine redidues become phosphorylated on the cytosolic tail. Proteins with SH2 domains, such as phospholiase C (PLC) and guanine-nucleotide release protein (GNRP), bind to the receptor. The binding of PKC results in its activation and cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG). Binding of GNRP activates Sos, a protein to which it is bound. Sos then activates the Ras protein, which initiates a cascade of reactions that results in the activation of transcription factors. The cytosolic domains of other receptors bind to and activate a G protein. The activated G protein dissociates from the receptor and transmits signals to one or more specific intracellular targets, including a specific PLC, phospholipase D (PLD), phospholipase A_2 (PLA_2) adenylyl cyclase (AC), or PIP_2. Effector molecules, such as cytokines and lipid derivatives, in turn initiate changes in specific transcription factors, including NF-*K*B. PA = phosphatidic acid; PC = phosphatidylcholine; PKA = protein kinase A; PKC = protein kinase C; RKR = receptor tyrosine kinase; TK = tyrosine kinase.

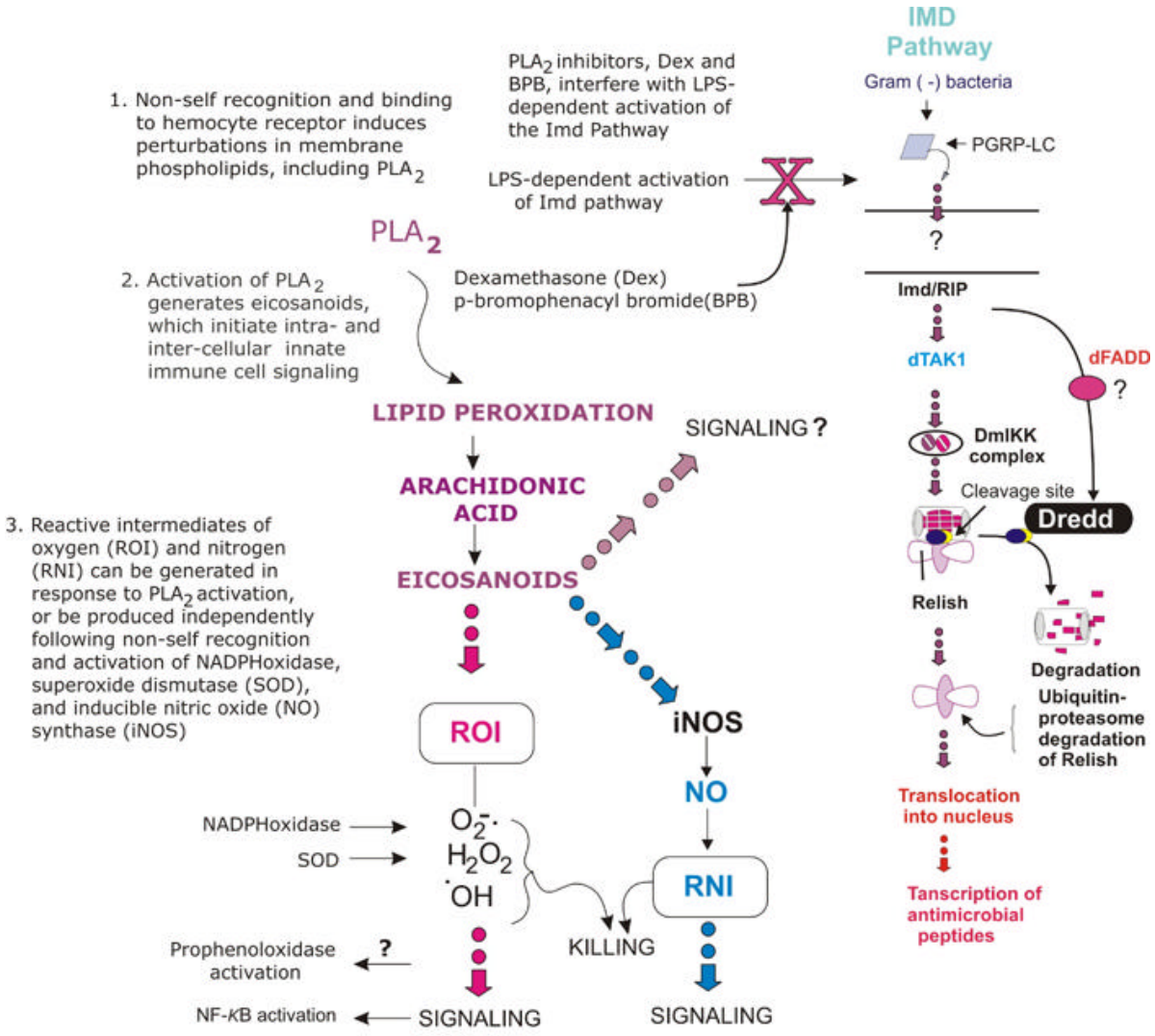

Fig. 10. Outline of the changes reported to occur following immune-induced perturbations in membrane phospholipids, and the involvement of PLA_2 or its derivatives, on the Imd pathway. The Imd pathways becomes unresponsive to LPS-activation following treatment with the PLA_2 inhibitors dexamethasone (dex) and p-bromophenacyl bromide (BPM), suggesting that alterations in membrane phospholipids play a role in insect innate immunity.

always exact, and challenges have been made to the application of these separate categories of caspases (Nicholson and Thornberry, 1997; Thornberry *et al*., 1997; Nicholson, 1999). It is likely that, depending on the origin and nature of the stimulus, and the type and developmental state of the cell, certain caspases can serve initiator or executioner functions, or both.

There is currently considerable interest in identifying the factors that regulate caspase-initiated apoptosis. It is still uncertain as to how these enzymes become activated in the numerous and diverse situations in which cells die. Many stimuli appear to promote caspase-mediated apoptosis in one of three ways: the apoptosome or mitochondrial pathway, the death receptor pathway, and by perforin-mediated trafficking of exocytosed cytotoxic granules from effector cells.

The mitochondrial pathway is triggered by cytosolic perturbations manifested in part by the loss of integrity of the mitochondrial membranes, and perhaps by

similar changes in the endoplasmic reticulum. Alterations in the mitochondrial transmembrane potential in response to such changes as heat shock, oxidative stress, and DNA damage, are known to cause the release of pro-apoptotic proteins, such as cytochrome c and apoptosis-inducing factor (AIF), alter cellular redox states, and promote the engagement of pro-apoptotic (e.g. Bax, Bad, Bak, Bid) and anti-apoptotic Bcl-2 family of proteins (e.g. Bcl-2, Mcl-1). The ratio between pro-and anti-apoptotic Bcl-2 family members determines, in part, the susceptibility of the cells to a death signal. Once released into the cytosol, cytochrome c interacts with Apaf-1 (apoptotic protease-activating factor-1) and ATP to form a complex (apoptosome) that converts procaspase 9 to active caspase 9, the initiator caspase that activates a downstream cascade of executioner caspases (Fig. 11). Endogenous inhibitors of apoptosis (IAPs), which render the caspases inactive, are negatively controlled by antagonists (e.g., Smac/Diablo) released from the mitochondrion by activated Bid.

The death receptor pathway mediating apoptosis in humans can be initiated either by death receptor ligation, a calcium-independent response, or via granule exocytosis, a calcium-dependent process. The binding of plasma membrane death receptors (e.g., TNFR, tumor necrosis factor receptor; Fas) with their specific death ligands (e.g., TNF; Fas ligand, FasL; Apo 3 ligand, Apo3L; TRAIL) recruits adapter proteins (e.g., FADD, Fas associated death domain; TRADD, TNF receptor-associated death domain; RIP, receptor interacting protein) and the initiator procaspase 8 to form a death-inducing signal complex (DISC). Caspase 8 can activate downstream executioner caspases directly, or by initially cleaving the pro-apoptotic protein Bid (Fig. 11). The cleaved Bid (tBid) is translocated into the mitochondrial membrane and promotes the release of cytochrome c. In the cytosol, the released cytochrome c associates with ATP, Apaf-1, and procaspase 9 to form a complex defined as the apoptosome, which activates downstream caspases. Ensuing changes include the systematic degradation of nuclear and cytoplasmic components, and the formation of apoptotic bodies that are removed by phagocytic cells.

The third pathway leading to caspase-mediated apoptosis is initiated by cytotoxic granules released from effector cells, such as cytotoxic T-lymphocytes (CTL) and natural killer (NK) cells, upon their encounters with rogue cells or those harboring pathogens (Fig. 12). NK cells constitutively possess cytolytic capacity, and in response to virus-infected cells secrete cytokines (e.g., IFN-ã and TNF-α) that limit viral replication and spread. Effector CTL possess receptors that manifest exquisite specificity, enabling them to specifically target infected cells that express viral peptides in association with appropriate surface MHC class I molecules. Cytotoxic T- lymphocytes can also release perforin, which forms transmembrane pores in target cells through which exocytosed granzymes attack specific intracellular components, such as Bid, to induce apoptosis through mitochondrial perturbation. Granzyme B can also activate caspases directly through the Fas-mediated death receptor pathway. Thus, the initiation of cell death by perforin-mediated trafficking of granzymes from cytotoxic T-lymphocytes into target cells represents an effective mechanism to ensure the apoptotic death of transformed cells (Hirst *et al.*, 2003; Trapani and Sutton, 2003), as well as cells harboring pathogens.

Following the activation of apical caspases, subsequent activation of downstream caspases initiate the orderly disassembly of cellular constituents and the formation of apoptotic bodies that are eventually removed by phagocytes. In many cell death programs, the specific sequence of caspase activation (i.e., caspase cascade), and the relative contributions of each of the participating caspases, are either partially defined or undetermined. Perhaps the most consistant data available to date suggests that the specific activating process in the mitochonrial pathway occurs within the Apaf-1 containing apoptosome, and that the cytochrome c/Apaf-1-initiated cascade is activated solely by caspase 9. In the death receptor pathway, activation occurs within the DISC complex, and caspase 8 is the sole apical caspase component of the complex. The interactions of various caspases with signaling systems that have been associated with apoptosis and cellular innate immunity are presented in Fig. 13.

Caspase-mediated apoptotic responses similar to those described in humans have been documented in *Drosophila* (Abrams, 1999; Bangs and White, 2000; Bangs *et al.*, 2000; Rodriguez *et al*., 2002). Some of the elements of the *Drosophila* apoptotic machinery are presented in Fig. 14. The *Drosophila* genome encodes seven caspases, two of which, Dronc and Dredd, are homologues to initiator caspases 9 and 8, respectively (Kumar and Doumanis, 2000; Kumar and Cakouros, 2004). Four *Drosophila* caspases (Dcp1, Drice, Decay, Damm) resemble effector caspases. The seventh caspase, Strica, bears no apparent homologies (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999, 2002; Zhou *et al.*, 1999). As occurs with caspase 9, the activity of Dronc might require the formation of a holoenzyme complex involving intimate associations

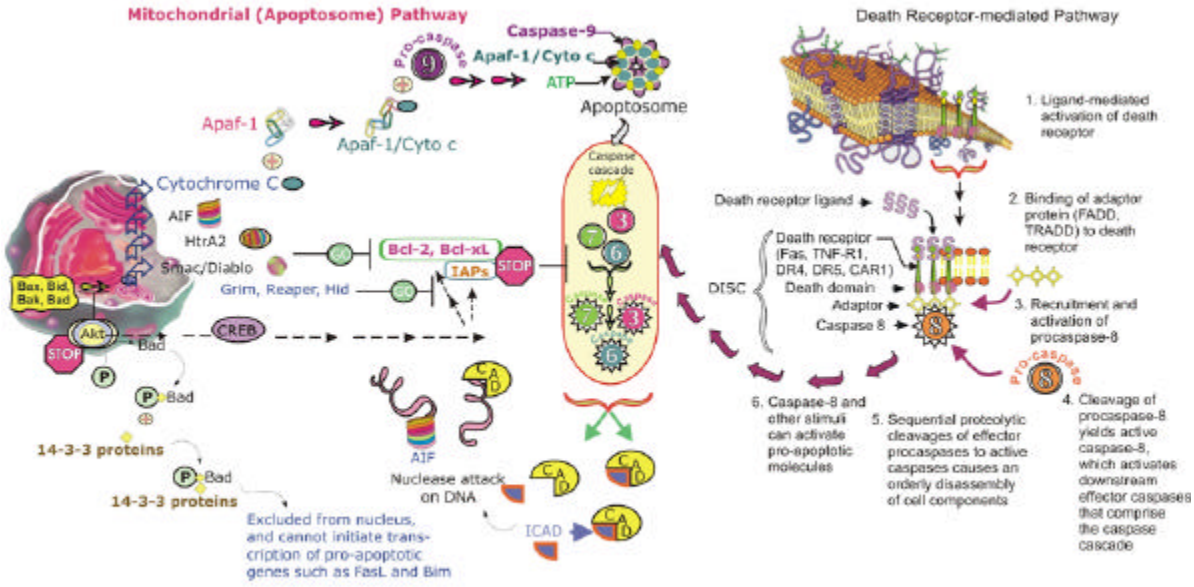

Fig. 11. Pathways of caspase-mediated apoptosis. A) The mitochondrial apoptotic pathway in humans involves caspase 9 as the initiating caspase, with upstream regulating processes mediated by members of the Bcl-2 family and Apaf-1. The signals are then transduced from caspase 9 to activate caspase 3 and other downstream executioner caspases (e.g., caspases 6 and 7), which in turn cleave specific substrates resulting in cell death. Inhibitors of apoptosis (IAPs), which render the caspases inactive, are negatively regulated in humans by the antagonists SMAC/Diablo and HtrA2, which are also released from the mitochondria by activated Bid. In *Drosophila*, Dronc and Dark are homologues of caspase 9 and Apaf-1, respectively. The specific antagonists that negatively regulate *Drosophila* IAP-1 (DIAP1) are Reaper, Hid, and Grim. Three additional *Drosophila* caspase inhibitors include DIAP2, dBruce, and Deterin. (B) The extrinsic or receptor mediated pathway is initiated with the binding of the death receptor Fas to its ligand FasL. This recruits the adaptor protein FADD (Fas associated death domain) and procaspase 8, which assemble to form a death-inducing signal complex (DISC). Within the complex, FADD forms a critical link with caspase 8 involving DED domains. This complex leads to the activation of caspase 8, which in turn can directly activate caspase 3 and other downstream effector caspases (e.g., caspases 6 and 7). Caspase 8 can also cleave Bid in some cells, which in tandem with Bax and Bak trigger cytochrome c release from the mitochondrion, assembly of the apoptosome (cytochrome c, Apaf-1, dATP, procaspase 9) and subsequent activation of caspase 9. Dredd is the *Drosophila* homologue of caspase 8, and the ortholog of FADD (DFADD), similarly binds to and regulates this initiator caspase.

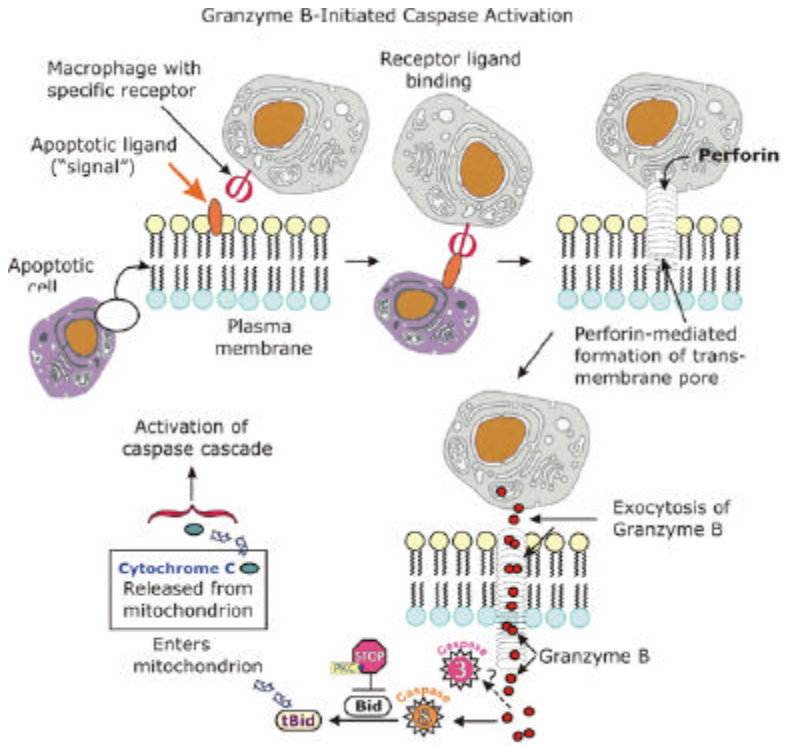

Fig. 12. Perforin-mediated trafficking of cytotoxic granules (e.g., Granzyme B) follows the binding of a phagocytic macrophage to an apoptotic ligand expressed on the surface of an apoptotic cell. Caspase 8 activated by this event engages Bid, and a cleaved component (tBid) enters the mitochondrion to initiate the release of cytochrome c.

with Dark, the *Drosophila* ortholog of the apoptosis protease-activating factor Apaf-1 (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999; Zhou *et al.*, 1999). Dredd appears to play a limited role in apoptotic signals, but it is known to be an essential element in transducing signals in the Imd pathway in response to certain microbial challenges, possibly by cleaving the NF-*K*B protein Relish (Stoven *et al.*, 2000, 2003). *Drosophila* Decay resembles caspases 3 and 7 (Dorstyn *et al.*, 2000), while Damm is most closely aligned with caspase 6 (Harvey *et al.*, 2001). Dcp1 and Drice resemble caspase 3 (Fraser *et al.*, 1996; Fraser and Evan, 1997; Fraser *et al.*, 1997; Song and Steller, 1999; Song *et al.*, 2000). While the sequential order of the *Drosophila* caspases remains to be elucidated, the data presently available suggest that Dronc is a critical initiator caspase (Meier *et al.*, 2000; Muro *et al.*, 2002; Yu *et al.,* 2002). *Drosophila* IAPs include DIAP-1, DIAP-2, dBruce, and Deterin. DIAP-1 binds to and thereby inhibits the action of the effector caspases, Dcp1 and Drice, and the initiator caspase. Reaper proteins, which include Rpr, Hid, Grim, and Skl, Dronc (Kaiser *et al.*, 1998; Wang, *et al.*, 1999; Meier *et al.*, 2000) serve as antagonists by derepressing IAPs, a process that may liberate caspases from their inhibitors (Meier and Evan, 1998; Goyal *et al.*, 2000; Hay, 2000; Lisi *et al.*, 2000; Meier *et al.*, 2000; Wu *et al.*, 2001; Ryoo *et al.*, 2002; Chai *et al.*, 2003; Ditzel *et al.*, 2003).

Phagocytic hemocytes possess receptors that respond to unique signals manifested on, or released from, apoptotic cells. The specific receptors on the phagocytic cell tether the apoptotic cell and initiate intracellular signaling pathways that lead to engulfment (Aderem and Underhill, 1999; Fadok and Chimini, 2001; Krieser and White, 2002). Similar cell-cell recognition processes, if not similar signaling molecules also, are likely to be essential features of effective cell-mediated immune and autoimmune defenses. Plasma membrane proteins implicated in mammalian systems as phagocyte receptors involved in apoptotic cell

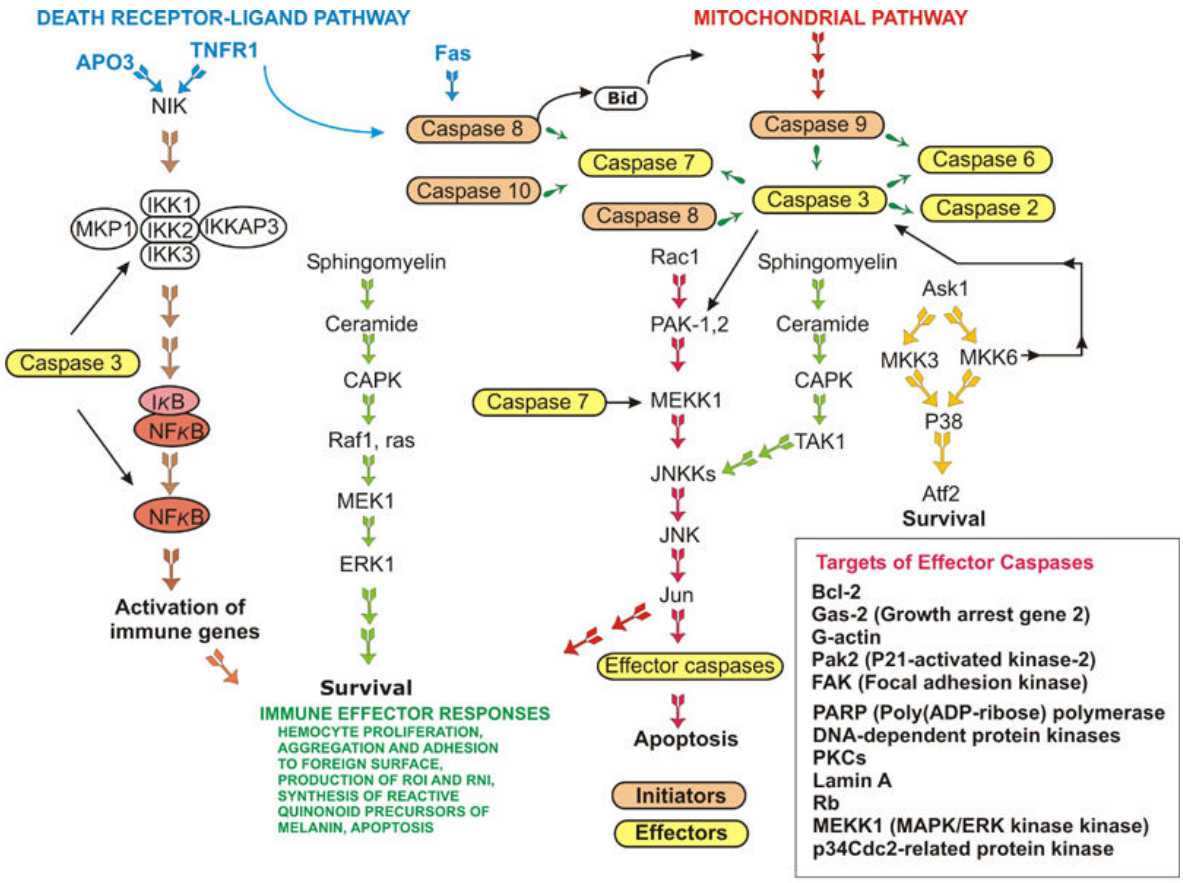

Fig. 13. Diagram illustrating possible caspase activation routes, and how these might integrate with signaling pathways that have been implicated in the modulation of apoptosis and innate immunity. Death receptor ligation promotes the recruitment and activation of caspase 8 within the DISC complex, whereas the mitochondrial pathway initiates activation of caspase 9, the sole apical protease in the cytochrome C/Apaf-1-mediated pathway. It should be noted that caspases cannot be categorized as exclusively initiator or effector, since certain caspases can serve dual roles under varied apoptotic stimuli.

recognition include the receptor tyrosine kinase Mer, $\alpha_V\beta_3$ integrin, CD14, CD91, CD36, and phosphatidylserine (PS) receptor. Mer is bridged to PS on the apoptotic cell surface by the adaptor Gas6. CD91 interacts with calreticulin, which is bridged to the complement pathway component C1q and the mannose binding lectin. CD14 on phagocytes binds to ICAM3 on apoptotic cells. $\alpha_V\beta_3$ integrin binds to the secreted milk fat globule-EGF-factor 8 protein (MFGE-8), which binds to PS on apoptotic cells. $\alpha_V\beta_3$ integrin is also functions in conjunction with CD36, which is bridged to apoptotic cells through thrombospondin. One modification in apoptotic cells that appears to be essential for their recogniton by phagocytes is the translocation of PS from the inner to the outer leaflet of the plasma membrane (Fig. 15) (Fadok *et al.*, 1998a; Fadok *et al.*, 2000; Fadok *et al.*, 2001a, b). A PS-specific receptor has been identified and found to be functional in phagocytosis of apoptotic cells. Human macrophages that use a PS receptor also require CD-36 for phagocytosis of apoptotic cells (Fadok *et al.*, 1998c)

Unfortunately, little is known of the receptors on *Drosophila* hemocytes that may serve in the recognition of apoptotic cells. As occurs in human apoptotic signaling, PS is also found on the surface of apoptotic cells in *Drosophila*. In humans, the phospholipid serves as a marker for phagocytosis, with recognition being made by PS-specific receptors. *Drosophila* Croquemort, the homologue of the human CD-36 receptor, has been implicated in the blood cell-mediated phagocytosis of

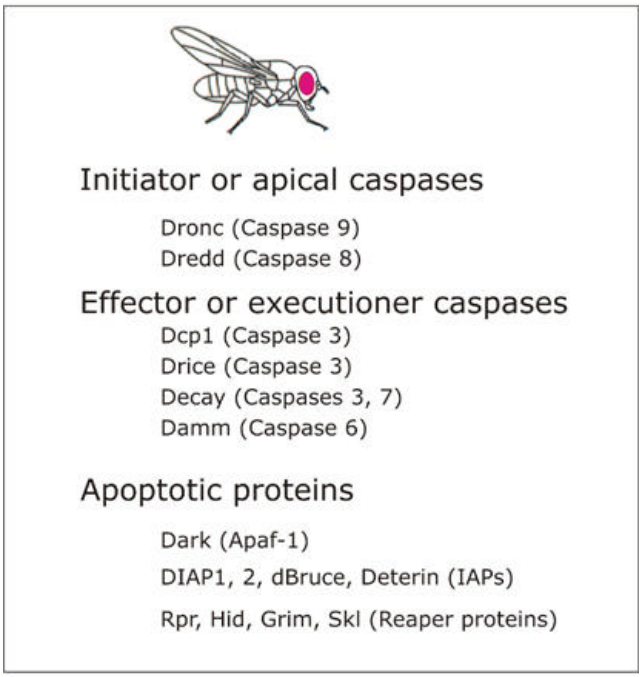

Fig. 14. Principal components in the *Drosophila* apoptotic machinery. Proposed mammalian homologues appear in parentheses.

apoptotic cells (Franc *et al*., 1996, 1999). It remains to be established, however, if Croquemort functions as a PS receptor. In humans, PS on apoptotic cells is believed to signal through Rac-1 or Cdc-42 to induce membrane changes required for engulfment (Krieser and White, 2002).

From an evolutionary perspective, apoptosis may represent the oldest energy-dependent cytotoxic response, initially involved in embryogenesis, then in protecting against rouge cells, and later in combating intracellular pathogens. It seems reasonable to assume that, as a counter strategy, intracellular pathogens evolved ways to circumvent host apoptotic responses, or at least delay them until the pathogen no longer requires the host cell to develop. It would be interesting to learn if the apoptotic machinery of insects is employed in the hemocyte-mediated melanotic encapsulation responses associated with melanotic tumors and the encapsulation of metazoan parasites. Of equal interest is to ascertain if virulent parasites possess mechanisms to suppress such host responses (Waterhouse and Trapani, 2002; Sutton *et al*., 2003; Trapani and Sutton, 2003). Among the many signaling pathways that respond to cell- death promoting stresses are the mitogen-activated protein kinase (MAPK) family members (Wada and Penninger, 2004). MAPKs are serine/threonine kinases that transduce signals from the cell membrane to the nucleus in response to a wide range of stimuli (e.g., including DNA damage, heat shock, ischemia, inflammatory cytokines, ceramide, and oxidative stress), and initiate compensatory changes in such processes as cell proliferation, motility, metabolism, and apoptosis. MAPKs family members include the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), and the p38-MAPKs. Both pro- and anti-apoptotic effects have been attributed to these pathways, and it appears the differing roles they manifest may depend on the cell type, the experimental conditions imposed on the cells, and the involvement of other interacting signaling systems. An outline of some signaling systems likely to interact in modulating caspase-dependent apoptosis is given in Fig. 13. Given the importance of these pathways in determining cell survival or apoptosis, it would be of considerable interest to investigate if the pathways regulating these processes represent likely targets for immune

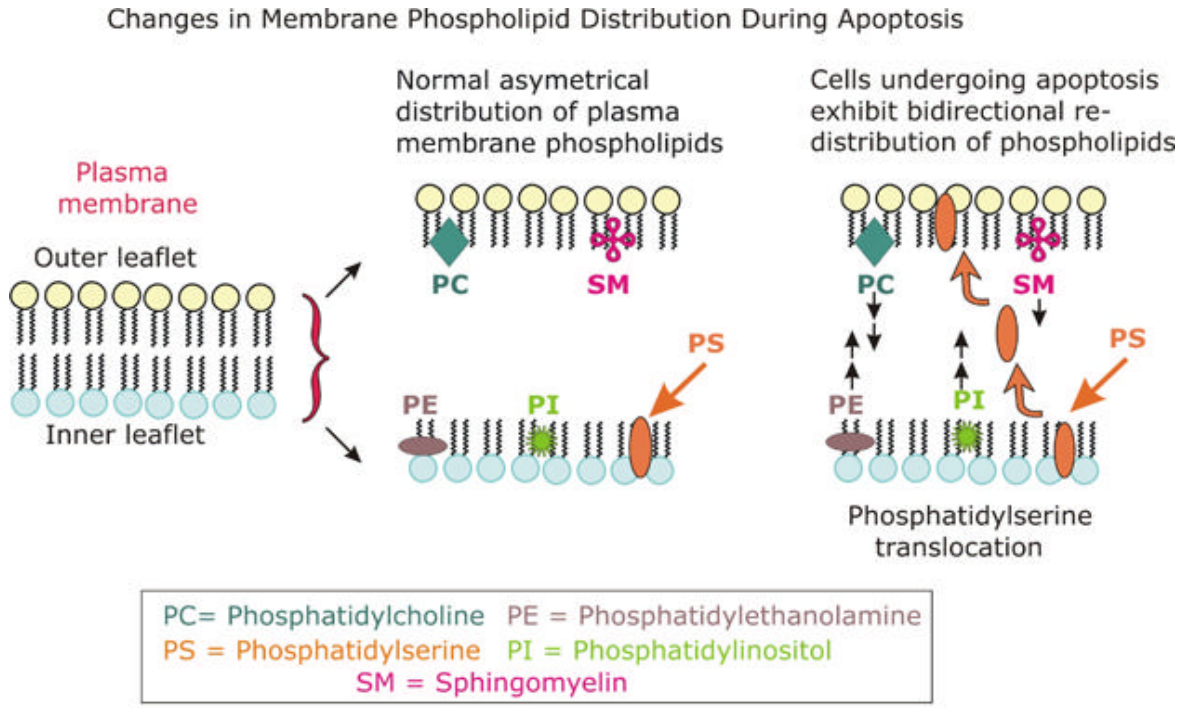

Fig. 15. Diagram summarizing the bidirectional transmembrane transfer of phospholipids during apoptosis in some cells. The translocation of PS to the outer leaflet of the plasma membrane serves as a signal for binding by phagocytic cells armed with a receptor for PS.

suppression by pathogens and parasites.

While there is ample justification to vigorously pursue efforts to understand how the caspase-mediated apoptotic machinery recognizes and eliminates aberrant cells and cells infected with pathogens, it may be instructive to consider these mechanisms as having evolved initially to serve developmental pathways. This would account for the observation that not all procaspase activation results in cell death. Moreover, caspase-independent cell-death processes have been described, and these are also likely to represent important elements in the defense strategy of a host (Abraham and Shaham, 2004).

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