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REVIEW

Insect immunity and its signalling: an overview

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Abstract

The innate immunity is the immediate and sole response of invertebrates for the protection against foreign substances and pathogens. In insects, it relies on both humoral and cellular responses that are mediated via certain recognizing receptors and activation of several signalling pathways. Fat body and hemocytes are the origins for the production and secretion of antimicrobial agents and activators/regulators of cellular response, while cell mediated immunity in insects is performed by hemocytes. In the last years, research has focused on the mechanisms of microbial recognition and activation of intracellular signalling molecules in response to invaders. In this review, we summarize the mechanisms of the innate immunity in insects and refer to potential interactions between humoral and cellular responses, combined with the involving signalling pathways and their cross talk.

Key Words: insects; innate immunity; signalling pathways

Introduction

Living creatures are surrounded by a basically hostile environment. In order to survive, they have developed several defense mechanisms, including the immune system. These mechanisms protect organisms against foreign substances and pathogen invasion. In case of such an invasion, the first line of defense is available immediately and involves mechanisms, either humoral or cellular, that are non specific. The discrimination between humoral and cellular responses is, up to a point, arbitrary, since they all share same signalling pathway that are activated by different stimuli (Lavine and Strand, 2002; Marmaras and Lampropoulou, 2009).These mechanisms are embraced under the term innate immunity, which is the sole immune response in invertebrates. Vertebrates have developed a second line of defense, the acquired immunity, which is highly specific and contains mechanisms targeting to a particular threat, each time.

Insects, the most widespread metazoans on Earth, have a well-developed innate immune system that allows general and rapid responses to infectious agents while they lack an acquired immune system. The protection against pathogens begins primarily with certain barriers such as cuticle, gut and trachea, tissues that are difficult to be penetrated, while immune response is originated by the fat body and the hemocytes.

Fat body is the largest organ of the hemocel, the insect body cavity, and is a major site for the production and secretion of antimicrobial peptides (Hoffmann, 2003). Hemocytes circulate in insect hemolymph. They derive from stem cells that differentiate into specific lineages. However, certain hemocyte types are not common in all insects and differ among species (Charalambidis *et al*., 1995; Meister and Lagueux, 2003).

The humoral immune response is based on the products of characterized immune genes induced by microbial infection and encode antimicrobial peptides, which are synthesized predominantly in fat body and released into hemolymph (Hoffmann, 1995; Gillespie *et al*., 1997; Nakatogawa *et al*., 2009; Shia *et al*., 2009). Hemocytes and epithelial layers of the integument and the gut are also sites for the synthesis of such molecules. These genes are either not expressed or are constitutively expressed at a low rate prior to infection (Hoffmann, 1995; Engström, 1998). In addition, humoral immune responses include activation of enzymic cascades that regulate coagulation and melanization of hemolymph, and production of reactive oxygen and nitrogen species (ROS-RNS) (Gilespie *et al*., 1997; Bogdan *et al*., 2000; Nappi and Vass, 2001; Hoffmann, 2003; Mavrouli *et al*., 2005).

Cellular responses are performed by hemocytes and include phagocytosis, nodulation and encapsulation (Schmidt *et al*., 2001; Nappi *et al*., 2004; Lamprou *et al*., 2005; Mavrouli *et al*., 2005; Sideri *et al*., 2007).

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There are a lot of important review papers in the literature which present, in details, specific groups of signalling pathways or mechanisms of innate immunity in insects. This paper is an overview of these mechanisms, describing, in general, humoral and cellular responses along with major signalling transduction pathways, emphasizing on their cross talk.

Origins of innate immunity in insects

Fat body

The larval fat body is the major site of the intermediate metabolism of insects and plays functions analogous to those of the vertebrate liver. It consists of thin layers or strings, generally one or two cells thick, or small nodules suspended in the hemocel and distributed throughout insect body (Roma *et al*., 2010). The majority of proteins of the hemolymph are synthesized in this tissue, which also serves as lipid, carbohydrate and protein storage. The fat body is a target tissue for all principal insect hormones such as neural hormones, juvenile hormone and ecdysone (Keeley, 1985) and is also a site of response to microbial infection. Characterized immune genes, in the fat body, are induced by microbial infection and encode antimicrobial peptides which are then released into the hemolymph (Hoffmann, 1995; Engström, 1998). In *Drosophila*, seven antibacterial peptides have been characterised namely, cecropin, attacin, defensin, drosocin, diptericin, metchnikowin and also an antifungal peptide called drosomycin (Lemaitre and Hoffmann, 2007). In addition, Lepidopreran fat body synthesizes and releases several other proteins, such as pattern recognition protein hemolin and two immulectins serine proteinases: prophenoloxidase activating proteinase and a serine proteinase inhibitor from the serpin family (Zhu *et al*., 2003).

Hemocytes

In insects, there are no blood vessels. Blood and interstitial fluid are indistinguishable and are collectively referred as hemolymph which bathes all internal tissues, organs and hemocytes, and facilitates the transport of nutrients, waste products and metabolites. The most common types of circulating hemocytes, in the hemolymph of lepidoptera (*Manduca sexta*, *Bombyx mori*) and diptera (*Drosophila melanogaster*, *Ceratitis capitata*) are granulocytes and plasmatocytes (Lavine and Strand, 2002; Kanost *et al.*, 2004). However, these haemocyte types are not common in all insect species (Lavine and Strand 2002; Meister and Lagueux, 2003; Lamprou *et al*., 2007). In addition, the terminology used to designate each haemocyte type is often different from one insect species to another (Ribeiro and Brehelin, 2006), although, there have functional similarities among different insect species.

In *Drosophila*, plasmatocytes are professional phagocytes and are the equivalent of mammalian cells from the monocyte/macrophage lineage. Phagocytosis permits rapid removal of dead cells, during embryogenesis and metamorphosis and pathogens during infections. Plasmatocytes also synthesize and secrete antimicrobial peptides and signal to the larval fat body, the functional equivalent of the mammalian liver, in response to an infection (Agaisse *et al*., 2003). Based on morphological criteria, hemocyte types similar to *Drosophila* have been recognised and classified in *C. capitata* larvae, although they may differ significantly in function. Medfly plasmatocytes, besides phagocytic activity, are involved in nodule formation and melanization, as they contain the precursors of the prophenoloxidase cascade (Mavrouli *et al*., 2005; Sideri *et al*., 2007; Marmaras and Lampropoulou, 2009).

Pattern recognition proteins/receptors

The first step for the initiation of immune response, either humoral or cellular, is the recognition of the pathogen. This is achieved by the pattern recognition proteins/receptors (PRPs), that recognize and bind conserved domains (patterns) located on the pathogen surface, which are called pathogen-associated molecular patterns, (PAMPs) (Medzhitov and Janeway, 1997) The most characterized PRPs are the type C lectins, the peptidoglycan recognizing proteins, the β-1,3-glucan proteins, the hemolin and the integrins (Bettencourt *et al*., 1997; Michel *et al*., 2001; Bettencourt *et al*., 2004). These proteins are present on the plasma membrane of fat body cells and hemocytes or they are soluble in the hemolymph. They bind on lipids and carbohydrates which are synthesized by microorganisms and are exposed on their surface, such as lipopolysaccharites (LPS) of Gram negative bacteria, lipoteichoic acids and peptidoglycans of Gram positive bacteria and β-1,3-glucans of fungi (Nappi *et al*., 2000). Insights on the characterization of PRPs have been obtained mainly from studies in *Drosophila*. Certain hemocyte protein recognition receptors appear to be unique to *Drosophila* whereas others have direct homologues to other insect species or even mammals (Marmaras and Lampropoulou, 2009). The binding of invaders' PAMPs on PRPs induces the synthesis of antimicrobial proteins or initiates the proteolytic activation of phenoloxidase cascade or activates cellular immune response, leading to phagocytosis, nodule formation and encapsulation of the invaders (Yu XQ *et al*., 2002; Marmaras and Lampropoulou, 2009).

Immunolectins

Lectins are sugar recognition molecules and play an important role in immune-related reactions enabling an organism to distinguish self from non-self or modified-self determinants. They are characterized by a wide range of binding activities. Nineteen genes were originally identified in Drosophila as members of the C-type lectin family, although the distinct function, for each one of them, had not been clarified (Theopold *et al.*, 1999). C-type lectins had been classified into seven groups based on their overall domain structure. Analyses of the superfamily representation in several completely sequenced genomes have added 10 new groups (Zelensky and Gready, 2005). In lepidopterans, immunolectins are involved in prophenoloxidase

activation, phagocytosis and nodule formation (Yu *et al*., 2003; Yu and Kanost, 2004).

Peptidoglycan recognizing proteins

Peptidoglycan (PGN) consists of a sugar backbone and a stem-peptide of three to five amino acids, found mostly on the surface of Gram positive bacteria (Kaneko and Silverman, 2005; Little and Cobbe, 2005). The peptidoglycan recognizing proteins (PGRPs) are small extracellular proteins (20 kDa) which are synthesized and secreted by the fat body, integument, gut, and in a minor degree by the hemocytes (Kaneko and Silverman, 2005). They are defined by a domain with homology to an enzyme called amidase. Several PGRPs, from flies and mammals, present amidase activity, and several others are predicted to have amidase activity. In addition, some PGRPs lack a critical cysteine required for the active site, and are therefore thought to function only as recognition proteins without enzymic activity (Mellroth *et al*., 2003). Recognition of PGNs on Gram-positive or Gram-negative bacteria, by circulating PGRPs, activates the Toll or IMD intracellular signalling pathways, respectively, leading to the nuclear translocation of two NF-κB/Rel proteins and drives anti-bacterial peptide gene expression. The details of these intracellular signalling pathway have been reviewed previously (Silverman and Maniatis, 2001; Aggrawal and Silverman, 2007).

β-1,3-Glucan recognising proteins

In *Drosophila* there is a Gram-negative bacteria-binding protein (DGNBP) family, (Kim *et al*., 2000). DGNBP-1 exists in both soluble and glycosylphosphatidylinositol-anchored membrane and functions as a pattern recognition receptor for LPS from Gram-negative bacteria and β-1,3-glucan from fungi and mediates innate immune signalling for the induction of antimicrobial peptide gene induction in cultured *Drosophila* immune cells. (Kim *et al*., 2000). Two biosensor for fungal and bacterial infection, called β-1,3-glucan recognition proteins (βGRPs), are present in the hemolymph of *M. sexta* (Wang and Jiang, 2010). Both βGRPs specifically recognize soluble or insoluble β-1,3-glucan and LPS, bind onto a hemolymph proteinase-14 precursor (proHP14) through specific protein–protein interactions and initiate proPO activation system.(Wang and Jiang, 2010). Similar activation of proPo system occurs in the beetle *Tenebrio molitor* (Zhao *et al*., 2007).

Hemolin

The hemolin is a member of the immunoglobin superfamily and is synthesized by the fat body. Hemolin has been found in the hemolymph of two lepidopteran species *Hyalophora cecropia* and *M. sexta* and its concentration increases 20-fold, upon bacterial infection, although it has no direct antimicrobial activity (Bettencourt *et al*., 1997; Eleftherianos *et al*., 2007). In *M. sexta*, hemolin recognizes and binds LPS on Gram negative bacteria and lipoteichoic acid on Gram positives bacteria, leading to their aggregation. (Daffre and Faye, 1997; Yu and Kanost, 2002). It must be noted that LPS and lipoteichoic acid bind on the same site on the hemolin molecule. Hemolin may also bind on glycolipids of the bacterial wall, showing that it acts as a wide range PRP against infection. In *H. cecropia*, hemolin binds on bacterial LPS and then binds on hemocytes, in a calcium dependent manner, thus activating protein kinase C, which initiates phagocytosis (Bettencourt *et al.*, 1997; Daffre and Faye, 1997).

Integrins

Integrins are surface proteins, widely expressed in metazoans (sponges to humans), and participate in adhesion, migration and tissue organization (Hughes, 2001). Integrins recognize and bind RGD motifs (amino-acid triplet Arg-Gly-Asp) in specific cell-surface, or extracellular matrix (ECM) or soluble proteins (collagen, laminins, fibronectins) (Hynes, 2002; Humphries *et al.*, 2004). Integrins are primary molecules for the recognition of foreign agents and the initiation of immune response. In the medfly *C. capitata*, they are involved in bacteria (Gram-positive and Gram-negative) phagocytosis by plasmatocytes, but not in LPS or abiotic targets uptake (Lamprou *et* al., 2007; Mamali *et al.*, 2009). In *M. sexta*, integrins play a key role in stimulating hemocytes adhesion leading to encapsulation (Zhuang *et al.*, 2008).

Humoral responses

The recognition of invading pathogen either as bacteria or fungi or even viruses is followed by the immediate *de novo* synthesis of antimicrobial peptides (AMPs) and their secretion into the hemolymph (Zasloff, 2002; Bulet *et al.*, 2004). These peptides are mainly synthesized by the fat body and in a lesser degree by the hemocytes, integument, gut, salivary glands and reproductive structures (Nappi and Ottaviani, 2000).

Antimicrobial peptides

Over 150 antimicrobial peptides (AMPs) have been isolated and characterized in insects. These molecules are small, 12-50 amino acids, cationic peptides, which bind anionic bacterial or fungal membranes leading to disruption and cell death (Zasloff, 2002; Yount and Yeaman, 2006). Although they have different structure and target organisms (bacteria or fungi), the AMPs are classified in four groups; a) cecropins, b) cysteine-rich peptides, c) proline-rich peptides, and d) glycine-rich peptides.

Cecropins were firstly isolated in *H. cecropia* after injection with bacteria (Hultmark *et al*., 1980; Steiner *et al*., 1981). These peptides are produced in response to septic injury by either Gram positive or Gram negative bacteria bacteria and affect on cellular proliferation by inhibiting the synthesis of proteins of the cell membrane.

Defensins and drosomycin are cysteine-rich peptides. Defensins, destroy mostly gram-positive peptides by forming channels in the plasma membrane which leads to cell lysis, while drosomycin has an antifungal activity. Diptericin is an antibacterial peptide that has been found only in diptera species and is induced upon Gram negative bacteria infection in a way similar to attacines (Nappi and Ottaviani, 2000). Lysozymes are enzymes that

degrade peptidoglycans of the bacterial cell wall. They are also found in other animals, plants, fungi and bacteriophages (Bulet *et al.*, 1999).

Enzymic cascades

Coagulation of hemolymph

Insects have developed mechanisms for the coagulation of hemolymph, in case of wounding, to prevent loss of body fluids (Theopold *et al.*, 2002).In the cockroach *Leucophaea maderae*, hemocytes secrete a calcium dependent transglutaminase that catalyzes the polymerization of lipophorins and vitellogenin-like proteins. These last proteins have a domain homologous to the Von Willebrand clotting factor in mammals (Bohn *et al.*, 1994). The most characterized mechanism is the one in *Lymulus polyphemus*, which appears to be similar in *Drosophila* (Vimlos and Kurucz, 1998). According to this, LPS and β-1,3-glucan trigger a serine protease chain reaction, finally leading to the coagulation of the hemolymph. In addition, serine protease activates melanization cascade (Nappi *et* , *al.,* 1995; Mavrouli *et al.*, 2005; Sideri *et al.*, 2007). It must be noted the dual role of serine protease in the insect immunity since intermediate metabolites of these two cascades, preclotting enzymes, melanin derivatives and reactive oxygen species, are toxic invading pathogens.

Melanization of hemolymph

Melanization, the pathway leading to melanin formation, has a central role in defense against a wide range of pathogens and participates in wound healing as well as in nodule and capsule formation in some lepidopteran and dipteran insects, (Lavine and Strand, 2001; Lavine and Strand, 2003; Mavrouli *et al.*, 2005). Melanization depends on tyrosine metabolism. Briefly, tyrosine is converted to dopa, an important branch point substrate, by activated phenoloxidase (PO). Dopa may be either decarboxylated by dopa decarboxylase (Ddc) to dopamine or oxidised by PO to dopaquinone.

Dopamine is also an important branch point substrate, because dopamine-derived metabolites either via PO or through other enzymes are used in several metabolic pathways, participating in neurotransmission, cuticular sclerotization, cross-linking of cuticular components via quinone intermediates, phagocytosis, wound healing and melanization in immune reactive insects (Fearon, 1997; Aderem and Underhill, 1999; Ling and Yu, 2005; Marmaras and Lampropoulou, 2009).

Cellular responses

Hemocytes are responsible for a number of defense responses in insects, among which phagocytosis, nodulation, encapsulation and melanization have been documented. These processes appear to be discrete immune responses in terms of gene expression and outcome. However, these certain immune responses share a number of common elements that function in concert to clear pathogens from the hemolymph. Below we have outlined the current data on these defense responses and their relationships.

Phagocytosis

Phagocytosis initiates with the recognition of the invading pathogens, engulfment and is completed with their intracellular destruction, by individual hemocytes. In insects, phagocytosis is achieved mainly by the circulating plasmatocytes or granulocytes, in the hemolymph (Gillespie *et al.*, 1997; Meister and Lagueux, 2003; Lamprou *et al.*, 2005, 2007). The uptake of a microbe by a phagocytic cell is an extremely complex and diverse process which requires multiple successive interactions between the phagocyte and the pathogen as well as sequential signal transduction events. Phagocytosis is induced when phagocyte surface receptors, are activated by target cells. It must be noted that the hemocyte response to various bacteria differs. For example, in *A. aegypti* hemocytes respond to *E. coli* with phagocytosis, whereas to *Micrococcus luteus* with melanization (Hernandez-Martinez *et al.*, 2002; Hillyer and Schmidt 2003a, b). Furthermore, differences exist in the efficiency and speed of phagocytosis among different bacteria. It has been shown that *E. coli* is more readily phagocytosed than *S. aureus*, in *A. gambiae* as well as in isolated medfly hemocytes (Levashina *et al.*, 2001; Moita *et al.*, 2006; Lamprou *et al.*, 2007). These results strongly suggest that several distinct molecular mechanisms regulate phagocytosis in insects.

Nodulation

Nodulation refers to multicellular hemocytic aggregates that entrap a large number of bacteria. Melanized or non-melanized nodules are formed in response to a number of invaders. Nodule formation appears to be related with eicosanoids in many insect species (Miller *et al.*, 1999) or prophenoloxidase (PO) and dopa decarboxylase (Ddc) in medfly hemocytes (Sideri *et al.*, 2007).

Encapsulation

Encapsulation refers to the binding of hemocytes to larger targets, such as parasites, protozoa, and nematodes. Encapsulation can be observed when parasitoid wasps lay their eggs in the hemocel of *Drosophila* larvae. Hemocytes after binding to their target they form a multilayer capsule around the invader, which is ultimately accompanied by melanization. Within the capsule the invader is killed, by the local production of cytotoxic free radicals ROS and RNS, or by asphyxiation (Nappi *et al.*, 1995; Nappi and Ottaviani, 2000).

Antiviral response

Viruses are intracellular pathogens that infect all forms of life. The first potent antiviral defense mechanism was identified in plants, through RNA silencing (Ding and Voinnet, 2007). Recently, RNAi was found to play an important role in the control of viral infection in *Drosophila*. This mechanism of gene silencing depends upon small RNAs that are 21-30 nucleotides.

Central to the RNAi mechanism are the slicing enzymes of the Argonaute (AGO) family, which mediate highly specific cleavage of target RNA molecules. The specificity of AGO enzymes is achieved by their association with small RNAs,

which guide them to complementary sequences. Three RNAi pathways, involving different members of the AGO family, have been defined in *Drosophila*: first, the small interfering (si)RNA pathway involves AGO-2, and is activated by double-stranded (ds)RNA. siRNAs are produced by the RNaseIII enzyme Dicer-2, which forms a complex with the dsRNA-binding protein (dsRBP) protein R2D2; second, the micro (mi)RNA pathway involves AGO-1, Dicer-1, and its dsRBD cofactor R3D1, and regulates expression of drosophila genes, in particular during development; third, the Piwi-associated RNA (piRNA) pathway involves the three other AGO proteins encoded by the drosophila genome, namely Piwi, Aubergine, and AGO3. piRNAs are involved in the control of mobile genetic elements, including the retrovirus gypsy, in the germ-line (Brennecke *et al.*, 2007; Ding and Voinnet, 2007; Kemp and Imler, 2007).

Signalling pathways in innate immunity

From the overview of the mechanisms concerning the innate immunity, three major responses can be summarized. The production of antimicrobial peptides due to specific receptors, either soluble or membrane, the internalization-phagocytosis, which follows the attachment of bacteria on the cell membrane and the role of RNA interference in the antiviral immunity.

The hallmark of the *Drosophila* humoral immune response is the production of antimicrobial peptides in the fat body and their release into the circulation (Aggarwal, and Silverman, 2008; Feldhaar and Gross, 2008). Two recognition and signalling cascades regulate expression of these antimicrobial peptide genes. The Toll pathway is activated by fungal and many Gram-positive bacterial infections, whereas the immune deficiency (IMD) pathway responds to Gram-negative bacteria. Both of these are initiated by peptidoglycan recognition proteins (PGRPs) and complete their action via the conserved NF-κB signalling cascades for the control of immune-induced gene expression (Aggarwal and Silverman, 2008).

Phagocytosis is triggered by certain transmembrane proteins on the hemocyte surface. The most common classes of such receptors in insect plasmatocytes are the scavenger receptors, the EGF-like-repeat-containing receptors, the integrins and the PGRPs (Feldhaar and Gross, 2008; Marmaras and Lamproulou, 2009). The key intracellular molecules that promote signals from pathogens that attach on cell-surface receptors, are the scaffold and adaptor proteins. Scaffold proteins are proteins that bind other proteins that usually function in sequence. Adaptor proteins are proteins that augment cellular responses by recruiting other proteins to a complex. These molecules function as organizing platforms that bring together both the enzymes and the substrate proteins, in the same complex (Marmaras and Lampropoulou, 2009).

We show that antimicrobial peptide synthesis and bacterial internalization share a lot of signalling molecules and pathways. Antiviral response, although it consists of a totally different procedure targeting to the degradation of viral nucleic acids by RNA interference it includes three classical immune signalling pathways (Toll, Imd, and Jak-STAT) responsive to infection by different viruses (Kemp and Imler, 2009; Sabin *et al.*, 2010).

The Toll pathway

Insects respond to Gram-positive bacterial and fungal infections via the Toll pathway. Its basic component is the transmembrane receptor Toll and the intracellular adaptors Tube and MyD88 (Lemaitre and Hoffmann, 2007; Aggarwal and Silverman, 2008), Toll is not a pattern recognition receptor since it does not bind pathogens or pathogen-derived compounds, directly and is activated by the extracellular cytokine Spätzle. To activate Toll pathway, microbial recognition must be preceded. The detection of Gram-positive bacterial peptidoglycans and fungal betaglucans by specific PGRPs and GNBPs, respectively, activate serine protease cascades from the fat body that culminate in Spätzle cleavage, thus, liberating the C-terminal 106 amino acids of Spätzle, the mature Toll ligand. The cleaved Spätzle binds the Toll receptor which recruits the Tube/Myd88 complex, followed by the kinase Pelle activation. Pelle kinase triggers an intracellular signalling cascade involving several factors resulting in the activation of the transactivator proteins Dorsal and Dif belonging to the NF-kB family. After their translocation in the nucleus they induce transcription of the respective genes encoding for instance defensins, drosomycin, cecropins. In addition to Dorsal and Dif a *Drosophila* IkB homolog called Cactus is activated which is an inhibitory factor that negatively modulates the Toll-mediated immune response (Feldhaar and Gross, 2008).

The Imd pathway

The Gram-negative bacteria activate antimicrobial peptide synthesis via the Imd pathway (Nappi *et al.*, 2004; Lemaitre and Hoffmann, 2007; Aggarwal and Silverman, 2008). This pathway was initially defined by the identification of a mutation named *immune deficiency* (Imd) that impaired the expression of several antibacterial peptide genes (Lemaitre and Hoffmann, 2007). The bind of bacterial monomeric or polymeric DAP-type PGN on the single-pass transmembrane cell surface receptor PGRP-LC, results the recruiting of the intracellular adaptor Imd (Aggarwal and Silverman 2008). Signal transduction leads to Relish cleavage and the Rel domain translocates to the nucleus, whereas the inhibitory domain remains stable in the cytoplasm. *Diptericin* gene is an Imd target in response to injection of *E. coli* (Gram-negative bacteria).

The JAK/STAT pathway

The JAK/STAT pathway, has three main cellular components: the receptor Domeless, the Janus Kinase (JAK), and the STAT transcription factor (Lemaitre and Hoffmann, 2007). Bacterial

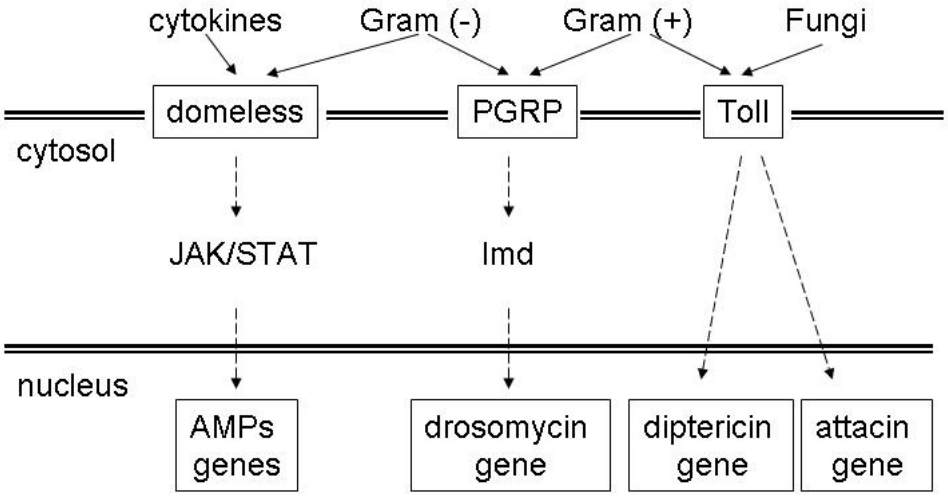

Fig. 1 Humoral immune response in insect fat body. Secreted cytokines as well as pathogens, either bacteria or fungi, bind on several immune-related receptors in a non specific way, among insect species. This leads to the expression of antimicrobial protein genes and secretion of their respective peptides, via certain cytoplasmic pathways either specific (JAK/STAT for domeless or Imd for peptidoglycan recognizing proteins-PGRP) or non specific (toll receptor) for each receptor.

infections induce hemocyte to produce cytokine Unpaired-3 (UPD3), which is the ligand of Domeless. The result of this pathway, after immune challenge, is the STAT protein accumulation in the nucleus and the activation of gene expression. The transcriptional regulation is complex, with additional inputs from both the Imd and MAPK (mitogen-activated protein kinase) pathways (Aggarwal and Silverman, 2008).

RNA interference pathway

RNA interference (RNAi) has been found to play an important role in the control of viral infection in Drosophila (Kemp and Imler, 2009). Central to the RNAi mechanism are the slicing enzymes of the Argonaute (AGO) family, which mediate highly specific cleavage of target RNA molecules. These enzymes associate with small RNAs, which guide them to complementary sequences. Three RNAi pathways, involving different members of the AGO family, have been defined in *Drosophila*:

- the small interfering (si)RNA pathway involves AGO-2, and is activated by double-stranded (ds)RNA. siRNAs are produced by the RNaseIII enzyme Dicer-2, which forms a complex with the dsRNA-binding protein (dsRBP) protein R2D2 (Ding and Voinnet, 2007)

- the micro (mi)RNA pathway involves AGO-1, Dicer-1, and its dsRBD cofactor, and regulates expression of *Drosophila* genes

- the piwi-associated RNA (piRNA) pathway involves the three other AGO proteins encoded by the drosophila genes, namely Piwi, Aubergine, and AGO3. piRNAs are involved in the control of mobile genetic elements, including the retrovirus gypsy, in the germ-line (Brennecke *et al.*, 2007)

Demonstration of the critical role of RNAi as a potent antiviral mechanism in drosophila is based on three lines of evidence: genetic data indicating that RNAi pathway mutants are hypersensitive to RNA virus infections, identification of viral suppressors of RNAi (VSRs), which counteract the immune defense of the fly, and the presence of siRNAs of viral origin in infected cells/flies (Kemp and Imler, 2009).

Integrin pathway

Integrins are heterodimeric transmembrane receptors consisting by an α and a β subunit. Integrins recognize and bind RGD motifs (amino-acid triplet Arg-Gly-Asp) in specific cell-surface, or extracellular matrix (ECM) or soluble proteins such as collagen, laminin and fibronectin (Hynes, 2002; Humphries *et al.*, 2004). This ability leads to intracellular signal transduction (outside-in signalling) via activation FAK/Src pathways and MAPK (Lamprou *et al.*, 2007; Marmaras and Lampropoulou, 2009). In the medfly *C. capitata*, integrins are involved in phagocytosis of bacteria, but not LPS, by hemocytes (Lamprou *et al.*, 2005; Lamprou *et al.*, 2007; Mamali *et al.*, 2009), due to the activation of p38 via Ras/Rho/actin remodelling pathway, while in *M. sexta*, they lead to stimulate encapsulation by the stimulation of hemocyte adhesion (Zhuang *et al.*, 2008).

Pathway cross talk

Signal transduction is based on several pathways which form a complicated network, cross talking to each other in order to lead to the appropriate response, due to extracellular stimuli. Such interactions may appear in every level of these pathways either in recognition (Fig. 1) or signal transduction or even the final response (Fig. 2) (Garcia-Lara *et al.*, 2005).

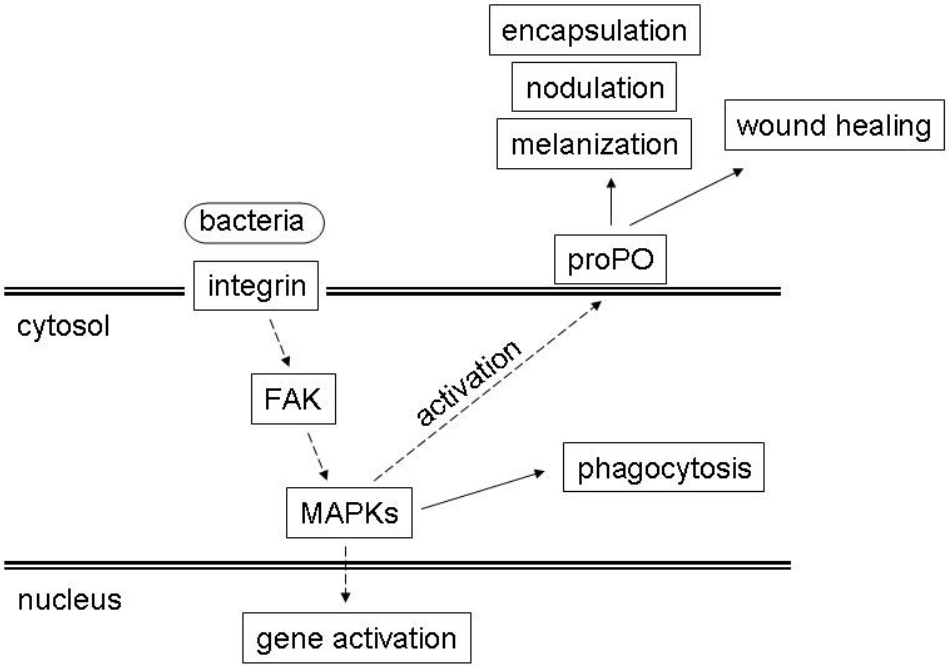

Fig. 2 Humoral and cellular immune response in insect hemocytes. The bind of bacteria on a β-integrin transmembrane subunit, triggers cytoplasmic signalling via focal adhesion kinase (FAK) and mitogen activated protein kinases (MAPKs) activation, major key point pathways. This leads to cellular responses such as phagocytosis, nodulation and encapsulation. The activation of FAK and MAPKs may also lead to humoral response such as melanisation and wound healing, through the activation of cell surface prophenoloxidase (proPO).

Different peptidoglycan recognition proteins (PGRPs) show strong preference, but not exclusivity, towards specific pathogen–associated molecular patterns (PAMPs) and on the other hand these pathogens may be concerted with other protein recognition patterns (PRRs). In *Drosophila* hemolymph, certain soluble PGRP, which recognizes not only a PGN, common to *S. aureus* and other Gram-positive bacteria but even a GNBP1, activate the Toll signal transduction pathway (Michel *et al*., 2001; Gobert *et al.*, 2003). The result is the synthesis of antimicrobial peptides (AMPs), such as drosomycin. On the other hand, a membrane PGRP (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002) and a soluble PGRP recognize peptidoglycans and activate the Imd pathway (Lemaitre *et al.*, 1997; Takehana *et al.*, 2002). Other PGRPs may also weakly recognize Gram-positive-type PGN or can bind with low affinity Gram-negative-type PGN as well as lipopolysaccharide (LPS) and lipotteichoic acid (LTA) (Dziarski, 2004).

It becomes obvious that an initial signal does not guarantee a specific outcome, since there is no exclusivity for the activated receptor. In addition, the transduction of the signal, from the cell membrane into the cytoplasm, does not necessarily follow a single pathway but it may change course to another, by unspecific intracellular pathways such as these of Src family or MAPKs namely, ERK, p38 and JNK (Garcia-Lara *et al.*, 2005). These enzymes appear to have overlapping and complementary functions in many pathways. Perhaps the function of these enzymes is to modulate the overall intracellular signalling network in the fat body and hemocytes, rather than operating as exclusive signalling switches for defined pathways. Thus, the final product differs among species and tissues. *Drosophila* responds to Gram-positive bacteria by the induction of Drosomycin synthesis, through the Toll pathway, while in Gram-negatives by the induction of diptericin, attacin and cecropin through the Imd pathway (Leclerc and Reichhart, 2004). However, it has also been reported that *S. aureus*, may induce the production of cecropins, while *E. coli* may induce the expression of drosomycin, giving additional evidence for a cross-talk between the two pathways (Hedengren-Olcott *et al*., 2004). The JAK/STAT pathway is activated in response to Gram-negative bacteria and appears to branch out from the Imd pathway in fruit flies and mosquitoes (Agaisse and Perrimon, 2004). The production of AMPs is not the only final response to the same initial stimulus. Other humoral as well as cellular responses may be triggered, indicating an extracellular response network of innate immunity, too.

Immune response cross talk

Fat body and hemocytes are the major components of the innate immune response in insects. They possess a diverse repertoire of receptors that allow cells to respond to external stimuli such as cytokines and pathogen-associated molecules. Signals resulting from these stimuli

activate the synthesis of antiviral peptides and the synthesis and secretion of antimicrobial peptides by the fat body. The functional responses of hemocytes are adhesion, cytokine release, melanization, phagocytosis, nodule formation and encapsulation. Hemocyte challenging, by a pathogen, triggers all these humoral and cellular responses, which do not function separately, but they seem to cooperate with each other, in order to block pathogen invasion (Fig. 2).

In medfly and mosquito, phagocytosis begins with the binding of *E. coli* on an integrin β-subunit of the hemocyte surface (Humphries *et al.*, 2004; Mavrouli *et* al., 2005; Moita *et al*., 2006; Mamali et *al.*, 2009). Integrins transmit signal to focal adhesion kinase/sarcoma (FAK/Src) and mitogen activated protein kinase pathways (Mavrouli *et al.*, 2005). This signal transduction leads to the secretion of serine proteases which convert the surface inactive prophenoloxidase to the active phenoloxidase and initiate melanization. In parallel, phagocytosis and nodule formation are triggered. Although these responses seem to be distinct, they appear to cooperate since blockade of one of them inhibits the other (Sideri *et al*., 2007). Abiotic latex beads and lipopolysaccharide (LPS) phagocytosis do not depend on proPO activation (Mavrouli *et al.*, 2005; Lamprou *et al.*, 2007).

The proPO activation system is composed of proteins recognizing several pattern-recognition proteins, serine proteases, proPO, as well as proteinase inhibitors that function as regulatory factors (Cerenius and Söderhall, 2004). ProPO is synthesized in hemocytes and appears to be distributed ubiquitously in the cytoplasm as well as on the surface of hemocytes (Ling and Yu, 2005; Mavrouli *et al.*, 2005). The proPO activation system is triggered by several microbial components, such as LPS and peptidoglycans. Activated PO catalyses the hydroxylation of tyrosine to 3,4-dihydroxy-phenyl-alanine (dopa). Dopa can be oxidized by PO to dopaquinone, which, via PO and the dopachrome conversion enzyme, ultimately result in melanin. Dopa may also be decarboxylated by dopa decarboxylase (Ddc) to form dopamine (Marmaras and Lampropoulou, 2009). Ddc is involved in wound healing, parasite defense, cuticle hardening and melanisation (Hodgetts and O'Keefe, 2006). A PO-based oxidation of dopamine leads to dopaminequinone and finally the cross-linking and melanization of proteins. The expression of Ddc mRNA in the hemocytes of *Pseudaletia separata* was enhanced by injection of an insect cytokine, growth-blocking peptide (Noguchi *et al*., 2003).

Melanization is the process that leads to melanin formation in both hemocyte-free hemolymph as well as on hemocyte surface after wounding or upon invasion with pathogens. Ddc is a key enzyme between melanization and phagocytosis, two unrelated procedures that are linked and facilitate each other. The activity of Ddc is elevated during melanotic responses in Drosophila and in the mosquito *Armigeres subalbatus* (Nappi *et al*., 1992; Huang *et al*., 2005). Melanization is also a critical process in defense against bacteria, and several reports link components of the melanization process with phagocytosis (Johansson and Söderhall, 1996; Hillyer *et al*., 2004; Mavrouli *et al*., 2005) It has been proposed that pathogens might be killed by toxic reactive oxygen metabolites produced in the process of melanisation (Nappi *et al.*, 1995). However, Ddc based melanization, appears to be distinct from the pathway leading to phagocytosis (Sideri *et al*., 2007). These two unrelated procedures share a number of substrates (tyrosine, dopa, dopamine) and enzymes (PO, Ddc).

Nodulation, as stated in the introduction, refers to multicellular hemocyte aggregates that entrap a large number of bacteria, and PO and Ddc are key enzymes in this process (Mavrouli *et al*., 2005). Nodules may be attached to tissue or surrounded by hemocytes. Nodule formation has not been fully characterized, although it is known that it is lectin-mediated. Melanization and nodulation are two distinct pathways which share a number of substrates and enzymes. Phagocytosis and nodulation processes are distinct from the melanisation process and questions have been raised whether branch-point substrates exist to differentiate phagocytosis from nodulation or whether they are processes in sequence (Sideri *et al*., 2007).

Conclusion

Innate immunity is an interesting and exciting field for research. Its evolutionary conserved mechanisms make insects first line tools for investigation. The study of immune responses and the relative signalling pathways have revealed their cooperation and the key components. Insect innate immunity appears to be an easy to handle tool to study how different stimuli activate the same receptor and how this receptor activates different pathways, leading to the same or different response.

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