

Review

Regulators and signalling in insect antimicrobial innate immunity: Functional molecules and cellular pathways

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ABSTRACT

Insects possess an immune system that protects them from attacks by various pathogenic microorganisms that would otherwise threaten their survival. Immune mechanisms may deal directly with the pathogens by eliminating them from the host organism or disarm them by suppressing the synthesis of toxins and virulence factors that promote the invasion and destructive action of the intruder within the host. Insects have been established as outstanding models for studying immune system regulation because innate immunity can be explored as an integrated system at the level of the whole organism. Innate immunity in insects consists of basal immunity that controls the constitutive synthesis of effector molecules such as antimicrobial peptides, and inducible immunity that is activated after detection of a microbe or its product(s). Activation and coordination of innate immune defenses in insects involve evolutionary conserved immune factors. Previous research in insects has led to the identification and characterization of distinct immune signalling pathways that modulate the response to microbial infections. This work has not only advanced the field of insect immunology, but it has also rekindled interest in the innate immune system of mammals. Here we review the current knowledge on key molecular components of insect immunity and discuss the opportunities they present for confronting infectious diseases in humans.

1. Introduction

Insects in nature are continuously exposed to pathogenic microorganisms. Part of the insect ability to occupy diverse habitats is attributed to their capacity to deal with pathogenic infections [1–4]. This is primarily due to the physicochemical properties of the cuticular surface of the integument and the linings of various body parts of ectodermal origin such as those of tracheae, alimentary canal, and reproductive ducts which provide a barrier against pathogen penetration [5–9]. Once these barriers are breached, the microbial intruders encounter a complex system of host defenses (Fig. 1), i.e., the innate immune response, which is activated when germline encoded specialized receptors recognize and bind to conserved pathogen molecular features [10,11].

Once recognition occurs, signal transduction pathways are activated, and lead to the regulation of distinct immune functions. Thus, innate immunity in insects comprises three major events: recognition of the nonself, transduction of down-stream signalling, and effector mechanisms [2,8,11].

Insect innate immune responses include a wide variety of responses. These responses have been classified according to different criteria and functional determinants including cellular, humoral, constitutive, induced, fast, slow, specific, and non-specific (Fig. 2) [2]. Cellular reactions refer to hemocyte-mediated immune functions, e.g., phagocytosis, nodulation, and encapsulation [12,13]. Humoral reactions include production of antimicrobial peptides (AMPs) [14], complex enzyme-cascades that regulate activation of precursors of immune-related

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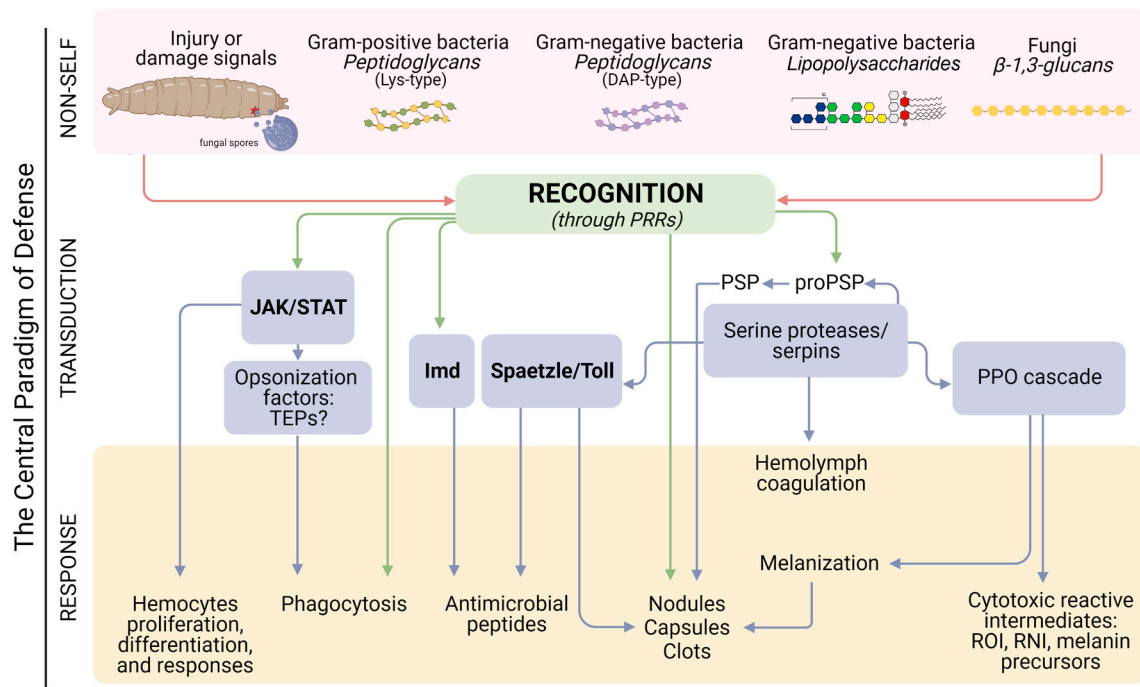


Fig. 1. An overview of insect innate immune response mechanisms and associated parameters. Damage-associated molecular patterns (DAMPs) or danger signals (e.g., actin, ATP, calreticulin, Egr (TNF- α), and reactive oxygen intermediates (ROI) generated via both sterile and septic injuries) or pathogen-associated molecular patterns (PAMPs) (e.g., the microbial elicitors β -1,3-glucans, lipopolysaccharides, and peptidoglycans) are recognized by pattern recognition receptors (PRRs) and in turn activate signalling pathways and immune factors (middle) that induce a large array of networked and synergetic host defense responses (bottom).

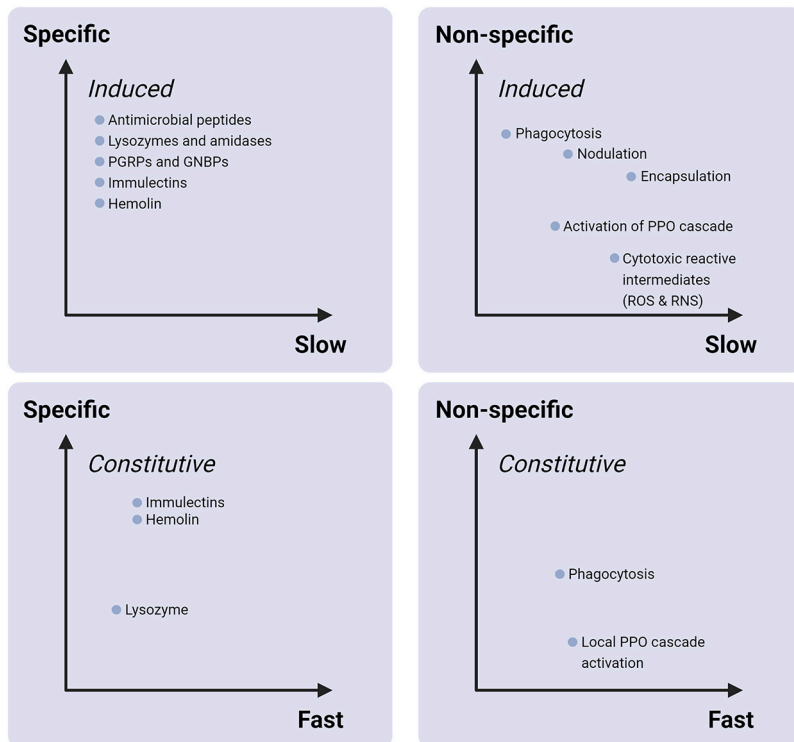


Fig. 2. Insect innate immune defense strategies. Immune responses in insects are categorized according to functional determinants and criteria related to expression level, specificity, and speed of action. Constitutive immunity is represented mostly by background activity of, e.g., the immune capability of the circulating hemocytes such as phagocytosis and the phenoloxidase (PO) and lysozyme activity. In addition, a rapid defense may also involve epidermal cells that locally activate the PPO-cascade to produce PO and release cytotoxic reactive intermediates of oxygen (ROI) and nitrogen (RNI) at a wounding site from redox-cycling melanin precursors associated with clot formation or melanotic encapsulation. Many of these responses have been characterized and are quite nonspecific, i.e., they are effective against a wide variety of immune challenges. By contrast, induced defenses depend on a specific challenge and are rather slower in activation, perhaps on the order of hours or longer. A typical delayed induced response in insects is the synthesis of antimicrobial peptides and proteins and soluble pattern recognition receptors. Modified after Fig. 1 in Reference [2] (reprinted with permission from Elsevier).

molecules such as prophenoloxidase (PPO) [15], pro-Spaetzle (proSpz) [16], and proplasmacyte-spreading peptide (proPSP) [17], production of humoral pattern recognition receptors (PRRs) and lytic enzymes [18,19], and reactive intermediates of oxygen (ROI) and nitrogen (RNI) [20]. This classification of immune responses into cellular and humoral is arbitrary since many humoral factors affect cellular functions and

several humoral molecules are produced by hemocytes.

This review provides current knowledge on the regulation of innate immune mechanisms in insects. In particular, we will expand on the recognition of pathogen-associated molecular patterns (PAMPs) by cellular and humoral PRRs and the specific molecules that participate in the detection of microbial invaders, the activation of evolutionary

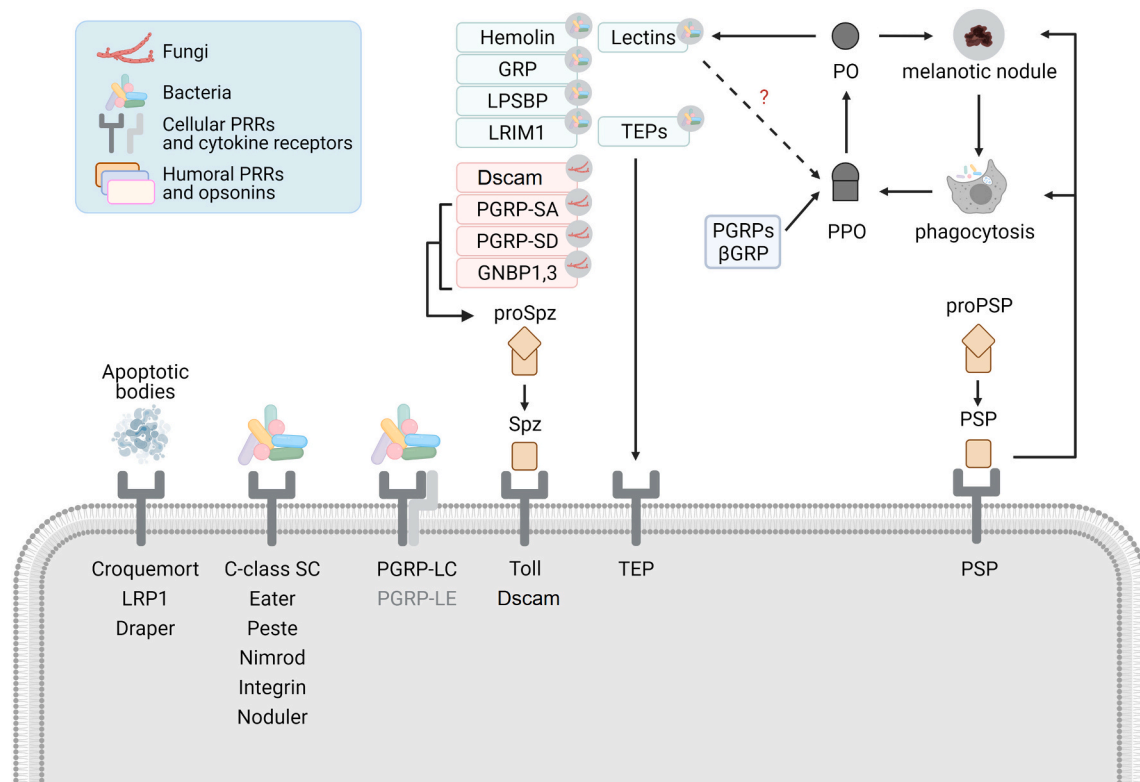


Fig. 3. Insect humoral and cellular pattern recognition, opsonins and cytokine receptors involved in microbial recognition. Humoral receptors GNBP1,3, LPSBPs, PGRP-SA and PGRP-SD aid recognition and opsonization processes of different microbes. Soluble circulating and cellular forms of Dscam and thioester-containing proteins (TEP) bind and opsonize microbial surfaces. Proteolytic processing of proSpaetzle (proSpz) to form the cytokine Spaetzle (Spz) is induced by binding of microbial PAMPs by PGRP-SA and PGRP-SD which in turn activates the Toll pathway. Hemolin, is a soluble receptor which is found only in Lepidoptera. Lepidopteran immunoglobulins bind bacteria and fungi, and form complexes with PO that enable specified deposition of melanin on the microbial surfaces. Eater, low-density lipoprotein receptor-related protein-1 (LRP1), Peste, and PGRP-LC with its co-receptor PGRP-LE cellular receptors in *Drosophila* bind bacteria. Uptake of apoptotic bodies in *Drosophila* is mediated by the CD36 homolog Croquemort, while LRP1 has a related function in *Anopheles gambiae*. In Lepidoptera, induced proteolytic processing of proPSP to the cytokine PSP induces adhesion of plasmatocytes to large microbial surface.

conserved signal transduction pathways, which ultimately lead to the induction of host defenses for the elimination of pathogens that threaten to disrupt host homeostasis. Understanding these complex processes is fundamental because it will allow us to determine the molecular details that control evolutionary conserved antimicrobial responses in invertebrates and vertebrates.

2. Pathogen associated molecular patterns (PAMPs), receptors, and recognition

Recognition of nonself that initiate defense responses in the innate immune system is regulated by pattern-recognition receptors (PRRs) which comprise secreted proteins and cell membrane-bound proteins [10,11,21,22]. PRRs bind to specific molecular motifs called PAMPs which are found on the surface of microorganisms such as bacteria and fungi. Therefore, these PAMPs function as potent immunostimulatory factors for both cellular and humoral immune responses [23].

As a major constituent of the cell wall of virtually all bacteria, peptidoglycan (PGN) represents a main target for innate immune recognition. PGN chains are polymers of alternating N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) in a β -1,4-linkage [24,25]. These chains are cross-linked by short peptide stems composed of alternating L- and D-amino acids via lactylamide bond between muramic acid and the peptide stems. Given that the carbohydrate backbone is conserved among bacteria (except for de-N-acetylated or O-acetylated variants), the peptide moiety displays considerable diversity. According to the residue at position 3 of the peptide stems, PGNs are divided into two major categories [26]: L-lysine type (Lys-type) and m-

diaminopimelic acid type (Dap-type). Usually Dap-type PGN stems are directly cross-linked, whereas Lys-type PGN stems are interconnected by a peptide bridge that varies in length and amino acid composition in different bacteria. Moreover, bacteria differ widely in the extent of crosslinking, thus introducing additional diversity in PGN structure [24].

Humoral PRRs for the major PAMPs have been identified in insects; however, little is known about cellular PRRs [3,27]. Recognition of a microbial invader is followed by the activation of down-stream signal transduction and effector responses [22,28]. After binding of humoral PRRs to PAMPs, the foreign microbe is recognized by activating receptors on the surface of hemocytes, a process known as opsonization. The invader can also be recognized directly by the hemocyte-surface receptors. In addition, certain receptors acting as regulators are involved in both humoral and cellular immune processes, corroborating the hypothesis that these receptors are not characterized by specificity and their classification into humoral or cellular receptors is rather arbitrary [29,30].

2.1. Humoral PRRs

Humoral PRRs are secreted proteins circulating in the hemolymph (Fig. 3). The main sources of these humoral molecules are the hemocytes and the fat body [28]. The main humoral PRRs include:

2.1.1. Lectins

In mammals, C-type lectins (CTLs) represent a superfamily of calcium-dependent carbohydrate-binding proteins that contain

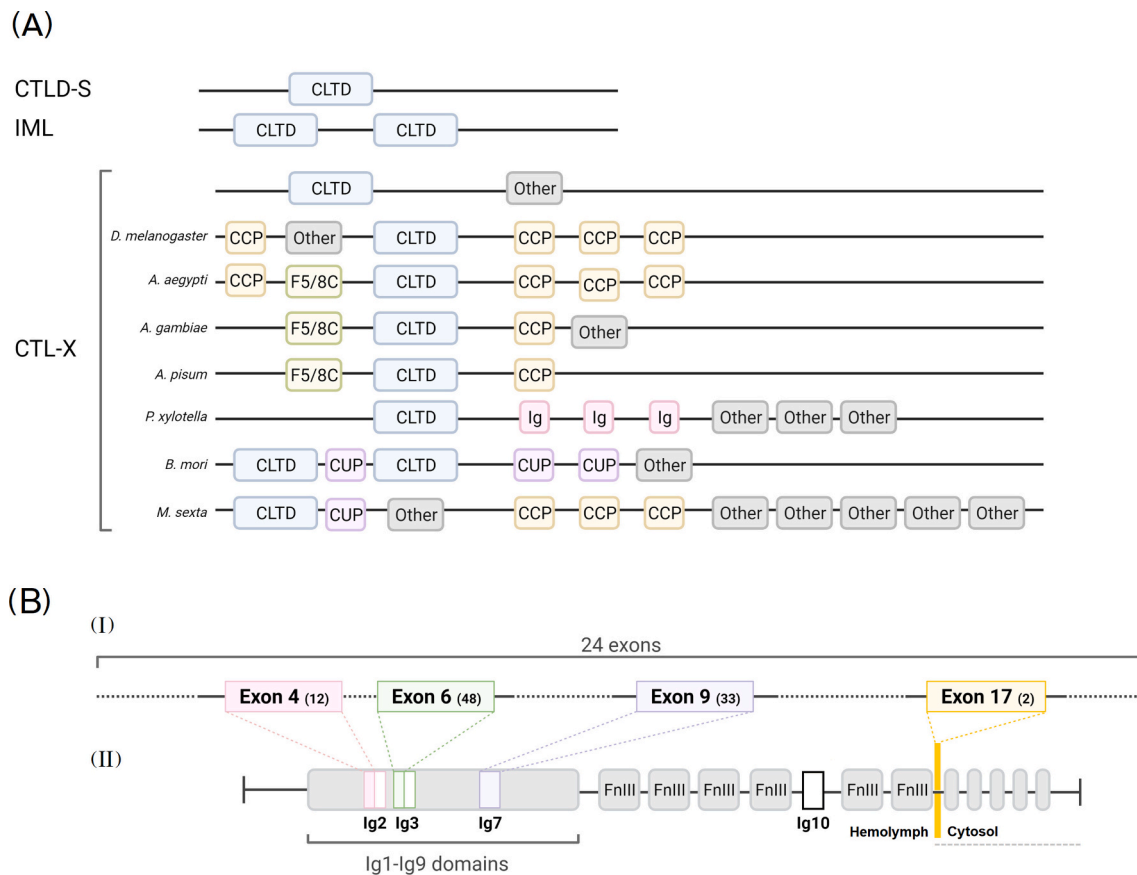


Fig. 4. Structural features of insect immune-related receptors. A. Domain architecture of selected insect C-type lectins (CTLs)/C-type lectin like domain (CTLTD)-containing proteins from seven insect models are modified after Fig. 1 in Reference [44] (reprinted with permission from Elsevier). CTLTD: C-type lectin-like domain; CCP: complement control protein domains (also known as short consensus repeats, characteristic to the complement system proteins); CUB: 110-residue protein motifs exhibiting a β -sandwich fold and mediating protein-protein interactions in various extracellular proteins; Ig: immunoglobulin-like domain (Ig-like receptor); F5/8C type C domain: discoidin domain (also known as C2-like domain). “Other domain”: = EGF-like domain: epidermal growth factor-like domain, or CBM: chitin-binding domain, or FN3 (fibronectin type 3 domain), or LDLRAD1: Low-density lipoprotein (LDL) receptor class A. Most insect CTLs contain single carbohydrate-recognition domain (CRD or CTLTD-S as depicted in the Figure), and CTLs with dual-CRD (the imlectin (IML) group) and containing one CRD with other functional domains (the CTL-X group) have also been identified in insects. In the CTL-X group, in addition to the CRD domain, other functional domains, such as CCP modules, EGF, CUB, Ig, F5/8 type C domain, and CBM), were identified. Comparing the structures of CTL-X, the CCP module is a common domain in the CTL-X group, however, the CUB, EGF and Ig domains are specific to lepidopteran insects of *B. mori* and *M. sexta*, while the F5/8 type C domain is widespread in dipterans and aphids. The CUB and Ig domains act in cell recognition or protein interactions, and the F5/8 type C domain is a module of blood coagulation factors. All these domains may assist and improve the binding capability of CTLs, and the domain organization of CTLs containing other functional domains may be species-specific due to evolution and selection pressure [44]. The domain and protein sizes are not in proportion. B. The Dscam in *Drosophila melanogaster*. Schematic genomic (I) and mature protein structure (II) after alternative splicing that generates hypervariable sequences in the 2nd, 3rd and 7th Ig domains and locations of alternative exon clusters [117]. Dscam contains 4 clusters of exons, each with array of possible exons. The number of exon cluster variants is indicated. These are spliced into the mRNA in an exclusive fashion, so that only one of each of the possible exons is represented. If all combinations of these exons are used in alternative splicing, Dscam can produce 38,016 different proteins [117]. Exons encoding for Ig domains are illustrated in coherent colors purple, green, pink, and orange for N-terminal halves of Ig2 and Ig3 domains, the full Ig7 domain and transmembrane (TM) domain, respectively. Two choices for the TM domain sequence also result from alternative splicing. All Dscam protein isoforms share the same overall domain structure as shown, but differ in the sequence in the colored regions. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

characteristic modules of carbohydrate-recognition domains (CRDs), functioning in pathogen recognition, cellular interactions, and innate immunity [31–34]. Immulectins in plasma of Lepidoptera are members of the C-type lectins with a dual carbohydrate-receptor domain [35–38]. They can bind to LPS, lipoteichoic acid, and β -1,3-glucan [39,40]. Immulectins have been shown to participate in innate immune responses, including phagocytosis, nodulation, encapsulation, and activation of PPO [38,41–44]. The roles of different insect CTLs in the antimicrobial immune response and in maintaining gut microbiome homeostasis have been reviewed elsewhere [38,44]. Here, a focus is given on domain organization, diversity and functionality of CTLs, e.g., immulectins, unique to insects (Fig. 4A).

Four C-type lectins, named immulectins (IML), have been purified from the plasma of bacteria-challenged *Manduca sexta* larvae. Each of these proteins contains two tandem C-type lectin carbohydrate recognition domains [39–41,45]. The lepidopteran IMLs differ from most animal CTLs, which contain only a single CRD, by having two CRDs [35,44]. CTLs with dual CRDs are also found in *Tribolium castaneum* [46] and crustaceans [47], but it is unknown whether they share a common ancestor or arose independently. The four *M. sexta* IMLs function as PRRs involved in PPO activation and encapsulation [38]. Among these, IML-2 is constitutively expressed at low levels in the fat body and its synthesis is induced higher than the other IMLs by injection of Gram-negative bacteria or LPS, but not by Gram-positive bacteria or yeasts.

IML-2 binds specifically to LPS and apparently contains two distinct binding sites, perhaps corresponding to its two domains. When IML-2 complexes with LPS, it stimulates the activation of PPO in plasma [39]. IML-1 is less specific in its binding properties and can bind to Gram-positive and Gram-negative bacteria, and to yeasts and cause these microorganisms to aggregate [41]. IML-1 is not detectable in plasma of most naive insects. Its mRNA levels increase after microbial challenge. IML-3 and IML-4 bind to *N*-acetylgalactosamine and glucose. IML-3 and IML-4 are expressed constitutively and remain unchanged in response to infection [40,45]. While IML-3 agglutinates *E. coli* via a calcium-dependent manner and does not facilitate melanization, binding of IML-4 to LPS always in a calcium-dependent manner enhances encapsulation and melanization.

LPS-binding protein (LPSBP) is a C-type lectin, which has been isolated and characterized from *Bombyx mori* [48]. It is a pattern recognition protein that recognizes the lipid A of LPS and is implicated in several cellular immune responses [49]. *B. mori* multibinding protein (BmMBP) constitutes two C-type lectins, BmLBP and BmMBP, too [50]. They were both purified from *B. mori* plasma, identified as humoral PRPs, and play a role in nodulation.

2.1.2. Thioester-containing proteins (TEPs)

Humoral factors such as the complement-like proteins represent a major component of the insect innate immune response against invading microbes [51–53]. TEPs are secretory glycoproteins, related to mammalian α 2-macroglobulins, that form covalent links to bacterial surfaces through their thiol-ester motifs [51]. Thus, they act as opsonins that promote phagocytosis, lysis, and melanization of microbes [52,54–56]. TEPs are expressed in the fat body and presumably secreted into the hemolymph and play a role in the regulation and activation of Toll, Imd, JAK/STAT, and JNK signalling [55,57,58]. *Anopheles gambiae* TEP1 (AgTep1) (ca.160 kDa) is processed upon infection with Gram-negative bacteria and the resultant C-terminal 80 kDa polypeptide binds to the bacterial cell surface via the γ -glutamyl ketone group of the peptide (the unique intrachain β -cysteiny- γ -glutamyl thioester), hence, it serves as a complement-like opsonin that promotes phagocytosis by plasmatocytes [59,60]. AgTep1 is secreted as an inactive protein in the hemolymph, and, upon activation, two leucine-rich repeat proteins are produced and circulated to regulate and promote the function of AgTep1 in pathogen recognition and killing [52].

2.1.3. Peptidoglycan recognition proteins (PGRPs)

PGRPs are highly conserved innate immune molecules that were first discovered in *B. mori* [61] and subsequently in other insects [62–64]. The PGRP family includes members having a homologous PGN-binding domain of ~165 amino acids long [65]. PGRPs can be categorized, based on their function, into catalytic or non-catalytic PGRPs [66]. Catalytic PGRPs have amidase activity that removes peptides from PGN chains and function as modulators of immune signalling pathways by sequestering PGN released by bacteria, e.g., PGRP-SB1, -SB2, -SC1A/B, -SC2 and -LB in *Drosophila* [67–70]. In the PGRP-LB structure, the catalytic Zn²⁺ binding site is formed by the side chains of His⁴², His¹⁵², and Cys¹⁶⁰ (the zinc-coordinating residues) and most likely through a water molecule by the conserved Tyr⁷ [71]. Non-catalytic PGRPs, such as PGRP-SA, -SD, PGRP-LC, and -LE, comprise the large majority of this protein family members [21]. They lack amidase activity and instead initiate a cascade of immune responses by recognizing and binding to PGN but do not hydrolyze them. This non-catalytic property is due to the absence in their binding groove of a key cysteine residue (zinc-coordinating residue; Cys¹⁶⁰) for Zn²⁺ binding, found in the catalytic counterparts. However, most non-catalytic PGRPs retain residues corresponding to His⁴² and His¹⁵² of the catalytic PGRP-LB [72].

Insect PGRPs are also grouped, according to their transcript length, into: short PGRPs (PGRP-S: -SA, -SB, -SC, and -SD), which are small (20–25 kDa) extracellular proteins, and long PGRPs (PGRP-L: -LA, -LB, -LC, -LD, -LE, and -LF) of up to 90 kDa, which can be intracellular,

extracellular, or transmembrane [67,69]. Many insect PGRPs are expressed in immune related tissues, such as the fat body cells, gut epithelia, and hemocytes. In most cases, PGRP expression is upregulated by exposure to PGN or bacteria [21,73].

Insect PGRPs are involved in the activation of innate immune signalling pathways. *Drosophila* PGRP-SA and PGRP-SD recognize bacterial Lys-type PGN which leads to the activation of Toll receptors [74,75]. Toll activation initiates a signal transduction cascade that results in the expression of drosomycin and other AMPs, which are primarily active against Gram-positive bacteria and fungi [10,74]. PGRP-LC acts as a transmembrane receptor of Gram-negative and bacilli DAP-type PGN upstream of the Imd pathway, with the contribution of PGRP-LE [76,77]. Both polymeric and monomeric DAP-type PGN can activate the Imd pathway and the minimal PGN motif for its effective induction is a disaccharide tetrapeptide known as tracheal cytotoxin (TCT, or GlcNAc-MurNAc(anhydro)-L-Ala- γ -D-Glu-meso-DAP-D-Ala) [78,79].

PGRPs are also responsible for triggering the PPO cascade, a proteolytic pathway in the hemolymph that leads to localized wound healing and melanization [16]. Several PGRPs, including PGRP-S from both *B. mori* [61] and *Tenebrio molitor* [80], and PGRP-LE from *Drosophila* [76,80], activate the PPO pathway following PGN recognition. In addition, PGRP-1 from *Holotrichia diomphalia* triggers this pathway by sensing β -1,3-glucan, a component of fungal cell wall [81].

2.1.4. Gram-negative bacteria-binding protein (GNBPs)

These proteins can bind to the cell wall of Gram-negative bacteria. *Drosophila*, for instance, has three different GNBPs, i.e., DmGNBP-1, -2, -3 [82], with GNBPs-1 and -3 that contribute to activation of the Toll pathway [83,84].

2.1.5. β -1,3-Glucan recognition protein (β GRP)

β GRPs are humoral PRRs which can interact with C-type lectins and PGRPs that bind to polysaccharides on the surface of microorganisms [82,85,86]. Their interaction leads to the activation of initiator peptidase(s) of the PPO activation cascade [86,87].

2.1.6. Hemolins

Hemolins have been initially isolated from *Hyalophora cecropia* and *M. sexta* [88,89]. They are hemolymph proteins belonging to the Ig supergene family (IgSF) and are composed of repeated immunoglobulin domains [90]. Hemolin synthesis in the fat body is strongly induced in bacteria-challenged *H. cecropia* and *M. sexta*, and hemolin gene expression is upregulated in bacteria-challenged *Hyphantria cunea* [91]. Hemolins have been observed to bind to fat body, Malpighian tubules, and hemocyte surfaces; thus, they may serve to recruit hemocytes to these tissues. *M. sexta* hemolins bind to Gram-negative and Gram-positive bacteria and promote aggregation. At the same time, hemolins can also bind to hemocytes. These properties suggest that hemolins might function as pathogen recognition proteins to modulate hemocytic immune responses [92,93]. Therefore, the ability of hemolins to interact with both bacteria and the surface of hemocytes suggests that they might work as opsonins to increase the efficiency of phagocytosis. The binding affinity of hemolins to glycolipids, that are widely present on Gram-negative and Gram-positive bacteria, suggest that they operate as PRRs with broad specificity in the defense against bacterial infection. Hemolins bind to LPS from Gram-negative bacteria and to lipoteichoic acid, a major component of the surface of Gram-positive bacteria [92,94]. It has been proposed that hemolins have two binding sites for LPS: one interacting with carbohydrate moieties in the O-antigen (cell wall antigens of Gram-negative bacteria which are components of LPS) and outer core regions of LPS, and the other interacting with the phosphate groups in its lipid A component [94,95]. Hemolins also mediate humoral immune activities by activating the PPO response [96].

2.2. Cellular PRRs

Cellular (membrane-bound) PRRs are implicated in cell-mediated immune responses by insect hemocytes against invading microbes [11,22,97]. They may also participate in few cases of humoral immune responses; for instance, the Toll and PGRP-LC receptors [98]. Furthermore, PGRP-SD upregulates the Imd pathway by promoting peptidoglycan re-localization to the cell surface [99,100]. Some of the hemocyte-surface receptors have direct mammalian homologs, whereas others appear to be exclusive to insects [101,102]. Cell surface receptors with roles in opsonin-independent phagocytosis, nodulation, and encapsulation include:

2.2.1. The Scavenger receptor SR dSR-CI

It belongs to class C scavenger receptors and is expressed in *Drosophila* hemocytes during embryonic development [103]. It recognizes Gram-positive and Gram-negative bacteria, but not yeasts. SR-CI expression is upregulated in larvae after exposure to bacteria [104]. It is conserved from insects to human and may represent one of the most primitive forms of bacterial recognition [105].

2.2.2. Peste

It is a class B scavenger receptor identified in S2 *Drosophila* cells that can bind certain intracellular bacteria [106].

2.2.3. Eater

It is a transmembrane protein with epidermal growth factor (EGF)-like repeats in its extracellular domain [107]. It is the first EGF-like repeat receptor shown to be involved in microbial recognition. Eater is expressed in plasmatocytes, appears to recognize a broad range of pathogens, and it mediates phagocytosis in a broad range of bacteria in *Drosophila* [107–109].

2.2.4. Nimrod

The Nimrod proteins are transmembrane proteins with 10 EGF-like repeats that are similar to those found in Eater and Draper from *Drosophila* [11,110]. Nimrod is a phagocytic receptor in *Drosophila* plasmatocytes and is involved in the phagocytosis of bacteria [111]. Nimrod-like genes are conserved in other insects, including the mosquito, *An. gambiae* [112], and the honeybee, *Apis mellifera* [113].

2.2.5. Noduler

It is a protein identified in *Antheraea mylitta*. It participates in the clearance of bacteria through a nodulation process [114]. Noduler specifically binds LPS, lipoteichoic acid, and β -1,3-glucan components of microbial cell walls, and mediates nodulation via p38/MAPK signalling [115].

2.2.6. Down syndrome cell-adhesion molecule (Dscam)

It is an Ig superfamily receptor [116]. The transmembrane forms of Dscam mediate efficient phagocytosis of bacteria in *D. melanogaster* (117) and *An. gambiae* [118]. Dscam can produce numerous isoforms in these insects via alternate splicing with distinct extracellular proteins. For instance, alternative splicing of the *D. melanogaster* Dscam generates hypervariable sequences in the second, third and seventh Ig domains (Fig. 4B). Therefore, Dscam is sufficient to provide a wide range of microbial recognition receptors in *Drosophila* [117]. Dscam soluble forms have been detected in hemocytes and cell-free hemolymph [119]. Dscam1 is involved in binding and uptake of *E. coli* by hemocytes and hence might act as an opsonin [119]. It is considered as a key immunological surveillance factor and might function similarly to antibodies [120,121]. The exact role of Dscam in the fly immune response still awaits further investigation, as a recent study did not identify any changes in Dscam1 splicing upon infection [122]. However, this contradicts another more recent report documenting the alternative splicing of Dscam in response to infection in the Chinese mitten crab, *Eriocheir*

sinensis [123].

2.2.7. Integrins

They represent a potential group of cell surface PRRs involved in microbial recognition. They are dimeric transmembrane receptors consisting of α - and β -subunits, and mediate hemocytic immune responses like phagocytosis and encapsulation [124–126]. In insect hemocytes integrins contain Arg-Gly-Asp binding motifs, which are the primary adhesive motifs present in many extracellular matrix molecules [127]. In *Drosophila* hemocytes, integrins are required for the proper encapsulation of wasp eggs [128,129]; in *Ceratitis capitata*, they are involved in phagocytosis of bacteria by plasmatocytes [130]. In plasmatocytes and granulocytes of the soybean looper, *Chrysodeixis* (= *Pseudoplusia*) *inclusens*, they take part in the recognition of both abiotic and microbial targets [131]. In *Pseudaletia separata*, tyrosine phosphorylation of the integrin β subunit and the adaptor protein P77 are critical for the activation of plasmatocytes and their spreading activity against bacteria upon induction through growth-blocking peptide signalling [132]. Integrin-antibody reactive proteins with possible $\alpha 5$ and $\beta 1$ epitopes can modulate immediate hemocyte function in *Galleria mellonella* [133].

2.2.8. PGRP-LC and (its coreceptor) PGRP-LE

These receptors are required for the activation of the Imd pathway by Gram-negative bacteria [21]. PGRP-LC also participates in phagocytosis of Gram-negative, but not Gram-positive, bacteria by *Drosophila* S2 cells [134]. Likewise, mosquito PGRP-LC is involved in the phagocytosis of bacteria and malaria parasites [135,136]. The *Drosophila* Imd pathway, which regulates the expression of dipterin and other AMPs that act against Gram-negative bacteria, is triggered by the binding of Dap-type PGN to the transmembrane receptor PGRP-LC in cooperation with PGRP-LE [137,138]. PGRP-LC has three alternative splice isoforms (PGRP-LCa, -LCx and -LCy) that have somewhat different specificities, whereas PGRP-LE exists as either a soluble intracellular or extracellular protein. PGN binding to PGRP-LC induces the formation of PGRP-LCa–PGRP-LCx heterodimers on the cell surface, which in turn induce downstream signalling [21].

2.2.9. Toll

In *Drosophila*, Toll consists of an extracellular leucine-rich repeat. The ligand of Toll receptor is Spaetzle (Spz) which is produced by its pro-Spz form after cleavage by the Spz-processing enzyme (SPE). This processing event leads to the activation of Toll signalling pathway, which controls the expression of AMPs that act against fungi and Gram-positive bacteria [29]. Toll receptor of *M. sexta* (MsToll), is also a typical single-pass transmembrane protein containing characteristic domain architecture of Toll and Toll-like receptors, including an extracellular domain comprising of leucine-rich repeats and a cytoplasmic TIR domain [139,140]. MsToll is expressed in the epidermis, fat body, hemocytes, Malpighian tubules, and midgut, and it may play a role in innate signalling in response to pathogenic invasion by activating the expression of AMP genes [139,141].

2.2.10. Croquemort and Drapper

Croquemort is a class B scavenger receptor, a CD36 homolog, and is a plasmatocyte-specific receptor that is present in several insect species [142]. It is expressed exclusively in plasmatocytes during embryogenesis and is mainly localized at the membrane surface of subcellular vesicles that contain apoptotic cell debris [142–144]. It participates in apoptotic cell recognition and apoptosis in *Drosophila* embryos and in phagocytosis of *Staphylococcus aureus*. In addition, Draper is a single-pass membrane protein with EGF repeats, which appears to be a receptor for phagocytosis of apoptotic cells, *S. aureus*, and *E. coli* by hemocytes in *Drosophila* [108,145–147]. Bacterial lipoteichoic acid has been identified as a ligand for Draper in *S. aureus* engulfment by plasmatocytes [147].

2.2.11. Plasmatocyte-spreading peptide (PSP) cognate receptor

PSP is an insect cytokine, first identified in *Chrysodeixis includens* [148]. The pro-PSP is activated by cleavage into a 23-residue PSP that mediates spreading of plasmatocytes. PSP acts through its cognate receptor [149]. PSP also acts in other insects, including *S. exigua*, in a dose-dependent manner [150]. In Lepidoptera, parasitoids and other encapsulation targets can induce proteolytic processing of proPSP producing the cytokine PSP, which induces changes of plasmatocyte morphology and behavior to rapidly adhere and spread across foreign surfaces [148–151]. Silencing PSP expression leads to impaired hemocytic antibacterial activity, as well as a reduction in overall cellular immune function in *M. sexta* [17]. PSP was proposed to act via a plasmatocyte-surface receptor, which stimulates the biosynthesis of prostaglandins (PGs) responsible for mediating plasmatocytes spreading [152–153].

2.2.12. The transmembrane LDL receptor-related protein 1 (LRP1)

LRP1, also known as CD91, is a large endocytic multidomain transmembrane receptor that belongs to a family of the multiligand low-density-lipoprotein receptors (LDLR) involved in regulation of cellular physiology and intracellular signalling [154]. In vertebrates, the cytoplasmic tail of LRP1 contains two NPxY motifs that can function as docking sites for cytoplasmic adaptor scaffolding proteins involved in signalling events, as well as sorting signals to endosomes and lysosomes [154]. LRP1/CD91 engage apoptotic cells and promote phagocytosis [155]. The related LDLR lipoprotein receptor-1 (LpR1) is acting in Toll-mediated immune response of *Drosophila* as an enhancer of the immune response [156]. LpR1 acts in scavenging of serpin/proteinase complexes via taken-up of the Necrotic serpin, a critical step in the regulation of proteolytic cascades. Knockdown of *An. gambiae* LRP1 decreases phagocytosis of *E. coli* by ca 80% and of *S. aureus* by 50% [135].

2.2.13. Fibrinogen-related proteins (FREPs)

The fibrinogen-like (FBG) domain, is named after the globular portions of vertebrate fibrinogen molecules, consists of ca 200 amino acid residues, and has high sequence similarity to the C-terminal halves of fibrinogen β and γ chains [157]. FREPs are evolutionarily conserved and contain a common C-terminal FBG domain and diverse N-terminal domains. They are ubiquitously found in vertebrates and invertebrates and have been linked to defense where the FBG domains are predicted to function in the recognition of carbohydrate moieties and their derivatives on the surface of microorganisms [158]. Surprisingly FREPs are the largest pattern recognition protein family in *An. gambiae*, with 61 putatively identified variants, whereas 14 members were found in *D. melanogaster* [158]. The FBN9 of *An. gambiae* was found to interact with the surface of Gram-negative and Gram-positive bacteria and knockdown of FBN22 and FBN39 resulted in mosquitoes losing their bacterial clearance capability [157]. The FBG-domain-containing protein Aslectin (AL-1) in the mosquito *Armigeres subalbatus* recognizes and binds to *N*-acetyl-*D*-glucosamine (GlcNAc) and likely acts in the antibacterial response [159].

3. Signal transduction

Signal transduction is the detection of specific signals at the cell surface by regulatory mechanisms where these signals are transmitted into the cell leading to the expression of certain genes and/or changes in cell behavior [160,161]. Detection of a signal by PRRs, as a result of pathogen recognition, is propagated via a signal transduction pathway that terminates with the activation of certain immune responses [3,22,162]. Intracellular signalling molecules of these pathways are organized as communication networks that process, encode, and integrate internal and external signals, and their relay stations are formed by multiprotein complexes (scaffold proteins, adaptor proteins, protein kinases, etc.) [3161]. The scaffold proteins bind protein to protein that usually function in sequence. The adaptor proteins recruit proteins that usually contain protein-protein interaction domains, and also protein

kinases and their substrates to complex [163].

3.1. Signalling molecules in hemocyte-mediated immune responses

Several homologs of vertebrate intracellular signalling molecules appear to function in insect hemocyte-mediated immune responses:

3.1.1. The monomeric G-proteins Rho

Rho GTPases are a family of small signalling monomeric G-proteins, and a subfamily of the Ras superfamily, including Rho, Rac, and Cdc42 [164,165]. These molecules are the main regulators of the actin network dynamics [165,166]. Rho GTPases are actively involved in regulating phagocytosis in mammalian cells (reviewed in [167]). The Rho GTPases Cdc42, Rac1, and Rac2, the Arp2/3 complex and Profilin act in phagocytosis in *Drosophila* [54,106,168–170], and Rac1 and Rac2 are also crucial for the encapsulation of eggs of the parasitoid wasp *Leptopilina boulardi* [171,172]. Ras/Rho/MAPs pathway operates in phagocytosis in response to LPS and *E. coli* in *C. capitata* via Ras and Rho protein homologs [173].

3.1.2. The proto-oncogene tyrosine-protein kinase Src

It is a non-receptor tyrosine kinase protein encoded by *c-Src* gene in human. It belongs to a Src kinase family. Src pathway controls a variety of biological processes, from cell proliferation and differentiation to cytoskeletal rearrangements and is involved in many immune signalling pathways including immunoreceptor as well as integrin signalling [174,175]. *Drosophila* C-terminal Src kinase participates in maintaining epithelial integrity through C-terminal Src kinase regulation [175]. Draper-dependent phagocytic activity in *Drosophila* glial cells is promoted through Src and Syk kinase signalling family (via an ITAM-domain-SFK-Syk-mediated signalling cascade) [176]. The *c-Src* homologue *Src64B* is able to activate the *Drosophila* cellular immune response. Overexpression of *Src64B* disrupts the sessile-haemocyte banding pattern, induces lamellocyte formation, and triggers F-actin formation and Jun kinase activation in plasmatocytes [177]. Src kinases also activate distinct responses during *in vivo* epidermal wound healing in *Drosophila* [178]. The involvement of Src (FAK/Src and MAPK signalling pathways) in phagocytosis has been shown in medfly hemocytes, too [179].

3.1.3. Focal Adhesion Kinase (FAK)

FAK is a 125-kDa cytoplasmic non-receptor protein tyrosine kinase that localizes to focal adhesions and contributes to the processes of integrin-mediated cell spreading and migration through regulation of actin cytoskeleton remodeling [180]. FAK functions as a phosphorylation-regulated scaffold to recruit Src. Activation occurs as a result of integrin binding to pathogens, which causes FAK autophosphorylation at Y397, resulting in a conformational change, which in turn allows Src or Fyn kinase to bind FAK. This coordinated sequence of events leads to mutual phosphorylation and activation of FAK and Src or Fyn. The active FAK/Src species then catalyzes phosphorylation of multiple substrates, therefore initiating a signalling cascade that regulates a large variety of cellular functions such as focal adhesion formation/turnover, phagocytosis, proliferation, migration, and survival [181,182]. The ability of FAK to modulate F-actin dynamics and Rho GTPase activity also enables it to control formation of lamellipodia, cell protrusions, and migration [181]. An insect FAK homolog, DFak56, has been reported to be expressed in *Drosophila* with strong homology to mammalian FAK, particularly those domains with key roles in its function [183]. Tyrosine phosphorylation of FAK in *C. capitata* hemocytes is significantly increased after immune activation with *E. coli* and FAK/Src complex is involved in promoting phagocytosis of the bacterial cells by hemocytes [184]. Silencing FAK significantly decreases the phagocytosis of bacteria and latex beads by *C. capitata* hemocytes [179].

3.1.4. Spleen tyrosine kinase (Syk)

Syk is a cytoplasmic 72 kDa non-receptor tyrosine kinase that contains two SRC homology 2 (SH2) domains and a kinase domain, and is expressed in a wide range of haematopoietic cells. Syk is a key player in diverse biological functions, including immune receptor signalling, cellular adhesion, and innate immune recognition [185,186]. Syk, the Syk homologue in *Drosophila*, is essential for Draper-dependent glial phagocytic activity [176]. Although represented in the *D. melanogaster* genome [187], further studies are required to determine whether a rudimentary form of the SYK pathway does function in immunity in flies.

3.1.5. Protein kinase C (PKCs)

PKC is a key family of enzymes involved in signalling pathways by phosphorylating substrates at serine/threonine residues in a variety of target proteins when activated by diacylglycerols. A wide variety of cellular events have been linked to the activation of PKC, including cell proliferation, changes in the cytoskeleton, and regulation of gene expression [188]. Generation of microbicidal ROI through the NADPH dual oxidase (DUOX) is a first line of defense in the *Drosophila* gut epithelia [189]. PKC is required for Cad99C (the orthologue of human Usher cadherin PCDH15)-dependent endosome formation and subsequent DUOX activation. In this aspect, PKC induces Ca^{2+} release from the endoplasmic reticulum, which is necessary for membrane associated DUOX activation and ROI cellular production [189].

3.1.6. Mitogen-activated protein kinases (MAPKs)

MAPKs are evolutionarily conserved and ubiquitously expressed eukaryotic protein kinases signalling cascades that autophosphorylate their own dual serine and threonine residues or those found on their substrates, to activate or inactivate their target [190,191]. MAPKs regulate important cellular processes such as intracellular metabolism, cell proliferation, external stresses, apoptosis, and immune defense. MAPK superfamily comprises of three protein kinase families, their isoforms are grouped according to their activation motif, structure and function [190]: the extracellular signal-regulated protein kinases (ERK1/2), the c-Jun N-terminal kinases (JNK1/2/3), and the p38 family of kinases (p38 MAPK α , β , δ , and γ). In *Drosophila*, genes encoding MAPKs *Rolled* [192], *Basket* [193], and *Dp38* (dMAPK^{p38a} and dMAPK^{p38b}) [194], which are homologous to mammalian ERK, JNK, and p38, respectively, have been characterized. p38 MAPK-dependent phagocytic encapsulation of bacteria is not restricted to resistance mechanisms but confers infection tolerance in *Drosophila* [195]. Further, p38 MAPK pathway regulates *Drosophila* immune and intestinal stem cells gene expression, via Ras/MAPK signalling [192,196]. It is known that alternative signalling pathway(s) may also activate MAPKs in response to the same ligands. MAPKs function downstream of FAK, and their activation is important for phagocytosis by *C. capitata* hemocytes [179]. MAP kinases mediate phagocytosis and melanization via PPO activation in *C. capitata* hemocytes [197]. p38 MAPK activation is required for *B. mori* cytokine paralytic peptide-dependent defensive responses, including phagocytosis [198].

3.1.7. Phosphoinositide 3-kinase (PI3K)

PI3K is a heterodimer consisting of a 110-kDa catalytic subunit (p110 α , β or δ) and a regulatory subunit (p85 α , p85 β , p55 α , p50 α , or p55 γ). PI3K phosphorylates the inositol head group of phosphatidylinositol lipids on the 3' position, allowing these to serve as ligands and functional regulators for proteins at the inner leaflet of the plasma membrane and on intracellular membranes [199–201]. PI3K binds to FAK via Y397 *in vivo* and *in vitro*. PI3K controls several cellular responses including cell growth, survival, cytoskeletal remodeling, and trafficking of intracellular organelles [201]. PI3K regulates important aspects of immune cell development, differentiation, and function [202]. PI3K signalling pathway is crucial for phagocytosis in *Drosophila*, it contributes to phagocytosis of bacteria in *M. sexta* and endocytosis of LPS by

C. capitata hemocytes [173,203–205]. Class I, II, and III PI3K are also involved in apoptosis and autophagy in *Drosophila* [206–211].

3.1.8. *Caenorhabditis elegans* CED intracellular homologues

These are intracellular proteins that trigger the internalization of bacteria. For example, *C. elegans* CED-1 (cell death abnormality protein-1) is a transmembrane protein similar to human SREC (scavenger receptor from endothelial cells) [212]. These proteins were observed to act in phagocytosis of apoptotic bodies in *C. elegans* [213]. Draper is the *D. melanogaster* orthologue of CED-1 of *C. elegans*, which triggers the engulfment machinery in *Drosophila* and mammals [11,214,215]. In *An. gambiae*, it has been demonstrated that a number of genes homologous to CED of *C. elegans* significantly affect phagocytosis of bacteria [216]. In the same context, simultaneous inactivation of both CED5 and CED6 homologs of *An. gambiae* result in a 80% decrease of phagocytosis [135].

3.1.9. Janus tyrosine kinases (JAKs)

JAKs are signalling proteins that function downstream of many tyrosine kinase receptors and activate the transcription factor STAT, which translocates to the nucleus. Janus kinase/signal transducers and activators of transcription (JAK-STAT) signalling pathway takes place in the fat body and in lymph gland-derived hemocytes (described below) [30,217].

3.1.10. Eicosanoids

Eicosanoids are metabolites of some fatty acids, including arachidonic acid and two other 20C polyunsaturated fatty acids. They are biosynthesized by enzymatic oxygenation reactions of these fatty acids. Groups of eicosanoids include prostaglandins, lipoxygenase products, and epoxyeicosatrienoic acids. Eicosanoids play key roles in mediating and coordinating insect cellular, and several humoral immune reactions to infection and invasion [153]. Studies in different insect species have documented the involvement of eicosanoids in insect hemocyte immune responses to bacteria, including phagocytosis, nodulation, hemocyte spreading and microaggregation. In addition, eicosanoids mediate PPO cascade activation only at the post-transcriptional level by inducing the release of PPO from oenocytoids, via hemocyte rupture [218,219].

3.1.11. The prophenoloxidase (PPO) activation cascade

PPO is a key element of the immune system in insects [220–224]. It is composed of several proteins, including PPO, serine proteases [225–227], and their zymogens, as well as serine protease inhibitors or the serpins [228,229]. This cascade is activated via different pathogenic agents or via physical spiking of integument breaching the epidermis (abiotic stress). The PPO activation system represents a pleiotropic pathway that participates in several humoral and cellular defense reactions including melanization, phagocytosis, nodulation, encapsulation, wound healing/hemolymph coagulation as well as cuticular sclerotization process [223]. Through this cascade, cell-free and hemocyte-surface inactive PO is converted to active PO, with generation of reactive cytotoxic intermediates of quinones, oxygen (ROI) and nitrogen (RNI) that eventually target the foreign microbe entrapped in the melanotic nodules and capsules [20].

3.1.12. Dopa decarboxylase (Ddc)

Ddc is a multifunctional enzyme that decarboxylates dopa to form dopamine. Ddc is critically involved in wound healing, parasite defense, and cuticle hardening and melanization [15,230,231]. PO-deficient or Ddc-deficient mutant *Drosophila* are significantly compromised in their cellular immune responses, such as encapsulation [232]. Silencing *Ddc* gene significantly reduces the rate of melanization in mosquitoes [233,234]. Surface-associated Ddc-dependent pathways regulate phagocytosis, nodulation, and melanization in *C. capitata* haemocytes due to insufficiency of hemocyte-surface PPO for the bacterial uptake by the hemocytes [179,235].

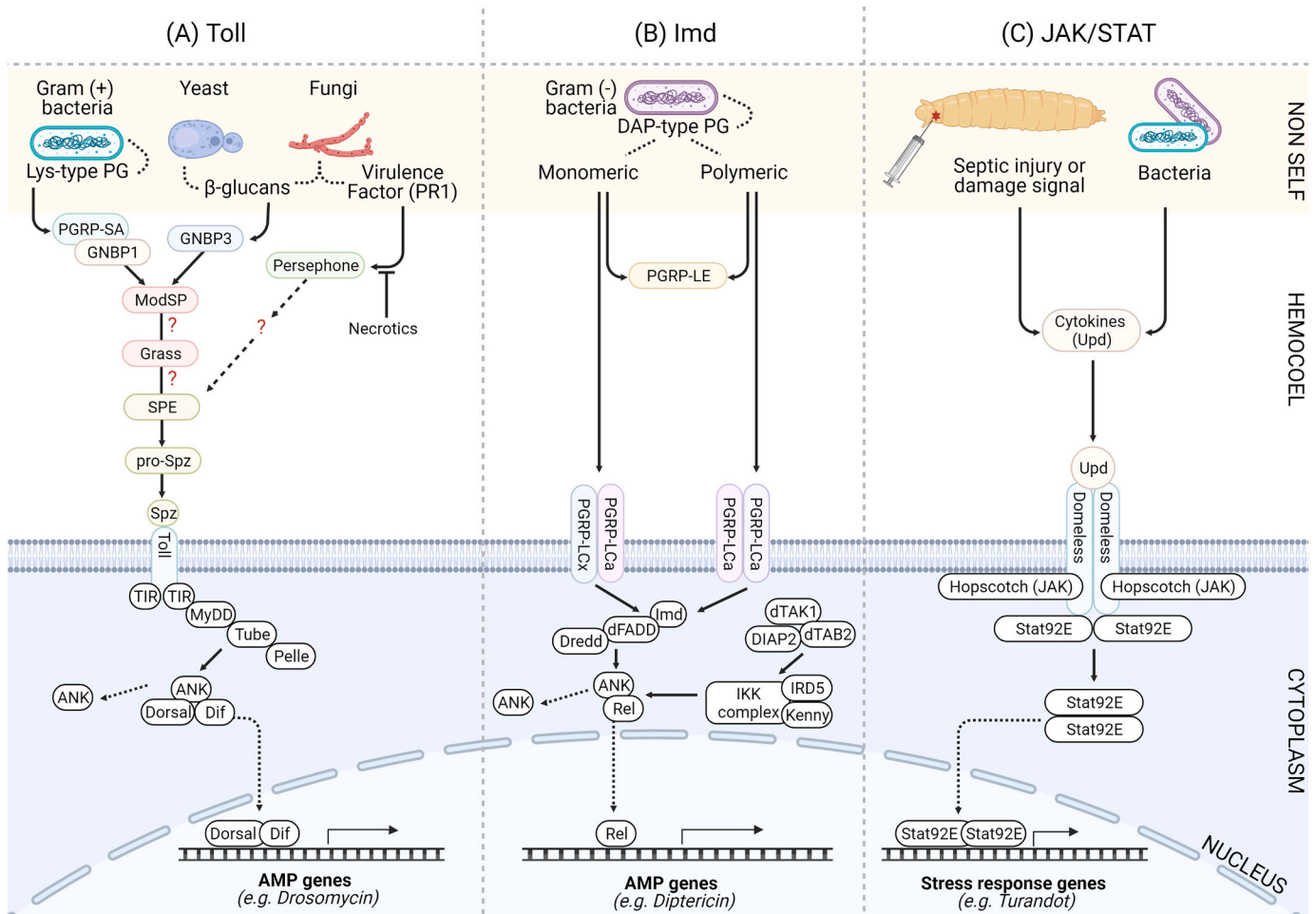


Fig. 5. Pathways of innate immune activation and regulation in insects. The three immune signal transduction pathways Toll (A), Imd (B), and JAK-STAT (C) in *Drosophila melanogaster* and the immune responses activated against bacteria and fungi. (A) The Toll receptor is activated after binding to the ligand Spaetzle (Spz), which is previously cleaved by the Spaetzle-processing enzyme (SPE). This endopeptidase acts downstream of pathogen recognition receptors PGRP-SA, GNBP1, protease Grass and Modular serine protease (ModSP) in response to Gram-positive bacterial infection, GNBP3 in response to yeast and fungal infection, and downstream of protease Persephone in response to fungal virulence factors and is regulated by Necrotics. Adaptor proteins myeloid differentiation primary response (MyDD), Tube and Pelle are recruited following Toll receptor activation. The ankyrin repeats (ANK) of Cactus bind to the transcription factors Dorsal and Dorsal-related immunity factor (Dif), and upon phosphorylation and degradation of Cactus, Dif or Dorsal dissociate and translocate to the nucleus where they induce the expression of antimicrobial peptide genes (AMP), such as *Drosomycin*. (B) The Immune deficiency (Imd) pathway is activated by Gram-negative bacteria through the pathogen recognition receptors PGRP-LC and PGRP-LE. Immune deficiency (Imd) is an adaptor protein that interacts with the *Drosophila* Fas-associated death domain (dFADD) and the Death related ced-3/Nedd2-like caspase (Dredd). Imd cleavage activates the TGF-beta activated kinase 1 (TAK1), TAK1-associated binding protein 2 (TAB2) and Death-associated inhibitor of apoptosis 2 (DIAP2) complex, which then induces the inhibitor of κ B kinase (IKK) complex that consists of Immune Response Deficient 5 (IRD5) and Kenny. The transcription factor Relish is cleaved by Dredd and the Rel domain is released from the ankyrin-repeat domain (ANK) and translocates to the nucleus to trigger the expression of antimicrobial peptide (AMP) genes, like *Diptericin*. (C) The Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway is induced by septic injury/damage signals or bacterial infection through binding of the Unpaired (Upd) cytokines to the cytokine receptor Domeless. Then, the JAK kinase Hopscotch phosphorylates Domeless and Stat92E, which is originally bound to the receptor. Stat92E disconnects from Domeless, dimerizes and then translocates to the nucleus to activate the expression of stress response genes, such as *Turandot*.

3.2. Signal transduction pathways

Drosophila can discriminate between classes of PAMPs by operating through two distinct NF- κ B pathways, Toll and Imd, which regulate AMP production by activating their NF- κ B transcription factors Dorsal/Dif and Relish, respectively [29,30,236–238]. In *Drosophila*, seven groups of distinct inducible AMPs have been identified [239,240]. Their activity is directed either against Gram-positive (Defensin) or Gram-negative bacteria (Diptericin, Drosocin, Attacins, Cecropins), as well as against filamentous fungi (Drosomycin, Metchnikowin). These AMPs are released into the hemolymph following their synthesis. The activation of the Imd pathway in response to bacterial challenge is rapid since signal transduction up to nuclear translocation of Relish occurs within minutes. Transcription of target genes, especially those encoding AMPs,

peaks within hours. In comparison, the Toll pathway is activated within hours, and the transcription of the target gene *Drosomycin* occurs for days. Therefore, quick response of the Imd pathway is likely more effective against fast-replicating bacteria [237].

3.2.1. The Toll-signalling pathway

This pathway is implicated in the regulation of genes encoding AMPs against Gram-positive bacteria (e.g., Defensins) and fungi (e.g., Drosomycin) (Fig. 5A) [28,29]. The activation of Toll signalling is not dependent on a direct binding of the membrane receptor Toll to PAMPs but is due to binding of a processed form of the cysteine-knot cytokine-like protein, the activated Spz [241–244].

Genes encoding Toll-related receptors have been identified in the *D. melanogaster* genome and share a similar molecular structure with an

ectodomain mainly composed of leucine-rich repeats and cysteine-rich flanking motifs [245,246]. Toll, or Toll-1, receptor is responsible for the induction of AMP genes via the Toll pathway. *Drosophila* Toll receptors have a cytosolic domain called Toll/IL-1R (TIR) domain, which interacts with adaptor molecules, thereby activating downstream signalling events [247–249].

In the *Drosophila* Toll pathway, extracellular recognition factors initiate peptidase cascades able to process the inactive precursor proSpz that leads to the activation of the Toll receptor ligand Spz [243,250,251]. This ligand is synthesized and secreted in the form of proSpz consisting of a prodomain and a C-terminal region (C-106) [251,252]. In non-signalling conditions, the prodomain of Spz masks a predominantly hydrophobic C-terminal Spz region. Activation induces proteolysis, which causes a conformational change exposing determinants that are critical for binding of the Toll receptor [243]. Remarkably, the prodomain remains associated with the C terminus and is only released when the Toll extracellular domain binds to the complex [243]. Two models for the binding of Spz to Toll have been suggested, the first of which implies that one Spz dimer binds to two Toll receptors [253]. The second model suggests that two Spz dimers, each binding to the N-terminus of one of the two Toll receptors, trigger a conformational change to activate downstream signalling [242].

A terminal serine peptidase called Spz-processing enzyme (SPE) is responsible for Spz cleavage [251]. For activation of this SPE, upstream cascades with various molecular components, depending on the invading microorganism, are present. Two peptidase cascades leading to the activation of the serine peptidase Grass are initiated by cell wall components of both fungi (β -1,3-glucan) and Gram-positive bacteria (Lys-type PGN) [254,255]. In addition, other serine peptidases, such as *spirit*, *spheroid*, and *sphinx1/2*, were identified in response to both fungal and Gram-positive bacterial invasion [255]. Upstream of Grass, a modular serine peptidase (ModSP), i.e., the most upstream serine peptidase of the *Drosophila* Toll cascade, which is conserved in insect immune reactions, plays an essential role in integrating signals originating from the PRRs GNB3 for fungi and PGRP-SA/GNB1 complex for Gram-positive bacteria and connecting them to the downstream serine peptidases, the Grass-SPE-Spz cascade [256]. An additional peptidase cascade leading to the activation of SPE is mediated by the peptidase Persephone, which is activated by Gram-positive bacteria and fungi, and is proteolytically matured by the secreted fungal virulence factor PR1 and Gram-positive bacterial virulence factors [250,254].

The recognition of the Gram-positive bacterial Lys-type PGN and the fungal β -1,3-glucan carried out by the PRRs PGRP-SA, PGRP-SD and GNB1 for Lys-type PGN, and GNB3 for fungi [250]. Upon Gram-positive bacterial recognition, PGRP-SA and GNB1 interact physically with a circulating PRR complex upstream of the Toll pathway [74,257]. Thereafter, activated GNB1, which has enzymatic activity, hydrolyzes the Lys-type PGN and produces new glycan reducing ends, which are presented to PGRP-SA [258]. An alternative suggestion is that GNB1 has no enzymatic activity and acts as a linker between PGRP-SA and ModSP [256]. Also, PGRP-SD has been proposed to function as a receptor for Gram-positive bacteria and it may further act in the recognition of DAP-type PGNs responsible for the activation of the Toll pathway by Gram-negative bacteria [75,259].

After binding of the processed Spz to the activated Toll receptor, the latter binds to the adaptor protein MyD88 via intracellular TIR domains (The Toll/interleukin-1 receptor homology domains) [260–262]. Upon this interaction, MyD88, Tube, and the kinase Pelle are recruited to form a MyD88-Tube-Pelle heterotrimeric complex through their death domains (DD)-mediated interactions in the same sequence [262–264]. A highly conserved Pelle/IL-1R-associated kinase (IRAK) interacting protein Pellino has been shown to act as a positive regulator of Toll signalling [265].

From the trimeric MyD88-Tube-Pelle complex, the signal proceeds after phosphorylation and degradation of the *Drosophila* I κ B factor Cactus, which is bound to the NF- κ B transcription factor(s) Dorsal and/

or Dif, inhibiting their activity and translocation into the nucleus [266]. This phosphorylation is achieved by Pelle [267]. Dorsal and/or Dif then translocate into the nucleus where they activate the expression of anti-bacterial and antifungal AMPs [268–274].

3.2.2. The Imd signalling pathway

This pathway is predominantly implicated in the regulation of genes encoding AMP against Gram-negative bacteria including, for instance, Cecropins, Drosocin, Diptericin and Attacins (Fig. 5B) [240–275]. This occurs through regulation of activity of the NF- κ B transcription factor Relish [276]. Similar to the Toll pathway, microbial recognition is the first step initiating the immune responses via the Imd pathway. This first step includes the detection of highly conserved microbial structures (PAMPs) via genome-encoded PRRs [23].

The Imd pathway components include the receptor PGRP-LC with the PGRP-LE as a coreceptor [134,137,138,277], the Imd [278], TGF- β (Transforming growth factor beta) activated kinase 1 (TAK1) [273,279–281], the *Drosophila* Fas-associated protein with Death Domain (dFADD) [282,283], the caspase-8 homolog Death-related ced-3/Nedd2-like protein (DREDD) [275,278,284], the *Drosophila* inhibitor of κ B kinase (IKK) complex [279], and the NF- κ B transcription factor Relish [276]. Imd signalling is supplemented by TAK1-associated binding protein (TAB2), the ubiquitination machinery components E3 ligase inhibitor of apoptosis 2 (IAP2), which associates with the E2 ubiquitin-conjugating enzymes Bendless (Ubc13), Uev1a (a ubiquitin conjugating enzyme variant), and Effete (Ubc5), and the transcription cofactor Akirin [281,285–288]. Furthermore, several negative regulators, which function at various steps in the pathway have been characterized [69,289–293].

The PAMP which triggers the Imd pathway is the DAP-type PGN of the cell wall of most Gram-negative bacteria, as well as some Gram-positive bacteria, such as *Bacillus* and *Listeria* species [73,77,78,294,295]. The PGRP-receptors implicated in the Imd pathway are the two non-catalytic PGRPs PGRP-LC on the plasma membrane, and the intracellular PGRP-LE, which binds specifically to DAP-type PGN. Once bound to PGN, these receptors likely dimerize or multimerize and subsequently intracellular signal is transmitted to the adaptor protein Imd [78,134,137,277,294,295].

PGRP-LC encodes alternative splice variants, i.e., *PGRP-LCa*, *PGRP-LCx*, and *PGRP-LCy* (in Flybase they are referred to as *PGRP-LC-RB*, *PGRP-LC-RA*, and *PGRP-LC-RC*, respectively) that have been characterized previously [296]. All the proteins encoded by these transcripts have identical transmembrane and cytoplasmic parts [79]. PGRP-LCx and PGRP-LCa are well-characterized and have differential binding specificities for different forms of DAP-type PGN [78,294,297]. PGRP-LCx binds polymeric DAP-type PGN in a deep binding cleft, and this polyvalent ligand may cluster multiple PGRP-LCx receptors; these clusters may trigger downstream signalling events [294,298]. On the other hand, the binding cleft of PGRP-LCa is occluded for binding polymeric PGN [298]. Monomeric fragment of DAP-type PGN, i.e., TCT, is able to interact with the binding cleft of PGRP-LCx, and subsequently PGRP-LCa interacts with this complex through both protein-protein and protein-PGN interactions to form a ligand-induced heterodimer [294,298,299,300,301].

PGRP-LE participates in DAP-type PGN recognition [76,77,294,297]. It is localized in the cytoplasm, where it recognizes DAP-type PGN that may enter the cytosol [300,302] or those introduced by cytosolic bacteria [297]. Moreover, PGRP-LE may be processed and released from cells and thereafter associates with the extracellular domain of PGRP-LC in recognition of the extracellular DAP-type PGN [77,303]. Upon TCT binding, PGRP-LE is known to form homopolymers, with atomic interactions nearly identical to those observed in the PGRP-LCx-PGRP-LCa heterodimer [294].

The mechanism by which DAP-type PGN binding to its receptors triggers the downstream signalling events is currently unknown [304]. The cytoplasmic N-terminal domains of PGRP-LC and PGRP-LE are

Table 1
Peptidoglycan recognition proteins (PGRP-) and their possible functions in *Drosophila*

Gene	Gene variant	localization of protein function	N-acetylmuramoyl-L-alanine amidase activity	Type of PGN recognized	Pathway	Function(s)	Reference (s)
-LA	-LAa (LA _D) ^a	transmembrane	–	Not bind to PGN ^b	Imd	Lacks the PGRP domain; Positively regulates the Imd pathway in barrier epithelia	[67], [305]
	-LAb (LA _E)	transmembrane	–	Not bind to PGN		Contains both a putative transmembrane domain and a PGRP domain	[67], [305]
	-LAc (LA _C)	transmembrane	–	Not bind to PGN		Encodes a short protein of 138 amino acids composed exclusively of a N-terminus-truncated PGRP domain	[63], [305]
	-LB	extracellular ^c	+	Dap-type	Imd	Modulates the magnitude of the Imd pathway activation (a negative feedback regulator); Controls the immune reactivity to ingested bacteria in the gut	[10], [69]
-LC	-LCa	transmembrane	–	Dap-type	Imd	Heterodimerization with PGRP-LC; induction of phagocytosis	[134], [301]
	-LCx	transmembrane	–	Dap-type	Imd	Recognition of monomeric Dap if heterodimerized with PGRP-LCa; Recognition of polymeric Dap-type LPS if alone; induction of phagocytosis	[134], [301]
	-LCy	transmembrane	–	Dap-type	Imd	Recognition of Gm ⁺ bacteria	[303]
-LD		transmembrane	–	Dap-type ^d		ND	[67]
-LE		transmembrane	–	Dap-type	Imd	Multimerizes in large complexes to recognize Gm ⁺ bacteria; activation of PPO cascade; induction of autophagy	[294], [303]
-LF		transmembrane	–	Dap-type	Toll	A specific negative regulator of the Imd pathway, possibly a decoy receptor	[290]
-SA		extracellular	–	Lys-type	Toll	Recognition of Gm ⁺ bacteria and acting with GNBPI	[74]
-SB-1		extracellular	+	Dap-type		ND	
-SB-2		extracellular	+	Dap-type ^d		ND	
-SC-1A		extracellular	+	Dap- and Lys-type	Toll	Recognition of Gm ⁺ bacteria, induction of <i>in vitro</i> cleavage of PGN; induction of phagocytosis	[68]
-SC-1B		extracellular	+	Dap- and Lys-type	Toll	Recognition of Gm ⁺ bacteria, <i>in vitro</i> cleavage of PGN; induction of phagocytosis	[68]
-SC-2		extracellular	+	Dap-type ^d		ND	
-SD		extracellular	–	Dap- and Lys-type	Toll	Enhances binding between PGRP-SA and GNBPI (trimerization)	[75]

^a Following Flybase nomenclature.

^b Structural predictions indicate that PGRP-LA would not bind to PGN, pointing to a regulatory role of this PGRP [305].

^c PGRP-LB lacks the signal peptide and transmembrane region, thus possibly making it a cytosolic protein [69].

^d Predicted PGN type recognized; ND, not determined.

important for signal transduction, however their structure and interaction mechanisms are poorly understood [304]. The interaction with Imd, and possibly other downstream components, may be triggered by multimerization or clustering of PGRP-LC or PGRP-LE, as mentioned above, or is due to a PGN-induced conformational change that is transmitted to the signalling domains, or is probably the result of both events [237]. Distinct functions of different PGRPs in Imd signalling are continuously evolving. For instance, the non-catalytic PGRP-LA encoded by a gene of the PGRP-LC genomic cluster was recently reported to fine-tune the Imd pathway in barrier epithelia [305]. The PGRP-S and -L variants identified in *Drosophila* as pattern recognition receptors are implicated in both Imd and Toll signalling pathways or they act as soluble extracellular effectors, beside other immune-related regulatory functions, as shown in Table 1.

Once DAP-type PGN is bound to the receptor, a signalling complex is recruited and formed. Imd recruits dFADD and the caspase DREDD. Imd interacts via its death domain with the *Drosophila* dFAAD which in turn recruits DREDD to the signalling complex [138,275,278,282]. DREDD is activated by ubiquitination by IAP2, which associates with the E2 ubiquitin conjugating enzyme [285,287]. Once activated, DREDD cleaves Imd just after Asp³⁰, removing the N-terminal fragment and exposing a highly conserved interaction (new binding) site for the E3 ubiquitin ligase IAP2, which is then further activated by K63-ubiquitination (a ubiquitin molecule is bonded by its C-terminus to a specific lysine residue, Lys⁶³) [286,287]. The K63-polyubiquitin chains recruit and activate TAK1 via the ubiquitin-binding domain of its regulatory partner TAB2. TAK1 is responsible for activating both the JNK and IKK/Relish branches of the Imd pathway [273,279,281]. Relish is activated by the phosphorylation of multiple sites at its N-terminus by the IKK complex [279]. The *Drosophila* IKK complex consists of two

subunits, i.e., the catalytic subunit called immune response deficient 5 (IRD5) and the regulatory subunit Kenny (which are homologous to mammalian IKK β and IKK γ , respectively) [306]. The IKK complex is required for the activation of Relish [279,306]. It was shown that phosphorylation of serine residues Ser⁵²⁸ and Ser⁵²⁹ of Relish is required for efficient recruitment of RNA polymerase II to the promoters of Relish target genes [306]. Relish consists of both an N-terminal NF- κ B Rel homology domain and a C-terminal ankyrin-repeat (IkB-like domain) [284]. The N-terminal transcription factor domain is released by endo-proteolytic cleavage [307]. The C-terminal part (Rel-49) remains in the cytoplasm and the active N-terminal part (Rel-68) translocates into the nucleus to activate the transcription of genes encoding AMPs [284,307,308]. Like in mammalian TNFR (the tumor necrosis factor receptor superfamily) signalling, the *Drosophila* Imd pathway forficates into the JNK pathway at the level of TAK1 and TAB2 [309–311].

The subcellular localization and interaction of the Imd pathway components and whether or not they change upon immune stimulation is not currently clear. PGRP-LC resides at the plasma membrane, yet Imd is mostly localized in the nucleus and can only be detected at the plasma membrane when the signalling pathway is activated [312]. In addition to Imd, and analogous to mammals, dFADD may be localized in the nucleus [313–315]. Therefore, the mechanism of Imd, dFADD, and DREDD recruitment to the signalling complex, and their presumed transport to the plasma membrane need further clarification.

3.2.3. The JAK-STAT-signalling pathway

The JAK-STAT cascade is a conserved, intracellular signalling pathway playing a central role in hematopoiesis, cell movement, cell proliferation and differentiation, and regulation of cellular immune reactions (Fig. 5C) [30,316,317]. It takes place in the fat body cells and is

Table 2
Diversity of selected immune-related genes and gene families across phylogenetically distinct insect orders

Order	Odonata	Orthoptera	Isoptera	Blattodea	Hemiptera	Hemiptera	Hemiptera	Hemiptera	Phthiraptera	Coleoptera	Coleoptera	Hymenoptera	Hymenoptera	Hymenoptera	Lepidoptera	Lepidoptera	Lepidoptera	Diptera	Diptera	Diptera
Insect species	<i>Coenagrion puella</i>	<i>Locusta migratoria</i>	<i>Zootermopsis nevadensis</i>	<i>Blattella germanica</i>	<i>Cimex lectularius</i>	<i>Acyrtosiphon pisum</i>	<i>Nilaparvata lugens</i>	<i>Bemisia tabaci</i>	<i>Pediculus h. capitis</i>	<i>Tenebrio molitor</i>	<i>Tribolium castaneum</i>	<i>Apis mellifera</i>	<i>Nasonia vitripennis</i>	<i>Camponotus floridanus</i>	<i>Bombyx mori</i>	<i>Manduca sexta</i>	<i>Spodoptera frugiperda</i>	<i>Anopheles gambiae</i>	<i>Aedes aegypti</i>	<i>Drosophila melanogaster</i>
Function																				
RECOGNITION																				
CTLs	–	14	23	69	7	5	9	5	9	12	16	12	31	10	21	34	9	22	39	34
PGRPs	4	14	6	8	1	0	2	1	1	6	7	4	12	4	12	10	10	7	8	13
GNBPs (βGRPs)	2	5	6	7	1	2	7	5	0	3	3	2	3	2	4	4	4	6	7	3
FREPs	–	–	4	3	2	2	4	–	4	–	7	2	0	4	3	4	–	61	37	14
TEPs	1	6	6	6	–	2	–	1	3	4	4	4	3	4	3	2	4	15	8	6
Hemolin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0
Dscam	1	99	12	1	3	1	9	5	1	–	1	1	–	50	1	1	3	1	1	3
Draper like	–	0	2	–	1	4	1	3	0	–	3	1	1	1	4	2	2	1	1	1
Nimrods																				
Eater	–	0	0	–	–	0	1	–	–	–	–	0	0	1	0	1	2	1	1	1
SIGNALING																				
Toll																				
Spaetzle	1	4	5	6	2	6	8	9	3	7	7	2	9	1	3	8	4	6	9	6
TLR	3	21	7	8	1	6	6	5	6	2	9	5	9	5	14	16	12	11	12	9
MyD88	1	1	0	0	1	1	2	1	1	1	1	1	1	1	1	1	2	1	1	1
Tube	–	0	2	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Pelle	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Cactus	1	4	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1
Dorsal	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dif	0	0	1	1	0	1	0	0	1	1	1	1	1	0	1	1	0	0	1	1
Imd																				
IMD	1	2	1	0	0	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1
FADD	–	1	1	1	0	0	–	0	0	1	1	1	1	1	1	1	1	1	1	1
DREDD	1	1	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
IAP2	1	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
TAK1	1	5	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1
Tab2	1	–	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
IKKγ (Kenny)	1	0	1	0	0	0	1	–	–	1	1	1	–	1	1	1	1	1	1	1
IKKβ (Ird5)	1	1	1	1	0	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1
Relish	1	1	1	1	1	0	1	0	1	2	2	2	6	1	1	2	1	1	1	1
JAK STAT																				
Dome (less)	1	2	2	0	1	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Hopscotch (JAK)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1
Stat92E	1	2	1	0	1	2	1	1	1	1	1	1	1	1	1	1	1	2	1	1
JNK																				
Bsk (JNK)	1	1	1	1	2	1	1	1	1	3	1	1	1	2	1	1	1	1	1	1
Hemipterous (Hep)	1	–	5	5	–	1	1	–	1	1	1	1	1	–	1	1	1	1	1	1
Jra/Jun	1	1	2	1	2	1	1	–	1	–	1	1	1	1	1	1	1	1	1	1
REFERENCE	[334]	[335]	[336]	[336]	[337]	[338]	[339]	[340]	[341]	[342]	[46,343]	[344]	[345]	[346]	[347]	[348]	[349]	[350]	[328]	[351]

–: not determined.

Additional searches in Ensembl Metazoa release 49: <http://metazoa.ensembl.org>, ImmunoDB: <http://cegg.unige.ch/Insecta/immunodb>, and NCBI: <https://www.ncbi.nlm.nih.gov/protein/>, VectorBase beta: <https://www.vectorbase.org> were also performed. The phylogenetic listing of insect orders is based on reference [333].

elicited upon septic injury as well as bacterial [318,319] and fungal [320–321] infections. This pathway is activated by elicitors that induce the expression of the cytokines unpaired (Upds) in hemocytes [321,322]. In *Drosophila*, the JAK-STAT pathway is comprised of the Unpaired-related ligands (Upd, Upd2, and Upd3) which are presumably released into the hemolymph and act as ligands to the receptor Domeless (Dome), the sole Janus kinase/Hopscotch (JAK/HOP), and the signal transducer and activator of transcription (STAT92E; hereafter referred to as STAT) [323–327].

The three Upd ligands Upd, Upd2, and Upd3 encoded in the *D. melanogaster* genome are glycosylated proteins secreted by hemocytes and are able to bind to the Dome receptor of fat body cells to activate the JAK-STAT pathway [328]. However, Upd is associated with the extracellular matrix [319], while Upd2 is freely diffusible [319,327] and both are involved in development, segmentation, and hemocyte proliferation and differentiation [318]. Upd3 is also freely diffusible and plays a role in responding to septic injury and Gram-negative bacterial infection [318,319]. In comparison to each other, Upd has been shown to be a more potent activator of a STAT-responsive promoter than Upd3 or Upd2 [319]. Binding of the appropriate Upd induces dimerization of the Dome receptor, allowing transphosphorylation of the JAK-HOP. Consequently, transphosphorylation of the conserved tyrosine residues in the STAT transcription factors occurs. Then, phosphorylated STATs dimerize and are translocated into the nucleus regulating the expression of downstream effector genes. Several splice variants of Dome, HOP, and STAT have been predicted in the *D. melanogaster* genome [328].

A subset of immune responsive JAK/STAT-dependent humoral effectors was identified as downstream effector genes. These include *Turandot* (tot) family members [318,329] and *Tep1* [59]. The JAK-STAT pathway induces the expression of *turandot A* (*totA*) during septic injury in *Drosophila* [318]. The expression of *totA* is activated in the fat body and is dependent on both Dome and Upd3. Also, *totC* and *totM* are thought to be regulated by the JAK-STAT pathway and respond to stress [329], although the specific function of these proteins is unknown. *Tep1* and *Tep2* expression in *Drosophila* is regulated by the JAK-STAT pathway and promotes tissue regeneration and wound healing [330,331]. *Tep1* is upregulated in response to septic injury and bacterial challenge in *An. gambiae*. Mutational analysis indicates that *Tep1* is involved in promoting phagocytosis [59].

3.2.4. The signalling pathway integrins/FAK/Src/MAPKs

In *C. capitata*, this signalling pathway can be stimulated by *E. coli* and *S. aureus* challenge. This stimulation leads to the activation of an Elk-1-like protein (transcription factor ELK-1 is a member of the ETS oncogene superfamily) and to the secretion of several bioactive molecules including protein activated serine peptidases [179,332]. Other stimuli, such as latex beads or LPS, also stimulate MAPKs, but through distinct pathways. Latex beads activate MAPKs via a yet unknown receptor(s) and FAK pathway, whereas LPS activates MAPKs through several yet undetermined signalling molecules. Therefore, in hemocytes of *C. capitata* and in response to different stimuli, distinct signalling pathways activate MAPKs [179]. Upon bacterial challenge, Elk-1-like protein is activated via MAPKs and is solely localized in the nucleus where is physically and functionally associated with FAK to regulate phagocytosis through a currently unclear mechanism [332]. Moreover, MAPKs mediate phagocytosis and melanization in *C. capitata* by activating the hemocyte-surface PPO [197], thus circulating PO/tyrosine is converted to dopa which is sequentially converted to dopamine via activity of hemocyte-surface Ddc. Dopamine-derived metabolites are likely involved in nodulation and melanization [235].

4. Conclusions

Various insect genomes have been fully sequenced and many of them having annotated immune genes. This paves the way for comparative genomic analyses of insect immunity and discovery of novel

mechanisms of immune defenses. We present a summary of immune genes across selected insect orders for the purpose of comparison among different insect models (Table 2). Obviously, Toll and Imd signalling pathways are well conserved across taxonomically diverse insect species. Insects and mammals share an ancient innate immune system comprising both humoral and cellular responses [352]. Understanding the structure and function of insect immune signalling pathways and their similarities with the innate immune system of mammals may increase the use of insects as *in vivo* models for studying the dynamics of host-pathogen interactions. For instance, the functional and structural homologues of the mammalian receptors Toll-like (TLRs)/Interleukin-1 (IL-1R), TNF α , β -1,3 glucan, and CTLs/macrophage mannose (CD206) in insects are Toll, Imd, β GRP, and CTLs/hemocytin receptors, respectively, and both mammals and insects share the NF- κ B and I- κ B transcription factors [101,102,353,354].

The insect innate antimicrobial response is a powerful system to unveil evolutionary conserved signalling pathways that control critical functions of host defense. This information is of paramount significance because it does not only enable us to speculate on the origins of the innate immune system, but it also facilitates the design of original tactics for targeting distinct components in the immune system of insects of agricultural or medical importance. Results from deciphering the connection between host innate immune signalling regulation and function and how these processes fine-tune the overall host antimicrobial protection can be extrapolated to interpreting the molecular events that take place during pathogenic infections in humans. Making such breakthroughs in biomedical research will lead to the development of health measures for detecting and curing infectious diseases.

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CRediT authorship contribution statement

Wei Zhang: Conceptualization, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **Gianluca Tettamanti:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Project administration. **Taha Bassal:** Conceptualization, Methodology, Resources, Writing - original draft, Supervision. **Christa Heryanto:** Software, Writing - review & editing. **Ioannis Eleftherianos:** Conceptualization, Methodology, Software, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Amr Mohamed:** Conceptualization, Methodology, Software, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

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