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Research article

INDUCED ANTIBACTERIAL PROTEIN IN INSECTS: IMPLICATION IN DEFENSE SYSTEM IN *DROSOPHILA*

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ABSTRACT : Despite of lacking the adaptive immunity that is found in higher vertebrates, insects are able to defend themselves from a large battery of pathogens by multiple innate immune responses using molecular mechanisms. These defense systems are strikingly similar to the innate immune responses of other multi cellular organisms, including humans. In insect, defense system relies on several innate reactions including phagocytosis of bacteria, nodule formation and encapsulation with the immediate onset of proteolytic cascades leading to localized blood clotting, melanization and finally the production of antibacterial protein. These antibacterial proteins channelized a line of innate defense system after 3hr of bacterial infection through different signaling pathways like Toll and Imd pathway. This review summarizes the recent studies on defense system in different insects and evaluates the mechanism of such signaling pathways in *Drosophila melanogaster*.
Keywords: Adaptive, innate, antibacterial protein, fat bodies, mRNA.

INTRODUCTION

Insects are one of the most successful groups of evolution accounting for nearly two million species and 10^{18} individuals [1]. They colonize all ecological niches except for the, seas. Consequently, they are confronted by an extremely large variety of potentially harmful microorganisms. During evolution, insects developed a complex and effective innate immune system, which apparently differs from the adaptive immune system of vertebrates. Vertebrates, including the human have both innate and adaptive immunity with 'immunological memory', whereas insects do not possess the ability to produce antibodies.

Innate defense systems in insect

Innate defense systems in insect haemocoel are comprised of cellular and humoral components [2]. These two immune systems cooperatively function in clearance of invading pathogenic microbes from the hemolymph. Hence, defensive arsenals of insects, like that of man contain both passive structural barriers against infection and a cascade of active responses to organisms that gain access to the haemocoel following injury to integument. The frontline of insect host defense is epithelial tissues such as the epidermis and trachea, which are not only act as mechanical barriers but also produce anti-microbial peptides.

Cellular defense system

The cellular defense of insects against pathogens and endoparasite is the prevention of infection via structural barriers such as rigid cuticle and peritrophic membrane that protects the mid gut. Even after this, if the bacteria persist in the system then initial hemolymph response is mediated by circulating hemocytes by the process of phagocytosis, nodulisation and encapsulation. However, if this innate mechanism of wiping out the antigen fails, synthesis of several bactericidal proteins occurs including lysozyme by the means of acquired immune system.

Phagocytosis

Cells with phagocytic activity usually represent a subpopulation of insect hemocytes. Both granulocytes and plasmatocytes are supposed to be primarily responsible for phagocytosis. Cell surface molecules described on phagocytic hemocytes exhibit striking similarities to the receptors found on mammalian phagocytic cells.

Insect proteins Malvolio and dSR-CI show homology to mouse natural resistance-associated macrophage protein-1 (NRAMP-1) and mammalian class-A macrophage specific scavenger receptors respectively [3, 4]. Croquemort is a member of the CD36 superfamily, mediates the recognition of apoptotic cells [5]. In *Drosophila*, another protein named peroxidasin is supposed to participate in the phagocytic breakdown of apoptotic cells [6]. Peroxidasin is homologous to mammalian macrophage peroxidases and contains four immunoglobulin-like C2 domains including a single IgA1 hinge region.

Nodule formation

Insect hemocytes aggregate to entrap bacteria during nodule formation. An insect lectin, named scolexin was found to be involved in the formation of nodules in the tobacco hornworm (*Manduca sexta*). Scolexin is produced by epidermal and mid gut cells upon wounding or bacterial infection [7]. In the medfly (*Ceratitis capitata*),a protein with molecular mass of 47kDa is secreted by hemocytes after lipo-polysaccharides (LPS) stimulation and aggregates *E. coli* cells by the presence of tyrosine and tyrosinase [8].

Encapsulation

Encapsulation is a multicellular defense mechanism where a capsule of overlapping layers of hemocytes is formed around protozoans, nematodes and eggs or larvae of parasitic insects. Encapsulation does not induce the expression of antimicrobial genes [9] but it may associate with melanization which contributes to the killing of the invader [10]. It is still unclear whether the reaction is mediated by a given subset of hemocytes or through an interaction between different subpopulations of immune cells [11, 12]. In both cases adhesion molecules are essential to the capsule formation. By analogy to vertebrates, the existence of various integrins in *Drosophila* raises the possibility that these molecules can participate in the cellular reactions of insects [13]. Moreover, the encapsulation response of the moth *Pseudoplusia includens* was found to involve an RGD (Arg-Gly-Asp)-dependent cell adhesion mechanism which is typical for integrins [14]. Parasites have developed various mechanisms to circumvent the encapsulation reaction of host insect. During oviposition endoparasitic wasps inject polydnaviruses which suppress the immune system of the host, thus ensuring successful development of the immature endoparasite [15,16]. In the genome of mosquitoes, quantitative trait loci (QTLs) involved in encapsulation process have been localized [17, 18].

Humoral defense system

Many species of insects possess an inducible humoral immune system, which is distinct from the system in vertebrate animals [19, 20]. The humoral responses depend on primary and secondary responses. The primary system involves reactions including activation of cascades of constitutive proteins present in the hemolymph, such as those in the prophenoloxidase (PPO) cascade [21], leading to melanization (deposition of melanin pigments onto pathogens and the wounded sites) which is believed to function in wound healing. The secondary humoral response requires the coagulation cascade, and the activation of intracellular signaling tissues and cells. These signaling activates gene for producing defense proteins such as anti-microbial peptides in the fat bodies, the immune tissue of insect which is functional equivalent of the mammalian liver. The reaction is usually linked to the induced synthesis of antimicrobial peptides and the stimulation of cellular immune responses such as nodule formation and encapsulation [6, 22].

PPO cascade and melanization

Primary humoral immune response initiated with the activation of PPO cascade leading to melanization. The process is induced by the cleavage of PPO to phenol oxidase (PO) by a serine protease, PPO-activating enzyme (PPAE). PO is an essential enzyme for the cellular immune responses but is also involved in other developmental and defensive processes such as wound healing and sclerotization [23, 24].The inactive proenzyme, PPO is synthesized in the hemocytes and after releasing by cell rupture it is either actively transported into the cuticle or deposited around wounds and encapsulated parasites [25,26]. The insect PPO enzyme contains a sequence with similarity to the thiol-ester region of the vertebrate complement component proteins C3 and C4 [27]. Hence, insect PPO cascade systems appear to be similar to the complement system of mammals. Several hemolymph proteins have been described as activators of PPO cascades in a variety of insects. Some of these proteins have been reported to play dual functions in hemolymph.

For instance, two hemolymph proteins, isolated from the wax moth (*Galleria mellonella*), ApoLp-III and Gm protein-24 have been tested on the insect humoral immunity and found that ApoLp-III enhanced the activity of antibacterial peptide such as cecropin however Gm protein-24 showed no effect on cecropin activity. Gm protein-24 and ApoLp-III, both involved in the activation of PPO cascade, which has been regarded as a critical immune reaction in insect hemolymph. On the other hand ApoLp-III which is a hemolymph protein plays a role in lipid metabolism in insects [28]. ApoLp-III participates in immune reactions as an LPS-binding protein (LBP) or as a potentiator of bacteriolytic activity of hemolymph other than lipid transport [29, 30]. LBP isolated from the hemolymph of *Bombyx mori*, *Manduca sexta*, *Drosophila melanogaster* and *Periplaneta americana* have been well characterized as immune factors to prevent infection by gram-negative bacteria [31, 32].

Signaling cascades

The secondary humoral immune process of insects' defense against the microbial infection is an evolutionarily conserved defense mechanism. The hallmark of the Drosophila humoral immune response is the rapid production of antimicrobial peptides in the fat body and their release into the circulation. Toll and Imd signaling cascades are two recognition and signaling cascades regulate expression of these antimicrobial peptide genes.

Toll and Imd signaling cascades regulating humoral and cellular responses in *Drosophila*

The fruit fly (*Drosophila melanogaster*) has been used to studying the basic principles of innate immunity because of the evolutionary conservation of innate immunity genes, pathways, effector mechanisms and the well-established techniques for manipulating its genetics. *Drosophila* is devoid of an adaptive immune system and relies only on innate immune reactions for its defense.

The microbial recognition and induction of anti-microbial peptides is mediated by the Toll and Imd (immune deficiency) pathways in *Drosophila*, which regulates antimicrobial peptide (AMP) expression in the fat body. Toll and Imd are pivotal molecules, mechanistically similar to the mammalian Toll like receptor (TLR) signaling pathway and tumor necrosis factor α receptor (TNFR) signaling pathways respectively [33,2]. The Toll pathway is activated primarily in response to fungal and some Gram-positive bacterial infections, whereas the Imd pathway is activated predominantly in response to Gram-negative bacterial infections [34]. Stimulation of the Toll pathway leads to activation of two NF-κB-like factors, Dorsal and Dorsal-related immunity factor (Dif), while the Imd pathway brings about the activation and nuclear translocation of the NF-κB-like factors [35-37].

Toll signaling cascade

In *Drosophila* Toll pathway is involved both in immunity and developmental processes [38]. In contrast to mammalian Toll like receptors (TLRs), *Drosophil*a Toll controls the dorsal-ventral (DV) patterning in embryos and upon binding with ligand Spatzle (Spz) it activates genes of antimicrobial proteins through the Toll-Dorsal signaling pathway [36]. *Drosophila* Toll does not appear to interact directly with pathogen-associated molecular patterns (PAMPs), but is activated by Spz, via a proteolytic cascade**.** Therefore, *Drosophila* possesses specific mechanisms to distinguish different pathogens.

Spatzle activation

In innate immunity, conserved molecular patterns of pathogens are thought to be identified by so-called pattern-recognition protein or receptors (PRPs) of the host defense systems (39). These proteins are produced in the fat body and secreted into the caterpillar's hemolymph such as peptidoglycan recognition protein (PGRP) was identified as a Gram-positive-binding protein followed by identification of the Gram-negative binding protein (GNBP), which binds to LPS and β-1, 3-glucan (40,41). The *Drosophila* genome contains 13genes encoding PGRP family proteins, and 3 encoding GNBP family proteins. Seven of the PGRPs are small (~20 kDa) extracellular polypeptides, whereas others are larger (30 to 90kDa) and either intercellular or membrane-spanning. GNBPs are 50 kDa proteins containing an N-terminal β-1, 3-glucan binding domain and a C-terminal β-glucanase-like domain [41, 42].

The recognition of the Gram-positive bacterial lysine-type peptidoglycan or the β-glucan from fungal cell walls is mediated by extracellular recognition factors. GNBP3 is responsible for yeast recognition [43]. The other identified factors, namely GNBP1, PGRP-SA, and PGRP-SD, appear to mainly recognize Gram-positive bacteria.

Upon Gram-positive bacterial recognition, PGRP-SA and GNBP1 physically interact and form a complex [44, 45]. Thereafter, activated GNBP1 hydrolyzes the Lys-type PGN and produces new glycan reducing ends, which are presented to PGRP-SA [46]. In contrast, Buchon *et al.* [47] showed that full-length GNBP1 had no enzymatic activity. They suggested a role for GNBP1 as a linker between PGRP-SA and modular serine protease (ModSP). PGRP-SD functions as a receptor for Gram-positive bacteria with partial redundancy to the PGRP-SA–GNBP1 complex [48]. It is reported that PGRP-SD can also recognize diaminopimelic acid (DAP)-type PGNs from Gram-negative bacteria, thereby activating the Toll pathway [49].

Drosophila Toll, the first identified Toll family member, consists of an extracellular leucine-rich repeat and intracellular signaling domains [50]. Spz, the ligand for Toll, is a secreted protein that is activated by proteolytic cleavage by a serine protease [51, 52]. In microbe recognition, Spz processing enzyme (SPE) is responsible for extracellular Spz cleavage [53]. Activation of SPE contains three upstream cascades depending on the activating microorganism (Figure 1). Two protease cascades leading to the activation of Gram-positive specific serine protease (Grass) are initiated by cell wall components of both fungi (β-glucan) and Gram positive bacteria (Lysine-type PG) [54]. Grass was originally identified to be specifically involved in the recognition of Gram-positive bacteria [55], but was later shown to be important also for the recognition of fungal components [54]. In addition, three other serine proteases, namely spirit, spheroide, and sphinx1/2, were identified in response to both fungi and Gram-positive bacteria [55]. Upstream of Grass, a ModSP, conserved in insect immune reactions, plays an essential role in integrating signals from the recognition molecules GNBP 3 and PGRP-SA to the Grass-SPE-Spatzle cascade [47]. A third protease cascade leading to the activation of SPE is mediated by the protease Persephone, which is proteolytically matured by the secreted fungal virulence factor PR1 [43] and Gram-positive bacterial virulence factors [54]. Similar detection mechanisms have been suggested to occur in mammals, in which TLRs or Nod-like receptors directly detect virulence factors or endogenous proteins released by damaged cells [56, 57].

Activation of Spz induces proteolysis, which causes a conformational change exposing determinants that are critical for binding of the Toll receptor [58]. Interestingly, the pro domain remains associated with the C terminus and are only released when the Toll extracellular domain binds to the complex [59]. Upon proteolytical processing, the Spz prodomain is cleaved, exposing the C-terminal Spz parts critical for binding of Toll. Spz binding to the Toll receptor initiates intracellular signaling.

After binding the processed Spz, the activated Toll receptor binds to the adaptor protein MyD88 via intracellular TIR domains [60, 61]. Upon this interaction, an adaptor protein, Tube, and the kinase Pelle are recruited to form a MyD88-Tube-Pelle heterotrimeric complex through death domain (DD)-mediated interactions. From the oligomeric MyD88-Tube-Pelle complex, the signal proceeds to the phosphorylation and degradation of the *Drosophila* IκB factor Cactus. In non signaling conditions, Cactus is bound to the NF-κB transcription factor(s) Dorsal and/or Dif in a context-dependent manner, inhibiting their activity and nuclear localization. So, the nuclear translocation of both Dorsal and Dif requires Cactus degradation [62]. After phosphorylation, nuclear translocation of Dorsal/Dif leads to activation of the transcription of several sets of target genes. This NF-κB like sites controls the synthesis of antimicrobial peptides in response to the presence of bacterial cell wall components in the insect blood.

The other role of Toll signaling like in early embryogenesis, the protease cascade gastrulation defective snake activates the protease easter, which cleaves full length Spz into its active C-106 form by a serine protease cascade [63, 64]. In addition, sulfotransferase Pipe is required independently of the protease cascade to activate easter.

Negative regulation in the Toll pathway

Toll pathway is repressed by an intracellular negative feedback loop. WntD (Wnt inhibitor of Dorsal) is a member of the wnt family of ligands. Activation of the Toll pathway leads to the transcription of wntD [65, 66]. WntD is able to block the translocation of Dorsal in cactus mutants. Therefore, WntD blocks nuclear translocation of Dorsal downstream of, or in parallel to Cactus. In addition to its role in embryonic patterning, WntD also regulates the Toll pathway in the context of immunity. For example, wntD mutants induce higher levels of some antimicrobial peptide genes. WntD mutants are also more sensitive to infection with Listeria *monocytogens*. It is hypothesized that WntD mutants have a higher mortality following infection due to the hyperactivation of Dorsal target genes [66].

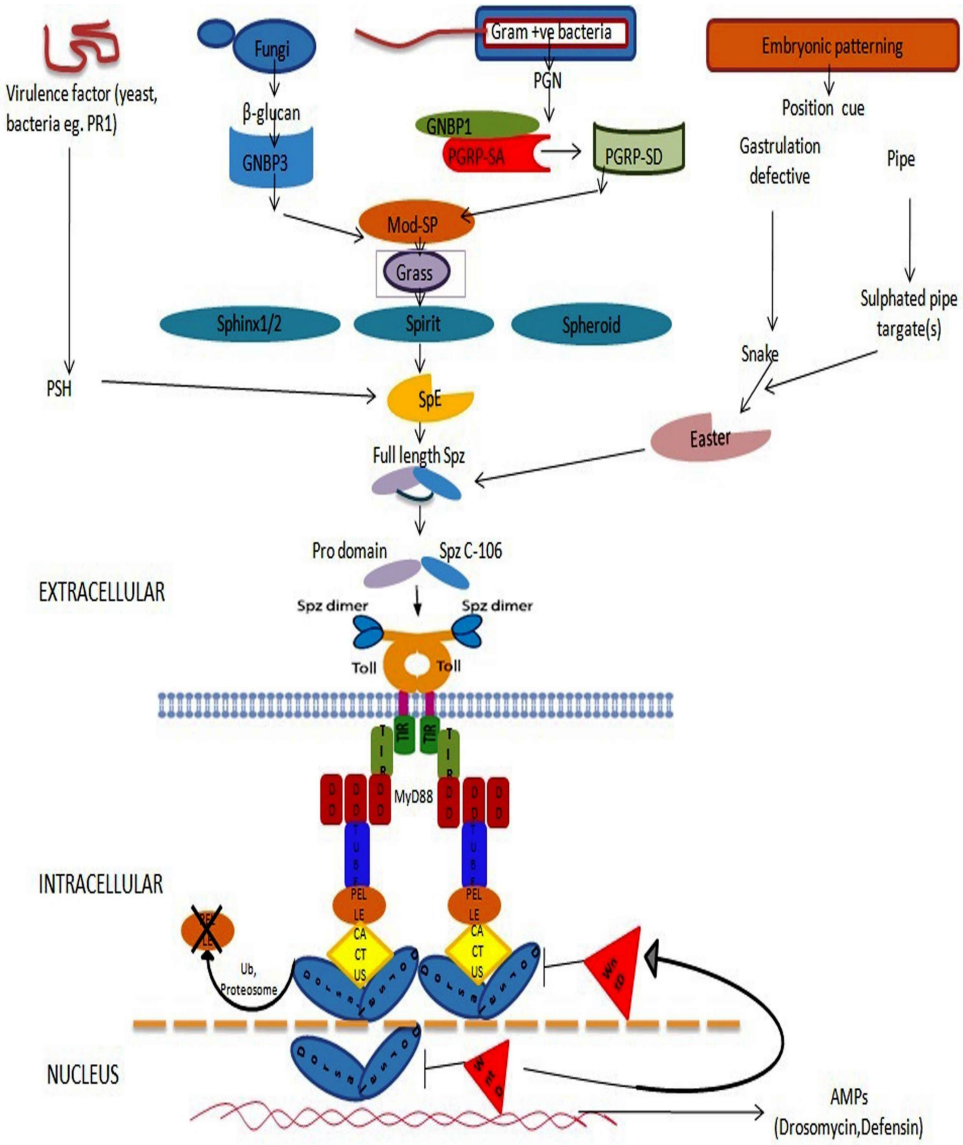

Fig. 1 Schematic representation of the Toll pathway including the three microbial recognition systems and the intracellular signaling cascade, while the black dark arrows and red triangle highlight the negative regulators and their likely targets.

Imd signaling cascade

Bacterial PG is a polymer consisting of glycan strands of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that are cross-linked to each other by short peptide bridges. PGs from Gram-negative bacteria and *Bacillus* species differ from other Gram-positive PGs in that lysine (Lys) is replaced with *meso*-diaminopimelic acid (DAP) at the third amino acid in the peptide chain.

The Imd pathway regulates the response to Gram-negative bacteria infection (Figure 2). Gram negative bacteria-derived DAP-type peptidoglycan is recognized by upstream receptors called PGRP-LC and PGRP-LE [67-70]. The recognition leads to activation of the cytoplasmic adaptor protein Imd, a homologue of the mammalian TNF receptor-interacting protein RIP [71, 72] which then activates the PPO cascade upstream of PPAE. Dimerise and multimerise receptor proteins activate other downstream adaptor protein of the Imd pathway include the DFADD (BG4) along with the caspase 8 orthologue DREDD [72-75]. Meanwhile, the Ser/Thr MAPK kinase, TAK1 and its partner TAB2 are activated possibly through the IAP2: Bendless (BEN):UEV1 a ubiquitin E3 ligase complex.

TAK1 and TAB2, in turn phosphorylate the IKKβ orthologue IRD5. Activated IRD5, in complex with IKKγ orthologue Kenny (KEY), phosphorylates the NF-κB orthologue Relish (REL). REL consists of an N-terminal nuclear factor containing domain (REL-68) and an inhibitory C-terminal domain (REL-49) responsible for anchoring REL in the cytoplasm. REL is then proteolytically cleaved by the caspase, DREDD, releasing the N-terminal domain REL-68. This translocates to the nucleus where it is able to activate transcription of genes encoding antimicrobial peptides such as *Diptericin* and *Attacin A* [76, 77].

Negative regulation in the Imd pathway

Tsuda *et al.* (2006) showed that plenty of SH3 (POSH) regulates the termination of Imd-JNK signaling. POSH mutant flies exhibit increased mortality following *E. coli* infection, possibly because of hyperactive immune responses [78]. POSH contains a RING finger, a signature ubiquitin E3-ligase motif, and is auto-ubiquitinated. Also, POSH immuneprecipitates with TAK1 and overexpression of POSH reduces the stability of TAK1. Thus, it is hypothesized that POSH negatively regulates the Imd -JNK pathway by regulating the stability of TAK1 via the ubiquitin/ proteosome degradation pathway. On the other hand, JNK signaling also inhibits Relish-mediated transcriptional activation, via the recruitment of a 'repressosome' to AMP genes [79, 80].

The Imd pathway may also be inhibited by another E3 protein, known as Dnr1, a conserved protein with an N-terminal ezrin/radixin/moesin domain and a C-terminal RING finger. Dnr1 appears to have a complex relationship with the caspase DREDD. Another negative regulator of Imd signaling is Caspar. Interestingly, Caspar is homologous to human Fas associated factor 1 (hFAF1), which associates with various components of the TNF/ NF-κB pathway such as FAS, FADD, caspase-8 and NF-κB [81, 82]. Over expression of Caspar inhibits AMP gene induction and causes decreased viability after infection with these same mildly pathogenic bacteria. It is hypothesized that Caspar blocks Relish cleavage by interfering with DREDD.

Other Signals and receptors

The innate immune processes of insects are triggered by a great variety of signals. Microbia, microbial substances, mitogens (arachidonic acid, phorbol esters and phytohemagglutinin [83] and the injury of the cuticle are exogen factors leading to the activation of both humoral and cellular defense mechanisms. Among microbial substances, LPS of gram negative bacteria,

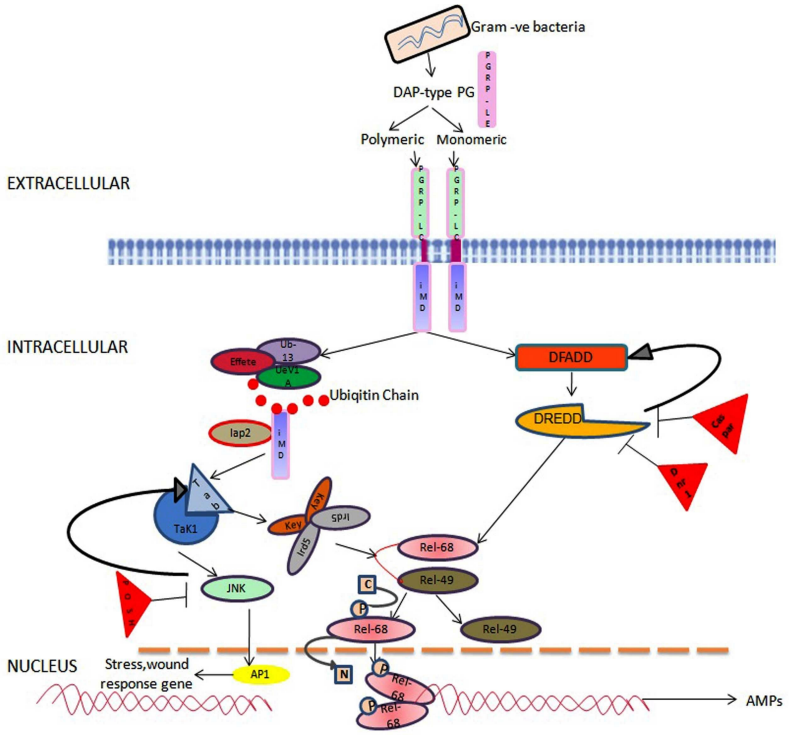

Fig. 2 Schematic representation of the Imd pathway while black dark arrows and red triangle highlight the negative regulators and their likely targets.

laminarin, 1, 3-β- D-glucans of fungi, PG of gram positive bacteria, zymosan and flagellin have been found to induce immune reactions in insects [84-87]. Hormones are best candidates for being the main modulators of these processes like 20-hydroxyecdysone enhances phagocytic activity of *Drosophila melanogaster* hemocytes *in vitro* [88]. *Drosophila* hemocytes synthesize the glycoprotein DS47 which is homologous to mammalian secretory proteins produced by activated macrophages [89]. The hemocytes of the silkworm release LPS during phagocytosis which leads to the activation of genes coding for antibacterial proteins [90]. Similarly to vertebrates, biogenic amines, cytokine-like factors (hemokines) eicosanoids and H_2O_2 can modulate insect cellular immune responses [91-93]. The hemocytes and fat body of the giant silk moth secrete a protein named hemolin that binds to the surface of bacteria and hemocytes and subsequently activates a signaling pathway involving protein kinase C and protein tyrosine phosphorylation [94, 95]. Hemolin is a member of the immunoglobulin superfamily and participates in a protein complex formation on the bacterial surface that is likely to initiate phagocytosis. The GNBP of insects shows serological cross-reaction and sequence similarity to the mammalian LPS receptor, CD14 [96, 97]. In insects, LPS binding proteins have been isolated from the hemolymph of the American cockroach [98] and of medfly [8]. The LPS binding protein of cockroach contains a carbohydrate- recognition domain of C-type animal lectins and acts as an opsonin [99, 100]. The receptors, hemomucin and FKBP39 of *Drosophila* bind *Helix pomatia* lectin (activator of T-lymphocytes) and the immunosuppressive drug FK506, respectively [101,102]. The membrane bound receptors for 5-hydroxytryptamine and LPS activate signal transduction events through adenylate cyclase and tyrosine phosphorylation, respectively [103,104].

Antibacterial proteins

All above signaling pathways lead to produce antibacterial protein. These antibacterial peptides were initially isolated in the 1980s from insect hemolymph after challenging Cecropia pupae with live bacteria [105]. More than 150 different antibacterial peptides and proteins have now been purified from different insect species [106-108]. These molecules are generally low molecular weight compound and mainly produced by the fat body and their mRNAs are detected simultaneously as early as 3hr after injection of bacteria [109].It is therefore thought that these molecules play a critical role in early immune defense against bacterial infection. It is reported that failure of early induction of these molecules may cause serious infection in insects [110]. On the basis of response to microbial infections, anti-microbial peptides in insects may be classified into five major groups: Lysozyme, Cecropin, Attacin, Defensin and Proline rich peptide; for eg. Morocin, Lobocin, Viresin, Diptericin, Drosocin, Drosomycin and Metchnikowin [111]. Lysozyme (EC 3.2.1.17) which is effective for Gram positive bacterial infection, includes higher molecular weight proteins and was first purified by Powning and Davidson, (1973) [112]. Cecropin is the family of small basic proteins, which were first isolated from the Cecropia silk moth in 1979 [113]. Cecropin may be induced in various kinds of insects by bacterial infection or simple injury [114]. Antibacterial proteins of this type have molecular weights of 4 to 5 kDa and show high bactericidal activity toward a wide variety of Gram-positive and Gram-negative bacteria.

Attacin which is nominated as third group, include proteins of larger molecular weights ranging from of 20 ~23 kDa. Attacin were firstly isolated from *Hyalophora cecropia* and showed activity against a few Gram-negative bacteria [115].

Defensin are highly effective against Gram-positive bacteria, including human pathogenic bacteria such as *Staphylococcus aureus*, however they do not exhibit strong activity against Gram-negative bacteria. Contrary to cecropins, defensins are more common in insects and have been isolated from several orders of insects such as dipteran, hymenopteran, coleopteran, trichopteran, and hemipteran. There are a total 6 AMPs that are found in the honey bee Defensin1 and Defensin 2 have been identified from a variety of insect species, whereas Apisimin and Hymenoptaecin have been reported only in the honey bee. All types of the antibacterial proteins have been reported in lepidopteran insects [116]. These peptides are secreted into the hemolymph from the fat body with different anti-microbial specificities [111].

Some antimicrobial peptides like Drosomycin, Metchnikowin and Cecropin are active against fungi, Defensin and Metchnikowin against Gram-positive bacteria and Attacin, Cecropin, Diptericin and Drosocin against Gram negative bacteria [117,118]. Diptericin and Drosocin induction is highly defective in Imd pathways mutants, Drosomycin and IM1 induction is highly defective in Toll pathway mutants, Attacin A, Cecropin A and Defensin induction is defective to different degrees in either Toll or Imd pathway mutants.

CONCLUSION

Toll and Imd provides homologs in several insect species such as an IKKβ homolog in an oyster and Rel homologs in dipteran insects but there are no molecular information on innate immune signaling found in other invertebrates. Drosophila host defense has paved the way for the search of homologs in mammalian innate immune responses. They offer unique opportunities to get closer to the roots of the mammalian innate immunity. As a result of the precise description of the innate immune system we may understand what makes an antigen immunogenic. Purification and Identification of such antibacterial proteins may permit the development of more effective vaccines and therapies for autoimmunity, tumors and infectious diseases in the future.

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