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Review

Overview of Innate Immunity in *Drosophila*

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Drosophila **protects itself from infection by microbial organisms by means of its pivotal defense, the so-called innate immunity system. This is its sole defense as it lacks an adaptive immunity system such as is found in mammals. The strong conservation of innate immunity systems in organisms from *Drosophila* to mammals, and the ease with which *Drosophila* can be manipulated genetically, makes this fly a good model system for investigating the mechanisms of virulence of a number of medically important pathogens. Potentially damaging endogenous and/or exogenous challenges sensed by specific receptors initiate signals via the Toll and/or Imd signaling pathways. These in turn activate the transcription factors Dorsal, Dorsal-related immune factor (Dif) and Relish, culminating in transcription of genes involved in the production of antimicrobial peptides, melanization, phagocytosis, and the cytoskeletal rearrangement required for appropriate responses. Clarifying the regulatory interactions between the various pathways involved is very important for understanding the specificity and termination mechanism of the immune response.**

Keywords: Antimicrobial peptide, Innate immunity, Phagocytosis, Regulatory circuits, Toll/Imd

Introduction

Innate immunity is a first-line defense system of multicellular organisms mounted in response to various microbial invaders (Kimbrell and Beutler, 2001; Hoffmann, 2003). After the initial proposal by Janeway that innate immunity is the key to early detection of, and defense against, infection in mammals (Janeway, 1989), much work has been done to identify the sensing molecules and downstream components involved. Recognition of pathogen-associated molecular patterns (PAMPs) by germline-encoded receptors is now known to initiate signaling cascades leading to the production of immune effectors such as antimicrobial peptides, cytokines, inflammatory mediators, and the activation of phagocytic and proteolytic cascades (Medzhitov and Janeway, 2000; Janeway and Medzhitov, 2002).

Only a restricted number of vertebrates have an adaptive immune system characterized by somatic rearrangement of immune receptor genes and clonal expansion of activated lymphocytes. Animals as well as plants lacking this type of adaptive immune system rely entirely on the innate system. *Drosophila* like other invertebrates has only an innate immune system, and has proved to be an ideal organism for screening for mutations in components involved in this system, many of which are common to all metazoa. The *Drosophila* system comprises three types of response; one is a humoral response dependent on the production of antimicrobial peptides in the fat body (the equivalent of the mammalian liver), the second a cellular response involving phagocytosis by plasmatocytes (*Drosophila* blood cell) and the third, melanization, which is believed to function in wound healing (Rizki and Rizki, 1984; Braun *et al*., 1998; Ferrandon *et al*., 1998; Tzou *et al*., 2000; Hoffmann and Reichhart, 2002). The sophisticated application of genetic methodology has permitted identification of at least two signaling pathways--the Toll and Imd pathways--that play pivotal roles in the production of the antimicrobial peptides by activating three NF-κB-like factors and initiating the innate immune response (Tzou *et al*., 2002; Hoffmann and Reichhart, 2002; Hultmark, 2003; Hoffmann, 2003). Stimulation of the Toll pathway leads to activation of two NF-κB-like factors, Dorsal and Dif (Dorsal-related immunity factor), while the Imd pathway brings about the activation and nuclear translocation of the NF-κB-like factor, Relish. In this review, we summarize recent advances in understanding innate immunity in *Drosophila*.

Antimicrobial peptides and humoral response There are seven inducible antimicrobial peptides in *Drosophila*. They are transcribed in the fat body within hours of an immune challenge and are secreted into the blood. Drosomycin, Metchnikowin and Cecropin are active against fungi, Defensin

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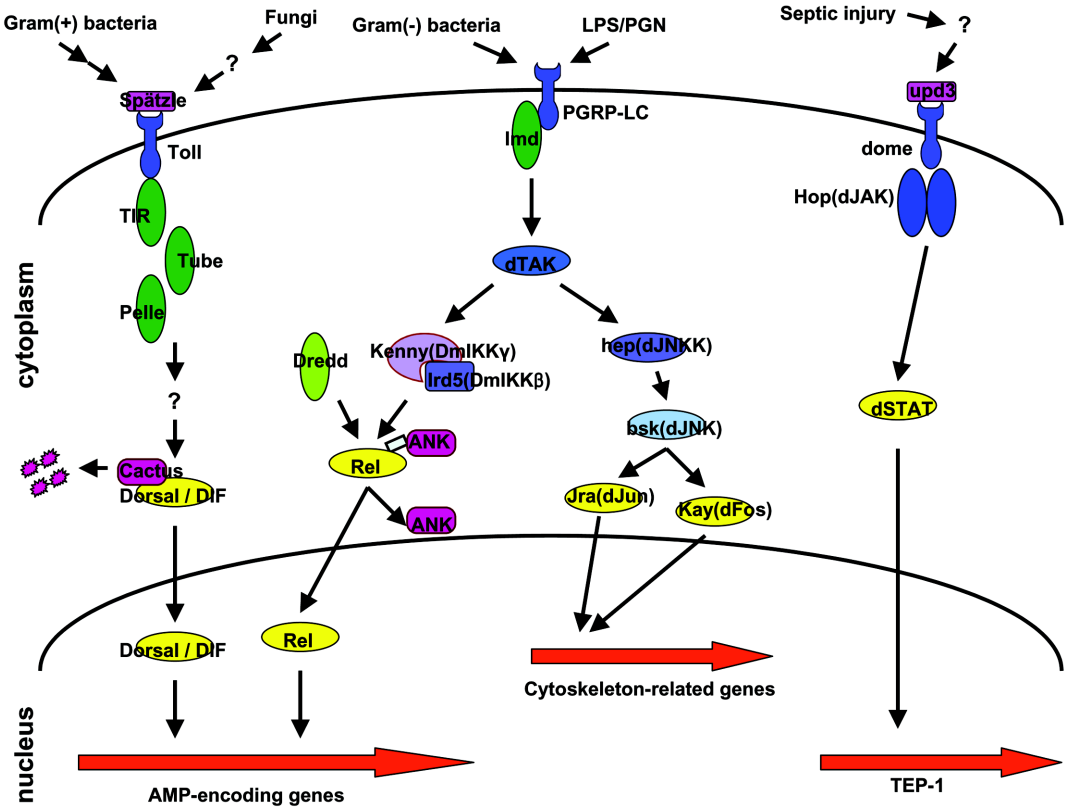

Fig. 1. The Toll, Imd and JAK-STAT pathways controlling the expression of genes related to physiological responses to infection. Spätzle is processed by a serine protease cascade upon infection and binds to Toll. This activates an intracellular signaling cascade culminating in degradation of Cactus and nuclear translocation of the NF-κB-like transcription factors, Dorsal and Dif. The Imd pathway branches into the JNK and IKK signaling modules at dTAK1. Relish, phosphorylated by the IKK complex, is cleaved to a smaller, active form with transactivating activity in the nucleus. The JNK pathway leads to activation of dAP1 (a homo- or hetero-dimer of dJun and/or dFos) via Hep and Bsk proteins which in *Drosophila* are dJNKK and dJNK, respectively. In addition, a STAT dimer is translocated to the nucleus as a result of activation of JAK kinase via the cytokine class I receptor, Domeless (Dome).

and Metchnikowin against Gram-positive bacteria and Attacin, Cecropin, Diptericin and Drosocin against Gram-negative bacteria (Bulet *et al*., 1999; Meister *et al*., 2000). These peptides are small and structurally diverse molecules that work in combination to block the growth of invaders by disrupting their membranes (Lemaitre *et al*., 1996). The promoter regions of the antimicrobial peptide genes contain a consensus sequence element similar to that of mammalian NF-κB. These κB-like sites control the synthesis of antimicrobial peptides in response to the presence of bacterial cell wall components in the insect blood, and mutations in these sites impair transcription of the corresponding genes (Engstrom, 1993; Kappler *et al*., 1993). Dorsal, the first *Drosophila* NF-κB-like factor identified, is activated by Toll and regulates dorsoventral patterning in the early embryo. The requirement for κB sites and similarities between the activation of Dorsal in the *Drosophila* embryo and the activation of NF-κB in cytokine-induced inflammation in mammals led investigators to envision a role for the dorsoventral regulatory cascade in immune defense (St. Johnston and Nusslein-Volhard, 1992; Morisato and Anderson, 1995; Belvin and Anderson, 1996).

Hemocyte differentiation and cellular responses Cellular responses also have important roles in protecting *Drosophila* against infection. *Drosophila* blood cells are of three types. Plasmatocytes are phagocytic macrophage-like cells comprising about 90% of the blood cells. Phagocytosis by them permits rapid uptake of infectious bacteria. Lamellocytes have a flattened shape and undertake the encapsulation of larger invaders such as parasite eggs, and crystal cells provide the enzymes required for the melanization reaction (Rizki and Rizki, 1984; Lanot *et al*., 2001). *Drosophila* hematopoiesis occurs during the embryonic and larval stages. Embryonic blood cells ingest apoptotic cells by phagocytosis, and engulf microbes injected into the embryo (Hartenstein and Jan, 1992; Tepass *et al*., 1994; Franc *et al*., 1999). Lymph glands, the larval hematopoietic organs, form along with the anterior portion of the dorsal vessel in the third instar larvae. These organs disappear at the pupal stage and no hematopoietic organs have been identified at later times (Rizki and Rizki, 1984; Rugendorff *et al*., 1994; Lanot *et al*., 2001).

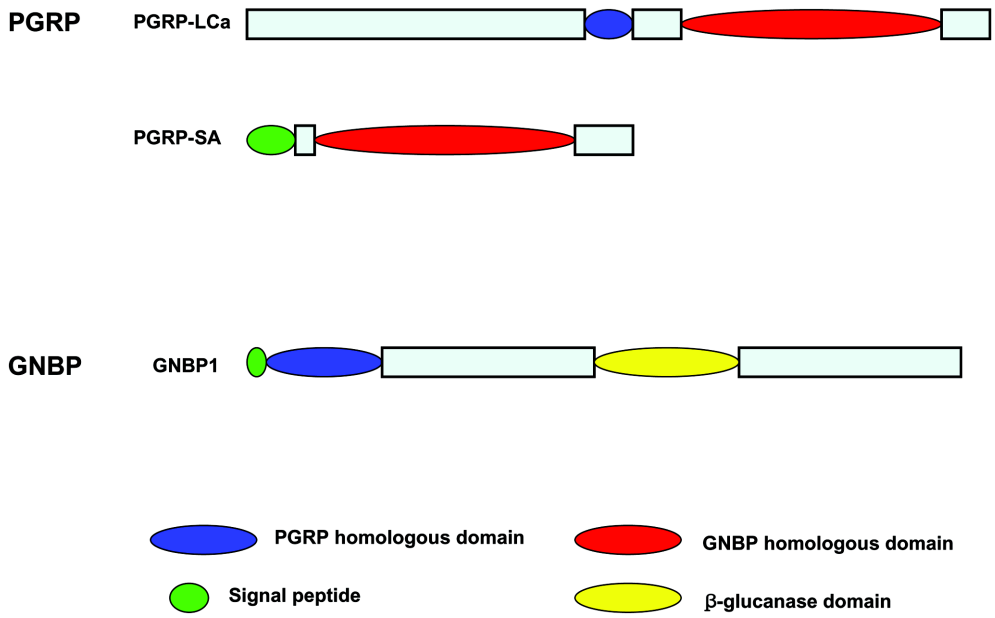

Fig. 2. Selected members of the PGRP and GNBP families. PGRP-LC, a transmembrane PGRP protein, is required for activation of the Imd pathway by Gram-negative bacteria (LCa is one of its isoforms). PGRP-SA and GNBP1 are small, secreted proteins that function in the activation of Toll by Gram-positive bacteria.

Commitment to the *Drosophila* hematopoietic lineage is controlled by the transcription factors Lozenge, Serpent, U-shape and Glial cell missing. The striking similarities between *Drosophila* Serpent, Lozenge and U-shape and the mammalian hematopoietic factors GATA, Acute Myeloid Leukemia-1 (AML1) and Friend of GATA (FOG), indicate that the molecular mechanism of blood cell lineage formation is conserved between *Drosophila* and mammals (Lebestky *et al*., 2000; Fossett *et al*., 2001).

Melanization When *Drosophila* is wounded by invaders and its cuticular barrier is broken, deposition of melanin and rapid clotting of the blood is induced (Söderhäll and Cerenius, 1998). Melanization contributes not only to wound healing but also to encapsulation of wasp eggs, and generates toxic intermediates such as reactive oxygen species (ROS) that may combat infection. The phenoloxydase required for production of melanin from dopamine is activated by a serine protease cascade (Söderhäll and Cerenius, 1998), and defects in this enzyme lead to increased sensitivity to infection (Braun *et al*., 1998; Rämet *et al*., 2002).

Signaling cascades regulating humoral and cellular responses The Toll signaling pathway was identified in parallel by biochemical studies of cytokine stimulation in mammalian systems and genetic investigations of dorsoventral patterning in *Drosophila* (Wasserman, 1993; O'Neill and Greene, 1998). In *Drosophila*, the spätzle/Toll/cactus dorsoventral regulatory gene cassette has been found to control the potent resistances to fungi and Gram-positive bacteria that depend on Dorsal and Dif. (Lemaitre *et al*., 1996; Meng *et al*., 1999; Wasserman, 2000). *Drosophila* Toll, the first identified Toll family member, consists of an extracellular leucine-rich repeat and intracellular signaling domains (Schneider *et al*., 1991). Spätzle, the ligand for Toll, is a secreted protein that is activated by proteolytic cleavage by a serine protease (Lavashina *et al*., 1999; Weber *et al*., 2003). Its binding to Toll activates the signaling cascade via dMyD88, the kinase, Pelle, a homolog of IRAK, and the adaptor, Tube. This leads to degradation of the IκB-like protein, Cactus, and nuclear translocation of the NF-κB-like protein, Dorsal, and Dif (Ip *et al*., 1993; Nicolas *et al*., 1998; Tauszing-Delamasure *et al*., 2002; Imler *et al*., 2002).

The response to infection by Gram-negative bacteria is controlled by a separate pathway, defined by mutations in the *imd* (immune deficient) gene. *Imd* flies have severe defects in resistance to Gram-negative bacteria but have normal responses to fungal and Gram-positive bacterial infections. The Imd pathway resembles the mammalian TNF-α pathway and culminates in the expression of antimicrobial peptides such as Attacin, Cecropin and Diptericin which confer substantial immunity to Gram-negative bacteria (Hoffmann, 2003; Brennan and Anderson, 2004).

The Imd pathway is initiated by activation of the peptidoglycan recognition protein (PGRP) (Choe *et al*., 2002; Gottar *et al*., 2002). After Imd was identified by studies of mutant flies with defects in the phenoloxidase cascade (Lemaitre *et al*., 1995), genetic analyses revealed other components of the pathway: a homolog of mammalian RIP (TNF-receptor-interacting protein), which is a death domain-bearing protein (Georgel *et al*., 2001); dTAK1 (TGF-β-activated kinase 1), a MAP kinase kinase kinase (MAPKKK)

(Vidal *et al*., 2001); two IκB kinase (IKK) complex components, namely IKKb (ird5) and IKKr (Kenny), which are homologs of mammalian IKK-β and IKK-ρ/NEMO and probably phosphorylate Relish, an NFn-κB family protein that is activated by proteolytic cleavage (Rutschmann *et al*., 2000; Silverman *et al*., 2000; Lu *et al*., 2001). In addition, FADD functions downstream of IMD, controlling the activity of Dredd, a *Drosophila* caspase-8 homolog, which acts with the IKK complex to fully activate Relish (Stoven *et al*., 2000; Hu *et al*., 2000; Leulier *et al*., 2002; Stoven *et al*., 2003).

The c-Jun N-terminal protein kinase (JNK; also known as the stress-activated protein kinase, SAPK) (Hibi *et al*., 1993), is a member of the mitogen-activated protein kinase (MAPK) superfamily; it participates in many processes in normal development and plays crucial roles in gene activation during the innate immune response (Stronach and Perrimon, 1999; Boutros *et al*., 2002). This JNK pathway is activated by cell stressors such as ultraviolet (UV) radiation, and pro-inflammatory cytokines and growth factors, and has been implicated in the control of diverse processes including morphogenesis, inflammation and apoptosis (Goberdhan and Wilson, 1998; Stronach and Perrimon 1999; Chang and Karin, 2001). In mammals, there is evidence for both pro-apoptotic and anti-apoptotic JNK activities (Lin, 2003), but extracellular stimuli generally activate the JNK pathway, resulting in programmed cell death (Verheij *et al*., 1996). In *Drosophila*, lipopolysaccharides (LPS) have been reported to activate JNK, which is required for induction of a subset of immune response genes (Sluss *et al*., 1996; Boutros *et al*., 2002), and homologs of many of the mammalian JNK pathway components have been identified; Hep, a MKK7 homolog, Bsk (DJNK), and Djun/Dfos, an AP-1 homolog, all of which function in dorso-ventral patterning and immunity. The JNK pathway branches from the Imd pathway at dTAK1 and participates in physiological processes such as morphological transitions during development, cell cycle regulation, apoptosis, phagocytosis and immunity.

There is recent evidence that mammalian NF-κB negatively regulates the JNK pathway in TNF-α-induced apoptosis (Tang *et al*., 2001; De Smaele *et al*., 2001). This type of negative crosstalk has also been found in a *Drosophila* cell line and in flies challenged with lipopolysaccharide (LPS) or gram-negative bacteria, and involves proteasomal degradation of dTAK1 by Relish-dependent genes (Park *et al*., 2004). In addition there is evidence that JNK also negatively regulates autocrine TGF-β signaling in a murine system in conjunction with AP-1 transcription factors (Ventura *et al*., 2004). Although there are some indications of the activity of JNK in *Drosophila*, investigation of the regulatory effect of the JNK signalosome in innate immunity is rendered difficult by the fact that loss-of-function mutations in this system are usually embryonic lethal (Glise *et al*., 1995; Sluss *et al*., 1996). However using RNA interference (RNAi), which mimics loss-of-function mutations, we have obtained evidence that the *Drosophila* JNK pathway inhibits the expression of NF-κB-dependent genes by recruiting the dHDAC complex to their promoters via dAP1 (Kim *et al*., 2005). The detection of reciprocal regulation between the *Drosophila* immunity pathways is very important for understanding the balance between initiation and termination of the immune response, and promises to provide insight into the mammalian counterpart of this regulatory interaction.

In mammals, local disturbances of physiological homeostasis lead to an acute and systemic reaction. In this process, IL6, produced in response to the presence of bacterial cell wall compounds such as LPS, triggers JAK-STAT signaling in hepatocytes culminating in translocation of STAT dimers to the nucleus where they activate transcription of target genes encoding acute phase proteins (Baumann and Gauldie, 1994; Alonzi *et al*., 2001). JAK-STAT signaling is required in the fat body of *Drosophila* to turn on genes, such as the stress-induced gene, *totA*, as well as *Tep1*, which encodes a thiolester-containing protein (Agaisse *et al*., 2003). Expression of *Tep1* has been shown to be constitutive in larvae with a gain-of-function mutation in JAK, and reduced in a corresponding loss-of-function mutation. This supports the evidence that the JAK-STAT pathway is activated upon immune challenge (Lagueux *et al*., 2000). The *Drosophila* hemocyte-specific cytokine-like protein, Unpaired 3 (Upd3), activates transcription of *totA* in the fat body via a homolog of the vertebrate cytokine class I receptor, Domeless (Dome), and this is followed by activation of the JAK-STAT pathway (Agaisse *et al*., 2003).

Recognition of pathogens In contrast to mammalian Toll-like receptors (TLRs), *Drosophila* Toll does not appear to interact directly with pathogen-associated molecular patterns (PAMPs), but is activated by Spätzle via a proteolytic cascade (Lemaitre *et al*., 1996). How does *Drosophila* recognize infection inducers and discriminate self from non-self? Two families of proteins have been implicated in pathogen recognition. First the peptidoglycan recognition protein (PGRP) was identified as a Gram-positive-binding protein and this was followed by identification of the Gram-negative-binding protein (GNBP), which binds to LPS and β-1,3-glucan (Lee *et al*., 1996; Kang *et al*., 1998; Kim *et al*., 2000; Werner *et al*., 2000). The *Drosophila* genome contains 13 genes 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 90 kDa) 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 (Werner *et al*., 2000; Ochiai and Ashida, 2000). PGRP-LC, a transmembrane PGRP, is required for activation of the Imd pathway. Loss-of-function mutants of PGRL-LC are susceptible to Gram-negative bacteria but not to Gram-positives and they fail to form the antimicrobial peptides (Choe *et al*., 2002; Rämet *et al*., 2002). Another PGRP, PGRP-LE, activates the Imd pathway when it is overexpressed (Takehana *et al*., 2002).

PGRP-SA, a secreted PGRP, functions in the detection of Gram-positive pathogens (Michel *et al*., 2001). Mutants of PGRP-SA were obtained by screening for flies defective in forming Drosomycin, which was known to be induced via the Toll pathway in response to both Gram-positive bacteria and fungi. PGRP-SA mutants are specifically susceptible to Gram-positive bacteria but resistant to fungi and Gram-negative bacteria; the induction of Drosomycin by Gram-negative or fungal infection is unaffected in these mutants, whereas its induction by Gram-positive bacteria is abolished (Michel *et al*., 2001; Leulier *et al*., 2003). The relatively large numbers of recognition molecules that have been identified help to account for the specific recognition of diverse pathogens.

Conclusion

During the last two decades, genetic and biochemical studies have uncovered the essential components of the Toll and Imd signaling pathways required for immune defense in *Drosophila*. However, the molecular mechanisms of other crucial responses to infection, including phagocytosis, encapsulation, melanization and coagulation, remain to be clarified. New techniques such as DNA microarrays and RNA interference, combined with the availability of the complete *Drosophila* genome sequence and forward and reverse genetic analysis, promise to yield insight into these other responses within the next few years.

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