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The Drosophila Imd Signaling Pathway

Henna Myllymäki,* Susanna Valanne,* and Mika Rämet*†‡§

The fruit fly, Drosophila melanogaster, has helped us to understand how innate immunity is activated. In addition to the Toll receptor and the Toll signaling pathway, the Drosophila immune response is regulated by another evolutionarily conserved signaling cascade, the immune deficiency (Imd) pathway, which activates NF-κB. In fact, the Imd pathway controls the expression of most of the antimicrobial peptides in Drosophila; thus, it is indispensable for normal immunity in flies. In this article, we review the current literature on the Drosophila Imd pathway, with special emphasis on its role in the (patho)physiology of different organs. We discuss the systemic response, as well as local responses, in the epithelial and mucosal surfaces and the nervous system. The Journal of Immunology, 2014, 192: 3455–3462.

Multicellular organisms constantly encounter potentially harmful microorganisms. Although insects lack an adaptive immune system in the classical sense, it has long been recognized that they too possess powerful means of fighting infections, such as the ability to phagocytose bacteria and encapsulate parasites. In addition, insects can mount a humoral response against intruders. This is characterized by the secretion of antimicrobial peptides (AMPs) into the hemolymph. This aspect was analyzed carefully first by Boman et al. (1), who eventually isolated the first AMPs from the silkmoth Hyalophora cecropia (2–4). The cloning of more AMPs from Drosophila and other insect models revealed conserved binding sites for NF-κB proteins in their promoter regions. This suggested that the mechanisms that induce AMP production in response to infection might be evolutionarily conserved (5–7). In addition to its function in development, the dorsal gene, coding for an NF-κB transcription factor, was discovered to mediate immune responses in adult flies, together with another NF-κB protein-coding gene, DIF, and an additional set of genes, including Toll, tube, and pelle, belonging to the Toll signaling pathway (8–13, reviewed in Ref. 14). Subsequently, genes coding for TLRs also were found in the human genome. The discovery of these pattern recognition receptors highlighted similarities between the human and Drosophila innate immune systems (15–18). However, the Toll pathway only controls the expression of a limited set of immune response genes in Drosophila. The immune deficiency (Imd) pathway, which regulates the activity of a third Drosophila NF-κB protein called Relish (19), controls the expression of most of the Drosophila AMPs and, thus, is indispensable for normal immunity (20).

Peptidoglycan-recognition proteins and activation of the Imd pathway

Microbial recognition is the first step in initiating the immune response via the Imd signaling pathway. For pathogen detection, the innate immune system uses specific, genome-encoded pattern recognition molecules that bind conserved structures, which are found on pathogens but are absent in the host (21). Peptidoglycan (PGN) is a good example of such a structure: it is present on most bacteria and is composed of conserved polymers of β-1,4-linked N-acetylglucosamine and N-acetylmuramic acid cross-linked by short stem peptides, which vary between different types of bacteria. PGN is recognized and bound by conserved host proteins, called PGN-recognition proteins (PGRPs), which share a 160-aa PGRP domain. Mammals have a family of four PGRPs, whereas insects have more (e.g., 13 genes coding for 19 proteins in Drosophila) (22, 23). The mammalian PGRPs are secreted proteins that bind bacterial muramyl peptides. Some mammalian PGRPs have an amidase activity, probably to eliminate the proinflammatory PGN, whereas others are more diverged from the insect genes and function directly as bactericidal proteins (24–26). It appears that mammalian PGRPs do not possess signaling activity.

Insect PGRPs can act both as enzyme-active amidases that degrade PGN and activate signal-transduction pathways and proteolytic cascades (Fig. 1). Insect PGRPs are classified as short (S) or long (L), according to their transcript size: short PGRPs have signal peptides and can be extracellular proteins, whereas long PGRPs can be intracellular, extracellular, and transmembrane proteins. Of the Drosophila PGRPs, PGRP-SA and PGRP-SD are needed for activating the Toll pathway (14, 27, 28). No immunological in vivo phenotype has been demonstrated for the amidases PGRP-SB1 and PGRP-SB2, even though PGRP-SB1 is strongly induced postinfection, and its bactericidal activity is shown in vitro (29–31). The function of PGRP-LD is unclear. The other PGRPs either positively or negatively regulate the Imd pathway.

*Laboratory of Experimental Immunology, BioMediTech, University of Tampere, FIN-33014 Tampere, Finland; †Department of Pediatrics, Tampere University Hospital, FIN-33521 Tampere, Finland; ‡Department of Pediatrics, Medical Research Center Oulu, University of Oulu, FIN-90014 Oulu, Finland; and §Department of Children and Adolescents, Oulu University Hospital, FIN-90029 Oulu, Finland

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Address correspondence and reprint requests to Prof. Mika Rämet, University of Tampere, FIN-33014 Tampere, Finland. E-mail address: mika.ramet@uta.fi

Abbreviations used in this article: AMP, antimicrobial peptide; Iap2, inhibitor of apoptosis 2; IKK, IκB kinase; Imd, immune deficiency; PGN, peptidoglycan; PGRP, peptidoglycan-recognition protein; TCT, tracheal cytotoxin.

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PGRP-LC is a transmembrane receptor that preferably binds a meso-diaminopimelic acid–type PGN found on Gram-negative bacteria and certain Gram-positive bacteria, such as *Bacillus* spp. (32, 33). It functions as the principal receptor for mediating the activation of the Imd pathway in a systemic infection and locally in the most anterior part of the midgut (34–37). PGRP-LC is spliced into several isoforms, three of which have been characterized. PGRP-LCx recognizes polymeric PGN; PGRP-LCa does not directly bind PGN, but it acts as a coreceptor with PGRP-LCx to bind monomeric PGN fragments called tracheal cytotoxin (TCT) (32, 38, 39). PGRP-LC mutant flies are still responsive toward TCT. This is due to PGRP-LE, which is found in two forms (40, 41). The short form is secreted and binds PGN in the hemolymph (42) and is thought to assist Imd signaling by presenting PGN to PGRP-LC, although the export mechanism of PGRP-LE has not been characterized. The full-length PGRP-LE remains in the cytoplasm, where it is thought to recognize TCT fragments that gain access to the cell. Binding of TCT leads to the oligomerization of cytoplasmic PGRP-LE in a head-to-tail fashion (39). Ectopic expression of PGRP-LE in the fat body is sufficient to activate AMP expression, in a cell-autonomous fashion, in the absence of infection. It also was shown that cytoplasmic PGRP-LE can activate the Imd pathway, independently of PGRP-LC, by interacting with Imd (40–43). PGRP-LE is the only intracellular pathogen receptor identified in *Drosophila*. The intracellular form is able to activate autophagy, whereas the transmembrane form can activate a prophenoloxidase cascade together with PGRP-LC (42, 44, 45).

PGRP-LF is a transmembrane protein that resembles PGRP-LC but lacks the intracellular signaling domain and does not bind PGN. PGRP-LF acts as an inhibitor of Imd signaling by binding PGRP-LC and preventing its dimerization (46–48). PGRP-LA is also predicted not to bind PGN and recently was shown to be dispensable for systemic infections. However, consistent with its expression profile, PGRP-LA appears to positively regulate the Imd pathway in barrier epithelia, such as the trachea and the gut (49). PGRP-LB, PGRP-SC1A, PGRP-SC1B, and PGRP-SC2 have an amidase activity and are shown to play somewhat redundant roles in down-regulating the Imd pathway during a systemic response. PGRP-LB is the major regulator in the gut. The amidase PGRPs digest PGN into short, nonimmunogenic or less immunogenic fragments and, therefore, prevent or reduce the activation of defense mechanisms (30, 31).

### Regulation of the Imd signaling pathway

The *Drosophila* Imd pathway is often compared with TNFR signaling in mammals, although the Imd pathway also shares similarities with the TLR signaling pathways. The Imd and TNFR signaling pathways are shown schematically in Fig. 2, with the conserved signaling molecules indicated by similar shapes and colors. In essence, activation of the Imd pathway and TNF-α target genes require activation of the transcription factor Relish and NF-κB, respectively (19). In mammalian NF-κB signaling, activation of the transcription factor is achieved by phosphorylation of the inhibitor protein IκB by the IκB kinase (IKK) complex. The phosphorylated IκB is degraded, and NF-κB is released.

![FIGURE 1.](http://www.jimmunol.org/) The *Drosophila* PGRPs and their roles in immunity. References are shown in parentheses. Lys-type PGN, lysine-type PGN.

![FIGURE 2.](http://www.jimmunol.org/) Schematic representation of the *Drosophila* Imd pathway and human TNFR signaling. Conserved components are indicated by similar shapes and colors. bend, bendless; eff, effete; Key, Kenny; K63 Ub, K63 polyubiquitination; NEMO, NF-κB essential modulator; TAB, TAK1-binding protein; TRADD, TNFR1-associated death domain.
However, like in the mammalian Rel proteins p100 and p105, IkB is contained in the C terminus of Relish. This is thought to mask the nuclear localization signal in the N terminus and inhibit Relish dimerization by the Rel homology domain. Therefore, in addition to phosphorylation, the activation of Relish requires cleavage of the inhibitory C-terminal part. This is likely accomplished by the caspase Dredd, because it was shown to cleave Relish in vitro (50).

Binding of PGN to the receptor results in recruitment of a signaling complex consisting of Imd, a death domain protein homologous to mammalian RIP1, the adaptor protein dFadd, and the caspase-8 homolog Dredd (20, 51–53). Dredd becomes activated by ubiquitination by the E3-ligase inhibitor of apoptosis 2 (Iap2) (54), which associates with the E2-ubiquitin-conjugating enzymes UEV1a, Bendless (Ubc13), and Effete (Ubc5) (55). Once activated, Dredd cleaves Imd, removing a 30-aa N-terminal fragment, and creates a novel binding site for Iap2, which can then K63-ubiquitinate Imd (54, 56). This leads to the recruitment and activation of the Tab2/Tak1 complex, which is responsible for the phosphorylation and activation of the Drosophila IKK complex (57–60). Relish is activated by the phosphorylation of multiple sites at its N terminus by the IKK complex (59). It was shown that phosphorylation of serine residues S528 and S529 is required for efficient recruitment of RNA polymerase II to the promoters of Relish target genes (59, 61). 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 coding for AMPs, such as Diptericerin and Cecropin (50, 62). Like in mammalian TNFR signaling, the Drosophila Imd pathway bifurcates into the JNK pathway at the level of Tak1 and Tab2 (63–65).

The activity of Imd signaling is fine-tuned at multiple levels by several molecules and mechanisms (66–68). For example, the ubiquitination state of various pathway components is delicately regulated. The E3-ligase Iap2 has been identified in many large-scale screens for Imd pathway regulators (60, 66, 69, 70), and it also was shown to be essential for Imd pathway-mediated AMP expression and bacterial resistance in vivo (60, 65, 71). In addition, a number of ubiquitinating and deubiquitinating enzymes have been implicated in the negative regulation of the signaling pathway. The ubiquitin-specific protease dUSP36/Scny prevents the accumulation of the activated, K63-ubiquitinated Imd and promotes its K48-linked ubiquitination and subsequent degradation (72). Iaf1 is a deubiquitinating enzyme that also was proposed to regulate the ubiquitination state of Imd (73). Tak1 is targeted for ubiquitination and degradation by the RING-finger protein POSH (74). CYLD is a deubiquitinating enzyme that down-regulates mammalian TNFR signaling by inactivating TRAF2. Drosophila CYLD interacts with the Ikk protein Kenny and also negatively regulates Imd signaling (75, 76). Activated Relish is targeted for ubiquitination and proteasomal degradation by the SCF complex (77), possibly promoted by the ubiquitin-binding protein dRYBP (78). Dredd is inhibited by the RING-domain containing protein Dnrl (79, 80), whereas Caspar, a homolog of the mammalian FAF1, inhibits the Dredd-mediated cleavage of Relish (81).

In addition to ubiquitination, another posttranscriptional modification that regulates signaling molecules is the addition of SUMO. Recently, it was shown that both Drosophila IKK proteins, Ird5 and Kenny, are associated with SUMO proteins, but only Ird5 is sumoylated on a conserved site, K152, which is needed for the activation of the Imd pathway (82). A proteomics analysis described in the same study identified 102 gene products that interacted with the core Imd pathway components and affected the activity of several reporter genes in response to a Gram-negative bacterial challenge in RNA interference assays. These gene products included a number of histone acetyltransferases and components of chromatin-remodeling complexes, such as the SWI/SNF complex. These discoveries indicate that the regulation of the Imd pathway involves DNA modifications (82). The Imd pathway is also positively regulated by a nuclear protein called Akirin (83). A recent study showed that, during myogenesis, Akirin colocalizes and genetically interacts with subunits of the SWI/SNF-class chromatin-remodeling complex to optimize gene expression by the transcription factor Twist. It remains to be studied whether Akirin provides a similar link between transcription factors and the chromatin-remodeling machinery in Imd signaling (84). Akirin shows high conservation in both sequence and function, and in addition to Drosophila, it was found to positively regulate NF-κB signaling in other insects, mammals, and reptiles (83). Akirin may even provide a target Ag for the development of a vaccine against insect vector-borne diseases (e.g., Ref. 85).

Moreover, the activity of the Imd pathway is negatively regulated at the transcriptional level by the transcription factors Zfh1 and the AP-1/Stat92E complex. Zfh1 strongly down-regulates the Imd pathway activity in vitro, but its role in vivo is more difficult to study because of developmental effects (83, 86–88). The JNK pathway transcription factor AP-1, the Stat92E transcription factor, and Dsp1 can form a complex that binds to the promoters of Relish target genes. Complex formation leads to recruitment of the histone deacetylase dHDAC1, the replacement of Relish, and target gene down-regulation, indicating that cross-talk between the Drosophila immune signaling pathways is needed for proper regulation of these genes (86, 87).

One of the target genes of the mammalian NF-κB protein is its own inhibitor, IkB. This creates a negative feedback loop, which is a major regulatory mechanism for the signaling pathways (89). Because Relish itself contains IkB, this method of regulation is not feasible in Drosophila. Instead, the Imd pathway is subject to other negative feedback mechanisms, such as the rapid induction of a gene called pirk (90, 91). Pirk expression appears to be induced by the Ras/MAPK pathway as well, suggesting that Ras/MAPK signaling functions in immunity by negatively regulating the Imd pathway (92). Pirk interacts with the receptors PGRP-LC and PCRP-LE and the adaptor protein Imd, thus likely interrupting signal transduction at the receptor complex (91, 93, 94).

20-Hydroxyecdysterone (ecdysone) is a steroid hormone known to coordinate tissue remodeling in flies undergoing metamorphosis, including the phagocytosis of dead cells. In addition, it is needed for the optimal phagocytic activity of hemocytes in response to bacteria (95). Ecdysone also was reported to regulate the activity of the Imd pathway in adult flies via its target genes, whose products are needed for PGRP-LC expression. In addition, some of these proteins appear to directly regulate the expression of a subset of the Imd pathway target genes (96).
The role of Imd-pathway activation in systemic and epithelial immunity

In addition to initiating the systemic response in the fat body, the Imd pathway is activated locally in several tissues (Fig. 3). Inducible immune responses in the epithelial and mucosal surfaces, such as the Drosophila tracheal airway system and the digestive system, are important, because these surfaces are constantly exposed to potentially pathogenic bacteria, fungi, and yeast (97, 98). In the trachea, an infection triggers the Imd pathway–mediated reactivation of a set of tracheal development genes, a survival reaction that remodels the epithelial tissue damaged upon infection (99). Also, AMPs are induced, and their expression is antagonized via a signaling route involving a Toll family member Toll-8/Tollo (100). Salivary glands have the potential to contribute to intestinal immunity by secreting soluble proteins and peptides into the saliva that rinses through the digestive system (101). Importantly, there are marked differences in the sets of genes induced in different tissues. For example, a microarray study comparing the immune response in the fat body, gut, and trachea showed that there is only a small set of “universal” Imd pathway target genes, mainly consisting of AMPs and pathway components; the rest of the infection-inducible genes are more tissue specific (49). Also, Imd pathway–mediated AMPs were shown to activate specific responses in the nervous system (e.g., Ref. 102).

The gut microbial community, or microbiome, is now recognized as an integral part of the host system with important metabolic activities (103). The Drosophila gut microbiome is a dynamic structure; its composition varies widely within and among Drosophila populations and species (104), and it is

**FIGURE 3.** Schematic representation of tissue-specific (dys)functions and the regulation of the Imd pathway. In Drosophila, systemic AMP expression is induced in the fatbody cells by PGRP-LC–mediated recognition of PGN fragments and monomers (TCT) and leads to the secretion of AMPs into the hemolymph. In the gut, the negative regulators Pirk, Caudal, PGRP-LB, and PGRP-LF maintain tissue homeostasis by preventing extensive Imd pathway activation by commensal microbes. In contrast, pathogenic bacteria are thought to secrete more PGN and, therefore, activate Imd pathway–mediated AMP expression. TCT molecules that cross the epithelial barrier and gain access to the hemolymph can also elicit a systemic response in the fatbody. PGRP-LA and Tollo regulate Imd pathway activity, specifically in the trachea (airway epithelium). In the brain, Dredd is activated independently of the upstream pathway components, and Relish-mediated AMP expression leads to neurodegeneration. Cad, Caudal; ECT4, ectoderm-expressed 4; Spz2, Spaetzle 2.
influenced by bacteria introduced into the gut by ingestion (105). The microbial community needs to balance a tolerance toward commensal bacteria and alertness toward pathogenic invasion, as well as the homeostasis between gut microbes and the host. There are two parallel systems in *Drosophila* that control this host–microbe homeostasis: the DUOX pathway(s) (106) and the Imd pathway. The Imd pathway is activated upon PGN binding by PGRP-LC and, specifically in the gut, by PGRP-LE (41, 107). Evidently, mechanisms for controlling the immune response play a major role in maintaining homeostasis. The first checkpoint is PGN sensing: pathogens that divide more rapidly than commensals shed more PGN upon cell division and, therefore, get recognized by the Imd pathway, unlike commensals (reviewed in Ref. 108). The second control mechanism is the expression of negative regulators that downregulate the Imd pathway at all of its levels. Amidase PGRPs (Fig. 1), especially PGRP-LB (30, 37), degrade and scavenge PGN in the gut lumen to prevent it from crossing the gut barrier. However, in some pathogenic infections, PGN fragments may translocate across the gut epithelium and trigger a systemic response (37, 109, 110) (Fig. 3). Some negative regulators directly bind components of the Imd pathway: PGRP-LF dimerizes with a PGRP-LCx isoform to form a competitive nonsignaling complex (46, 47). Pirk, which interacts with the receptor and Imd, is needed for normal immune tolerance in the gut (94). Furthermore, it was proposed recently that the transglutaminase protein inhibits Relish activity by catalyzing the polymerization of N-terminal Relish molecules (111). Another level of control in the immune system includes Caudal, the intestinal homeobox gene (112), which is expressed in a defined compartment in the adult fly midgut (113). Silencing of Caudal constitutively activates AMPs and leads to intestinal disorders, compromising the fitness of the animal (112, 113). Caudal, together with other gut-specific transcription factors, has important functions in patterning the expression of AMPs and other intestinal genes (113).

The Imd pathway in immune senescence and neurodegeneration

In humans, aging is associated with changes in immunity, such as upregulation of the innate immune system. This can lead to chronic inflammation, with immune functions actually deteriorating, making the elderly more susceptible to infections (114). In *Drosophila*, aging is also characterized by the increased expression of immune-related genes, including several antimicrobial peptides, PGRPs, and *Relish* (115–117), increased bacterial loads (118), and more persistent AMP activation upon infection (119). This suggests that flies might develop a chronic inflammation-like condition resembling that seen in humans. Genetic studies show that overexpression of *PGRP-LE* or *PGRP-LC* in the fat body activates the Imd pathway, which enhances the flies’ resistance against pathogens and has no acute effects on reproduction or general fitness. However, long-term activation of the Imd pathway creates an inflammation-like state and results in reduced lifespan, suggesting that maintaining an active immunity is physiologically costly in the long run (114, 120). Evidence supporting this hypothesis includes a delay in the age-related immune activation in flies reared on a calorie-restricted diet (116). Moreover, *Relish* mutant flies live longer than do wild-type flies on a calorie-restricted diet (121), and female *Relish* and *Imd* mutants do not show signs of reduced fecundity (number of eggs laid) after injection with killed bacteria (119). Thus, the Imd pathway appears to be involved in mediating chronic inflammation and immune senescence, but the exact mechanisms need to be studied further.

Many human age-related neurodegenerative diseases also are characterized by the activation of inflammatory pathways in neural cells, caused by the accumulation of aberrant protein aggregates, such as the β-amyloid fibrils in Alzheimer’s disease. Therefore, an elevation in TNF-α levels and NF-κB activity are often found to contribute to the disease pathology by inducing the expression of additional proinflammatory molecules or cell death (122). Similarly, involvement of the Toll or Imd pathways in neurodegeneration has been observed in *Drosophila*. An experimental bacterial infection in the brain causes progressive, age-dependent neurodegeneration at the injection site, together with locomotive defects. The phenotype is dependent on Relish, and strikingly, overexpression of individual AMPs alone in neurons or glial cells is sufficient to cause these symptoms. This suggests that AMPs could be directly cytotoxic to brain cells (123). *Drosophila* neurodegeneration disease models also have been used to study immune-related diseases in the nervous system. Ataxia-telangiectasia is a rare disease caused by a recessive mutation of the ATM gene, which encodes a DNA damage-responsive protein kinase. In *Drosophila*, a temperature-sensitive allele of ATM or the expression of an ATM RNA interference construct in glial cells leads to reduced longevity and mobility, increased death of neurons and glial cells, and increased expression of immune-response genes (124). These changes are dependent on Relish but not Dif or Imd (102). Furthermore, Dredd has been associated with retinal degeneration caused by a mutation in an eye-specific phospholipase C (*norpA*). In this model, the light-dependent endocytosis of rhodopsin, its accumulation in the late endosomes, and the resulting death of photoreceptor cells are not dependent on the developmental apoptosis machinery but require Dredd. Moreover, this retinal phenotype was rescued by mutations in *Relish* and *key*, but not *Imd* or *Fadd*, suggesting an alternative means for Dredd regulation. In addition, overexpression of active Relish during development has detrimental effects on various tissues and cell types, such as neurons, photoreceptor and eye cells, and the wing (125). Thus, several *Drosophila* models demonstrate the activation of Relish and its target genes in neurodegeneration. The neurotoxicity is dependent on Dredd but not the upstream Imd pathway components, and aberrant Dredd activation due to a mutation in the negative regulator Dnr1 is also sufficient to cause neurotoxicity (102, 123, 125).

The importance of the Toll pathway in *Drosophila* development is well established, and accumulating evidence suggests that the Imd pathway also has functions distinct from its conventional roles. Therefore, *Drosophila* appears to be an advantageous model for studying the role of innate immunity signaling pathways and inflammatory responses in various contexts, such as aging, and the function and dysfunction of the nervous system.

Conclusions

The research on *Drosophila* immunity has blossomed during the past 20 years. The Imd signaling pathway has been at the
center of this investigation. Today, the genes that are required for the systemic IMD pathway–mediated AMP response are relatively well known, and the understanding of their respective molecular mechanisms is constantly improving. How the IMD response is tuned (down) is still under active investigation, and studies may give clues to understanding the (mis)regulation of mammalian NF-κB activity. The gut immune response is arguably the most studied aspect of the Drosophila immune response. Comprehending the mechanisms that regulate the IMD path in this context can be expected to lead to discoveries related to chronic inflammatory diseases of the gastrointestinal tract. The importance of the IMD pathway in neurodegeneration is emerging, leading to increased research on the pathway in this context. For example, the trigger for the neurotrophic activation of Relish is yet to be found.

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