REVIEW

Sensing microbial infections in the *Drosophila melanogaster* genetic model organism

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Abstract

Insects occupy a central position in the biosphere. They are able to resist infections even though they lack an adaptive immune system. *Drosophila melanogaster* has been used as a potent genetic model to understand innate immunity both in invertebrates and vertebrates. Its immune system includes both humoral and cellular arms. Here, we review how the distinct immune responses are triggered upon sensing infections, with an emphasis on the mechanisms that lead to systemic humoral immune responses. As in plants, the components of the cell wall of microorganisms are detected by dedicated receptors. There is also an induction of the systemic immune response upon sensing the proteolytic activities of microbial virulence factors. The antiviral response mostly relies on sensing double-stranded RNAs generated during the viral infection cycle. This event subsequently triggers either the viral short interfering RNA pathway or a cGAS-like/STING/NF-κB signaling pathway.

Introduction

The study of invertebrate immunity can be traced back to the pioneering work of Elie Metchnikoff on the defensive role of phagocytes against blastomycetes infection in *Daphnia*, which ushered the study of immunity in both invertebrates and vertebrates (Metchnikoff 1884). Thus, the initial focus of studies on insect host defense against microbial infections was on cellular immunity. It was only in 1918 that Glaser published a study showing the existence of an inducible humoral immune response in caterpillars (Glaser 1918). The antimicrobial activities found in insects were however not identified in insects until the biochemical isolation of the first antimicrobial peptides (AMPs) by Hans Boman in the late 1970s (Steiner et al. 1981). One can easily figure out the advantages for the organism to induce an immune

response only when needed. This however implies that evolution has selected mechanisms that allow hosts to sense the presence of microbial infections. These have been identified in the *Drosophila melanogaster* model over the past 20 years (Buchon et al. 2014; Ferrandon et al. 2007; Hultmark 1993; Lemaitre and Hoffmann 2007).

The study of the humoral immune system has been slow as compared to that of the mammalian one, which was highly facilitated in the latter by the ease of working with antibodies. This however led to a view of the immune system that was largely centered on adaptive immunity at the expense of innate immunity, the importance of which was only recognized rather recently (Janeway 1989; Janeway and Medzhitov, 2002). Actually, one strength of invertebrate models is that they allow the dissection of innate immunity unencumbered by the additional layer of specific, albeit slow, protection provided by acquired immunity. Furthermore, the very large majority of extant species rely solely on innate immunity for host defense against infections. AMPs were initially isolated from the *Hyalophora cecropia* moth pupa that is almost as large as a mouse (Hultmark et al. 1980; Steiner et al. 1981). Additional early biochemical studies led to the discovery of further AMP families, both in Lepidopterans and in large Diptera. This allowed cloning of the AMP genes and the

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study of their promoters, which revealed the existence of NF-κB binding sites in the promoters of AMP genes. It was however transgenic analysis in *D. melanogaster* that rigorously established the importance of these sites for the inducibility of the immune response in vivo (Engström et al. 1993; Meister et al. 1994). The next step was the discovery in 1996 of the major role played by the Toll pathway in the host defense against fungal infections, even though the study of NF-κB signaling relied on the induction of the systemic immune response with bacterial and not fungal challenge (Lemaitre et al. 1996). Transgenic analysis as well as the study of already available mutant lines underscore the power of the *Drosophila* model that benefits from more than 100 years of genetic studies as well as the development of transgenesis in the early 1980s (Rubin and Spradling 1982). Nowadays, genetic engineering allows expressing or inactivating genes at will in a cell type/tissue and time-dependent manner with numerous useful stocks being obtainable from stock centers on several continents (Bellen et al. 2010). Thus, the study of a gene can be performed at multiple scales, from that of molecules to that of the organisms or even at the population biology and evolution level since the genome of more than 100 species of *Drosophila* is now available, e.g., in Kim et al. (2021). This has resulted in an important body of knowledge easily classified by gene product (flybase.org) ever since the *D. melanogaster* genome was sequenced in 2000 (Adams et al. 2000). However, the most powerful tool of *Drosophila* genetics remains the ability to perform large-scale screens, a strategy that besides the identification of the genes required for *Drosophila* development in the early 1980s (Nüsslein-Volhard and Wieschaus 1980) allowed the molecular identification of sensors of the immune response that activate the expression of AMP genes, more than 20 years after the isolation of the first AMPs (Choe et al. 2002, 2005; Gobert et al. 2003; Gottar et al. 2002, 2006; Michel et al. 2001; Pili-Floury et al. 2004).

Here, this primer provides a global view of the mechanisms used by *Drosophila* to perceive the presence of infectious microorganisms and that allow suiting at least to some degree the specificity of the response toward the microbial nature of the invader (Lemaitre et al. 1997). We shall first succinctly describe the different arms of *Drosophila* host defense. Next, we shall focus on the sensors that detect specific microbial compounds of the cell wall as well as those that perceive the enzymatic activity of virulence factors. We shall also discuss some microbial metabolites released by microorganisms in the gut lumen and that are thought to contribute to the distinction between beneficial or harmless microbiota versus pathogenic

germs. Finally, we shall briefly describe the receptors likely involved in the cellular immune response and will provide an overview of the sensing of viral infections.

Overview of the *Drosophila* host defense against microbial infections

To study and comprehend host defenses, the choice of a microorganism is not neutral. In plants, the deciphering of innate immunity initially rested on the study of host–pathogen interactions, which was grounded on the needs from agriculture to protect crops derived from selected cultivars. It initially led to a view in which virulence factors from pathogens, against which plants had evolved effectors to specifically neutralize them, were actually called avirulence factors (Jones and Dangl 2006). In contrast, the use of nonpathogenic or weakly pathogenic microorganisms profoundly affected our initial understanding of *Drosophila* innate immunity as these microorganisms are thought to be rapidly cleared by the immune response of wild-type flies. Indeed, this allowed deciphering basal innate immunity mechanisms that function against any microorganism, pathogenic or not, without the added complexity of host–pathogen relationships that are superposed onto the basal layer. The *Drosophila* systemic immune response relies essentially on two NF-κB pathways. Some pathogens such as *Salmonella enterica*, *Aeromonas salmonicida*, or *Aspergillus fumigatus* are able to neutralize NF-κB immune signaling pathways (Jones et al. 2008, 2012; Pahl et al. 1996), a phenomenon that would hamper the identification and dissection of such pathways. In addition, it provided a paradigm in which to study the action of virulence factors, for instance, by the transgenic expression of virulence factors from highly virulent pathogens such as *Yersinia pestis* (Harnish et al. 2021; Paquette et al. 2012).

The systemic humoral immune response against bacterial and fungal infections

Genetic analysis of existing mutations or of mutants isolated in large-scale screens identified two NF-κB pathways that together regulate the expression of most genes induced or repressed by systemic infections (De Gregorio et al. 2002a; Ferrandon et al. 2007; Ganesan et al. 2011; Lemaitre and Hoffmann 2007). The immune deficiency (IMD) pathway is mainly activated upon infectious challenges with Gram-negative bacteria and regulates the expression of multiple AMP genes such as Diptericin or Drosocin that are active essentially on this class of bacteria. It resembles to some extent the mammalian TNF-α signaling pathway. In contrast, the Toll pathway is preferentially stimulated by prokaryotic Gram-positive bacteria and eukaryotic fungi and is required

for host defense against these pathogens. It regulates the strong expression of an antifungal peptide, Drosomycin, active on filamentous fungi and also of a family of secreted peptides, the Bomanins, the function of which is just beginning to be understood (Lin et al. 2020). The Toll pathway presents analogies to interleukin (IL-1) (Gay and Keith 1991) or possibly Toll-like receptor signaling, even though Toll itself belongs to a sub-family of Toll receptors that is distinct from mammalian Toll-like receptors (Leulier and Lemaitre 2008). Importantly, it does not directly detect compounds of microbial origins but is activated by the Spätzle (SPZ) cytokine, which needs to be proteolytically matured into an active ligand upon the upstream detection of infections (Levashina et al. 1999; Weber et al. 2003).

Of note, some antimicrobial peptide genes such as those encoding cecropins, attacins, or defensin appear to be jointly regulated by both pathways whereas Metchnikowin can be induced independently by either pathway. As mentioned above, the use of nonpathogenic bacteria allowed the determination of a significant degree of specificity in stimulating one or the other pathway or their effectiveness against specific classes of pathogens. In contrast, the use of highly virulent pathogens in septic injury models such as the Gram-negative bacterium *Pseudomonas aeruginosa* revealed that not only the IMD but also the Toll pathway was activated and required to provide some protection against this lethal pathogen (Lau et al. 2003; Limmer et al. 2011). It follows that systemic infections with *Drosophila* pathogens lead to the stimulation of both NF-κB pathways, which allows the formation of Relish/Dorsal/DIF homo- or heterodimer combinations that allow the two pathways to synergize (Tanji et al. 2007, 2010).

In addition to the Toll and IMD pathways, the c-Jun N-terminal kinase (JNK), Map/Erk kinase kinase 1 (MEKK1), and Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathways are also activated during infection but appear to play apparently ancillary roles and likely mediate homeostatic responses to stresses occurring during infection rather than directly fighting off pathogens (Agaisse et al. 2003; Brun et al. 2006; Ekengren et al. 2001; West and Silverman 2018).

Melanization and prophenoloxidase activation

An important host defense found in arthropods is melanization (Soderhall and Cerenius 1998). Besides its role in pigmentation, it is known to play important roles in sealing off wounds in complement of coagulation as well as host defense against infections. Melanization involves initially tyrosine metabolites, notably dihydrophenylalanine (DOPA) and dopamine that become oxidized into corresponding quinones by the action of phenol oxidases (POs), which then assemble into polymers that form eumelanin (Sugumaran and Barek 2016). This set of reactions also produces cytotoxic molecules that are likely active on microorganisms although the exact mode of action remains to be determined (Nappi et al. 1995). It is an open possibility that the production of cytotoxic molecules may be at least partially uncoupled from the blackening reaction at the wounding site (Dudzic et al. 2019). As active PO is toxic for the fly, it is found in the form of inactive pro-forms called pro-PO (PPO) that needs to be matured by proteolytic cleavage by partially characterized immune protease cascades. Thus, similar to the activation of the Toll pathway, PO activation results from the wound or microorganism-triggered proteolytic cascades. Melanization has been reported to be required in the host defense against fungal and some Gram-positive bacterial infections (Binggeli et al. 2014; Dudzic et al. 2015, 2019; Neyen et al. 2015; Wang et al. in preparation). Three genes encode PPOs, and they appear to have somewhat overlapping functions, although PPO1 and the Sp7 protease have been reported to be specifically required to fight off *Staphylococcus aureus* (Dudzic et al. 2019). In contrast, we have found PPO2, and not Sp7, to be involved in the host defense against fungal infections and at least one intestinal infection with a Gram-negative bacterium (Wang et al. in preparation; Chen et al. in preparation).

Of note, coagulation also participates in host defense against bacterial infections by trapping pathogens in a clot (Loof et al. 2011; Theopold et al. 2004). The biochemistry of activation of coagulation by clip-domain-containing proteases downstream of sensors of cell wall components has been especially well-studied in the horseshoe crab (Muta and Iwanaga 1996). These proteolytic cascades are evocative of those at work in *Drosophila* for Toll pathway activation, including the structure of coagulogen, which is related to that of Spätzle (Bergner et al. 1997).

Local immune responses at barrier epithelia

Insects have an exoskeleton made up of a chitino-proteinaceous cuticle that provides a strong physical protection against wounds and infections. It also covers the tracheal respiratory system, the foregut, and hindgut, although the composition of the latter may be adapted to its physiological functions such as water, ions, and amino acid resorption. The *Drosophila* cuticle is shed at each larval molt and replaced by an initially soft cuticle prior to its tanning. Even though the cuticle constitutes a strong barrier to infections, entomopathogenic fungi have evolved strategies that allow them to enzymatically degrade the cuticle at specialized structures known as appressoria developed by the germinating fungus. The fungus then penetrates into the hemocoel by exerting a strong osmosis-driven physical pressure through microscopic holes.

The development of fluorescent reporter transgenes for the expression of AMP genes has revealed expression patterns that can either be constitutive or inducible (Ferrandon et al. 1998). An example of constitutive expression is that of *Drosomycin* in the spermathecae, organs that store the sperm from males after insemination. A partial, "spontaneous" expression of *Drosomycin-GFP* was observed in larval tracheae in culture vials. It was possible to induce its widespread expression throughout the tracheal system by dipping the larvae in a concentrated solution of the Gram-negative bacterium *Erwinia carotovora carotovora* (*Ecc15*) now called *Pectobacterium carotovorum.* Interestingly, the inducible responses were found to be regulated by the IMD and not the Toll pathway, even though *Drosomycin* is essentially controlled by the Toll pathway during the systemic humoral immune response (Davis and Engstrom 2012; Ferrandon 2013; Onfelt Tingvall et al. 2001; Tzou et al. 2000).

Finally, the Toll and IMD pathways are known to be activated in the nervous system but also under circumstances that are not directly related to immunity, e.g., trauma or sleep homeostasis (Blum et al. 2021; Cao et al. 2013). As in mammals, the blood–brain barrier is likely to form an efficient barrier against the dissemination of infections. Nevertheless, some pathogens able to cross this barrier have been recently identified and these novel models will likely allow a better understanding of immune responses in the nervous system, which are poorly understood at present (Benmimoun et al. 2020). Interestingly, a link between the nervous system and host defense against systemic infections in insect larvae had already been established in the 1930s by Serguei Metalnikov at the Institut Pasteur.

The cellular immune response

The cellular immune response in adult *Drosophila* mostly relies on phagocytosis (Melcarne et al. 2019a; Stuart and Ezekowitz 2008). A limited number of hemocytes is found in adults and correspond to mostly sessile cells (Lanot et al. 2001). Until recently, adult hemocytes were thought to be constituted mostly of macrophage-like plasmatocytes. However, some 10% of adult hemocytes express PPO1 and might therefore play a key role in melanization defenses (Boulet et al. 2021). The link between melanization and hemocytes needs to be more thoroughly investigated in adult flies. In larvae, two major cell types exist, plasmatocytes and crystal cells, the latter containing PPO2 crystals, which rupture upon infection through a JNK-mediated process and release their content in the hemolymph (Bidla et al. 2007; Binggeli et al. 2014). A third cell type, the lamellocyte, is inducible

by parasitoid wasp egg deposition within the hemocoel of the second instar larva and together with plasmatocytes encapsulate the egg prior to PPO2-PPO3-dependent melanization (Dudzic et al. 2015). The melanized capsule isolates and may ultimately kill the wasp egg. We shall focus below on the putative phagocytic receptors as well as potential opsonins.

The *Drosophila* antiviral defenses

Whereas in mammals the interferon response is a primary line of defense against viral infections in most cell types, it has not been selected as the main antiviral defense in all protostome phyla (Schneider and Imler 2021). Indeed, while some evidence for an interferon-like response in mollusks has recently been provided, insects do not encode major components of this pathway in their genomes, even though one *Drosophila* antiviral effector, Vago, has been proposed to function in an analogous manner by activating the JAK-STAT pathway in *Culex* mosquito cells (Deddouche et al. 2008; Lafont et al. 2020; Paradkar et al. 2012; Qiao et al. 2021). Instead, insect antiviral immunity appears to rely to a large extent on RNA interference (RNAi), as is the case for plants and for some mammalian cell types such as embryonic stem cells. This defense involves Dicer-2 (DCR-2) and Argonaute2 (AGO2). In brief, DCR-2 processes long viral dsRNA such as those produced during viral replication into small RNAs known as vsiRNAs. These siRNAs are then loaded onto the AGO2-containing RNA-induced silencing complex (RISC), and one strand acts as a specificity determinant for the cleavage of viral RNAs. These responses take place in infected cells. Recently, a systemic aspect has been revealed that relies on the production of virus-derived complementary DNAs (vDNA) that in turn produce secreted secondary siRNAs released in exosome-like vesicles. These vDNAs appear to be involved in priming the immune response against secondary infections and also in transgenerational antiviral immunity (Goic et al. 2013; Mondotte et al. 2018, 2020; Poirier et al. 2018; Tassetto et al. 2017). Besides the siRNA pathway, virus-inducible gene expression programs regulated by several signaling pathways have been reported. A first one is the single JAK-STAT pathway of *Drosophila*, which plays a role in host defense against Dicistroviridae RNA viruses such as the *Drosophila* C virus or cricket paralysis virus (CrPV). It is also required to control the DNA virus Invertebrate iridescent virus (IIV)-6. JAK-STAT pathway mutants are more susceptible to these viral infections (Dostert et al. 2005; West and Silverman, 2018). This pathway appears to be more involved in the resilience to cellular damages occurring during viral infections than to a direct antiviral activity since the viral burden

does not appear to be strongly affected when the JAK-STAT pathway is affected. Alternatively, viruses might release effectors suppressing JAK-STAT-inducible antiviral compounds or the JAK-STAT pathway itself, as appears to be the case for the recently described Kallithea DNA virus with respect to Toll signaling (Palmer et al. 2019). This however does not seem to be the case for the RNA viruses that induce the expression of the *vir-1* JAK-STAT pathway target gene (Dostert et al. 2005).

The NF-κB signaling pathways have also been reported to be involved in host defense against viral infections, as supported by the existence of viral suppressors (Palmer et al. 2019). Interestingly, the Toll pathway appears to be effective against several RNA viruses in an oral but not in a systemic infection model, even though it gets activated in the fat body in both types of infection. Of note, this defense does not involve the Dorsal-related immunity factor (DIF), which mediates the systemic antimicrobial response in adults, but its neighboring gene Dorsal, which mediates the developmental role of the Toll pathway. Dorsal migrates to the nucleus only in infected fat body cells and not in other tissues. However, blocking the Toll pathway by RNAi in the fat body or other tissues failed to lead to an enhanced susceptibility to the ingested virus (Ferreira et al. 2014). It may indirectly interfere with a step of the viral infection process occurring only upon intestinal infection, e.g., translocation of the virus particles across the digestive barrier.

As regards the IMD pathway, it may be involved in the regulation of apoptosis, an effective antiviral defense, in complex manners (Schneider and Imler 2021). The finding that several viruses have incorporated suppressors of the IMD pathway suggests that it may exert a selective pressure on viruses. Recently, it has been found that a noncanonical IMD pathway involving *Drosophila melanogaster* STING, IKKß, and the NF-κB transcription factor Relish is required for host defense (Goto et al. 2020) and bears some resemblance to the antiviral response mediated by STING in mammals. How exactly it gets activated will be described further below.

Behavioral immunity

Insects anticipate to some extent the exposure to potential pathogens and adapt their behavior accordingly. Repulsive volatile compounds such as geosmin can be detected through specific odorant receptors, whereas others such as commercial lipopolysaccharide (LPS) (notoriously known to be heavily contaminated by other cell wall compounds such as peptidoglycan) are perceived through gustatory receptors (Soldano et al. 2016; Stensmyr et al. 2012). Interestingly, the

sensing of infections in the gut leads to the production of the Unpaired3 IL-6-like cytokine that metabolically reprograms the ensheathing glia and thereby affects their metabolic coupling to neurons. Thus, signaling from the gut leads to an enhanced avoidance of *Ecc15*-laced food (Cai et al. 2021). This aspect of innate immunity has been recently reviewed (Montanari and Royet 2021).

Sensing of Gram-negative bacterial infections: detection of DAP type peptidoglycan

The Gram-negative cell wall is a composite structure that comprises the cytoplasmic membrane, a gelatinous periplasm that includes a thin but rigid layer of peptidoglycan (PGN) and a lipid bilayer that forms the outer membrane (OM). The inner part of the OM is made of phospholipids and lipoproteins that connect to the PGN layer whereas the outer layer is made up of LPS and also integrates proteins such as porins as well as surface proteins. LPS is also known as endotoxin and triggers the mammalian inflammatory response at very low concentrations. Even though LPS is exquisitely detected in Limulus species, chelicerate arthropods, the cell wall compound sensed by the *Drosophila* innate immune system during bacterial infections is PGN, be it of Gram-positive or Gram-negative origin (Kaneko et al. 2004; Leulier et al. 2003). PGN is a complex polymer made of long glycan chains of alternating ß-(1,4) linked N-acetylglucosamine and N-acetyl muramic acid monomers cross-linked by peptide chains of three to five amino-acids that form a mesh-like layer. In Gram-negative bacteria, the third amino-acid of the linker peptide is meso-diaminopimelic acid (DAP), whereas in many Gram-positive bacteria, a lysine (LYS) is found at the same position. An amidated DAP is found in Gram-positive bacilli. Tracheal cytotoxin (TCT) is a glycan dimer with an anhydro form of muramic acid coupled to a DAP-containing peptide linker found at the end of PGN chains. TCT can be released during bacterial division and growth when PGN gets remodeled. Both DAP-PGN and TCT are detected by the *Drosophila* immune system and trigger the IMD pathway whereas LYS-PGN is a better inducer of the Toll pathway than DAP-PGN. PGNs are essentially sensed, or enzymatically inactivated, by members of the peptidoglycan recognition protein (PGRP) family (Fig. 1) (Ferrandon et al. 2007; Royet and Dziarski 2007).

The *Drosophila* genome encodes 13 PGRP family members that share a common 160 amino acid PGRP domain with similarity to N-acetylmuramoyl-L-alanine amidases

such as the T7 bacteriophage lysozyme (Werner et al. 2000). Only a few members of the family (PGRP-SCs and PGRP-LB) have maintained a catalytically active enzymatic site that allow them to cleave the lactyl-amide bond between the glycan chain and the first amino acid of the stem peptide of PGN (Bischoff et al. 2006; Charroux et al. 2018; Costechareyre et al. 2016; Garver et al. 2006; Gendrin et al. 2009; Guo et al. 2014; Mellroth et al. 2003; Paredes et al. 2011; Zaidman-Remy et al. 2006, 2011). Most of the other family members are involved in sensing PGN (Choe et al. 2002; Gottar et al. 2002; Iatsenko et al. 2016; Kaneko et al. 2006; Michel et al. 2001; Ramet et al. 2002; Takehana et al. 2002, 2004). PGRPs can either be short proteins noted PGRP-S that are secreted or long proteins (PGRP-L). Most PGRP-Ls include a transmembrane domain. For instance, PGRP-LC is a type II transmembrane domain receptor whereas full length PGRP-LE is cytosolic. The complexity of the family is enhanced by the existence of multiple splice isoforms that have been shown to have important functional properties as regards PGRP-LC and PGRP-LB.

The crystal structures of several PGRPs are available and revealed the major mechanism of binding to PGN and how the DAP- and LYS-PGN are being discriminated by distinct family members. The structures of PGRP-LB, PGRP-SA and human PGRP-Iα revealed that PGN is bound to the sensor through a large hydrophilic L-shaped cleft (Chang et al. 2004; Kim et al. 2003; Reiser et al. 2004). The DAP-type PGN sensors PGRP-LC and PGRP-LE complexed to TCT have been crystallized and their structures solved (Chang et al. 2006; Lim et al. 2006). These studies identified specific amino-acids including an arginine buried at the bottom of the docking groove that is required for forming a bidentate salt bridge with the carboxylate end of meso-DAP. These residues are not conserved in PGRPs that bind preferentially to LYS-PGN. These studies also revealed the head-to tail infinite polymerization of PGRP-LE bound to TCT, since the binding of the first PGRP-LE monomer induces the formation of a second binding site in which the disaccharide moiety is present at the dimer interface and not the peptide stem that is bound at the first binding site (Lim et al. 2006). In the case of PGRP-LC, the PGRP-LCx isoform complexes TCT whereas PGRP-LCa binds only to the TCT-PGRP-LCx complex (Fig. 1), as it is unable to directly bind to PGN because its binding groove is interrupted by two helical insertions (Chang et al. 2005). In contrast, modeling studies suggest that PGRP-LCx alone is able to bind to polymeric PGN by forming arrays in which molecules binds to
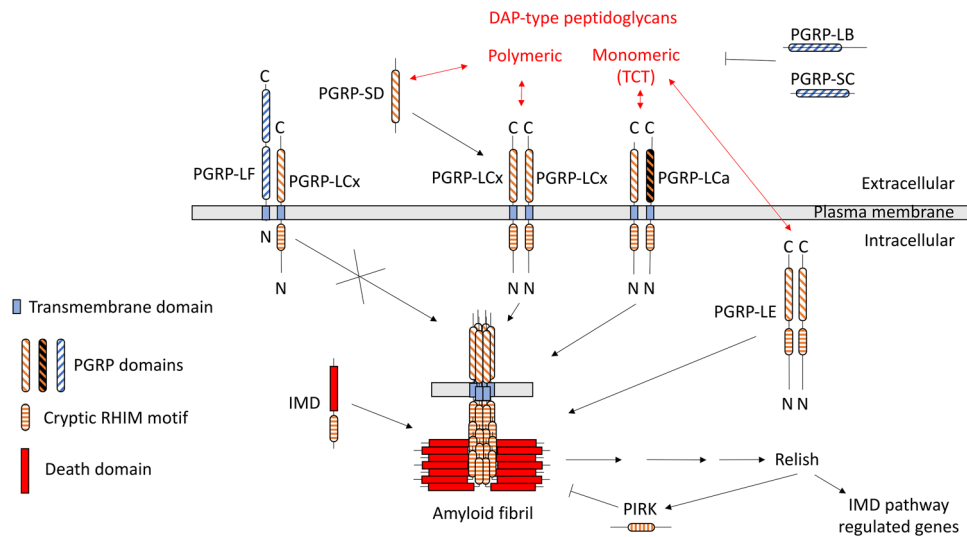
Fig. 1 Sensors of bacteria activating the IMD pathway. The IMD pathway is activated after recognition of Gram-negative bacteria, through the DAP-type peptidoglycans (PGNs) present in their cell wall and that may be released during bacterial growth and division or upon attack by AntiMicrobial Peptides (AMPs). DAP-type PGNs are sensed, or enzymatically inactivated, by members of the PeptidoGlycan Recognition Protein (PGRP) family. Polymeric DAP-type PGNs can be detected by PGRP-LCx whereas the monomeric DAP-type PGNs tracheal cytotoxin (TCT) is sensed by a PGRP-LCx-PGRP-LCa dimer. PGRP-LE functions as an intracellular sensor of TCT in barrier epithelia such as the gut or the Malpighian tubules and also in octopaminergic neurons. The cryptic RIP Homotypic Interaction Motif (cRHIM) domain found in PGRP-LCx, -LCa and -LE allows their oligomerization that seeds the formation of amyloid fibers, that the IMD adapter elongates through its own cRHIM domain. The amyloid fibers triggers downstream IMD pathway signaling that ultimately activates the Relish transcription factor. This process can be negatively regulated by the induction of the PIRK repressor. PGRP-SCs and PGRP-LB prevent detrimental IMD pathway activation by degrading polymeric or monomeric PGNs into metabolites unable to trigger the IMD pathway, a process that is counteracted by the binding of PGRP-SD to PGN. PGRP-LF may prevent the formation of PGRP-LC multimers and hence negatively regulate the initiation of IMD pathway signaling. PGRP-LA may enhance IMD pathway activation solely in barrier epithelia (not shown)

a PGN disaccharide every five units of the chain (Chang et al. 2006).

As mentioned above, only the PGRP-LCx isoform is able to bind to PGN whereas the PGRP-LCa is required to sense TCT both in vitro and in vivo. It was possible to test the role of each PGRP-LC isoform in vivo by complementation experiments of a null PGRP-LC mutant (Neyen et al. 2012). The PGRP-LCx alone was sufficient to confer resistance against *Ecc15* and also immune-responsiveness to this bacterium whereas the LCa and LCy other isoforms were not active, alone or in combination. Indeed, like PGRP-LCa, PGRP-LCy is structurally unable to bind to PGN but in contrast to the former is not required to sense TCT. More recently, PGRP-LC isoforms with an alternative first coding exons have been identified, which encode alternative cytoplasmic tails for LCx, LCy, and LCa. These rPGRP-LC alternative forms are more induced by an immune challenge than the other isoforms. They play a negative role and target the receptors for endosomal degradation via the ESCRT machinery in a negative regulatory loop. Thus, the function of these isoforms is to down-regulate and terminate IMD pathway signaling upon sensing PGN released by killed bacteria but not upon TCT challenge. This system may allow the organism to adapt to the level of the threat presented by live but not killed bacteria (Neyen et al. 2016).

PGRP-LE belongs to the PGRP-L family yet does not appear to have a signal peptide, even though a shorter form consisting essentially of the sole PGRP domain has been detected in the hemolymph. Its overexpression has been reported to induce melanization, presumably by triggering the proteolytic cascades in the hemolymph that activate PPOs (Takehana et al. 2002). Functionally, PGRP-LE is dispensable for triggering the IMD pathway in the systemic immune response induced by *Ecc15* but not *Escherichia coli*. It is however required for full activation of the systemic immune response induced by the ingestion of *Ecc15* bacteria in a sensitized *PGRP-LB* genetic background (see also below) (Neyen et al. 2016). Further studies have shown that PGRP-LE functions as an intracellular sensor of TCT in barrier epithelia such as the gut or the Malpighian tubules and also in the brain (Bosco-Drayon et al. 2012; Charroux et al. 2018; Kaneko et al. 2006; Kurz et al. 2017).

TCT can be sensed intracellularly by cytosolic PGRP-LE, which raises the question as to how TCT produced by extracellular bacteria reaches the cytoplasm. While it had been shown that the *Drosophila* SLC15 transporter family Yin is able to enhance the import of muramyl dipeptide and its subsequent sensing by NOD2 when ectopically expressed in mammalian cells, further studies in *Drosophila* demonstrated that this transporter does not play a significant role in transporting TCT intracellularly for PGRP-LE sensing (Capo et al. 2017; Charriere et al. 2010). In contrast, the SLC46 family CG8046 transporter is required for such a role, at least in Malpighian tubules (Paik et al. 2017). Another possibility for TCT delivery to the cytosol might involve the fusion of TCT-containing outer membrane vesicles secreted by bacteria, a phenomenon not investigated in *Drosophila* so far.

The intracellular part of PGRP-LC isoforms (rPGRP-LCs excepted) contain a cryptic RIP Homotypic Interaction Motif (cRHIM) domain, which is also found in PGRP-LE, the IMD adapter, and PIRK, a negative regulator of IMD pathway signaling. RHIM domains in mammalian RIP kinase (RIPK) proteins form functional amyloids during necroptosis (Grootjans et al. 2017; Liu et al. 2017). The PGRP-LE, PGRP-LC, and IMD proteins form amyloid fibers in vitro and also in *Drosophila* S2* cells when overexpressed (Kleino et al. 2017). The disruption of these amyloid fibers led to an impaired IMD immune response in S2 cells and in vivo in flies as well. It is thought that PGRP-LC and PGRP-LE when bound to their ligands seed the formation of amyloid fibers, whereas the much more abundant IMD adapter would then elongate the fibers (Kleino et al. 2017). This process can be negatively regulated by the induction of the PIRK repressor that poisons the formation of novel fibrils and might possibly disassemble existing fibrils. This later possibility remains however to be demonstrated.

Together with sensors of viral infections, PGRP-LE is one of the few known *Drosophila* cytosolic receptors that trigger innate immune responses. Interestingly, it has also been shown to target intracellular *Listeria monocytogenes*, a DAP-PGN containing Gram-positive bacterium, for xenophagy (Yano et al. 2008).

Modulation of PGRP-LC signaling by other PGRP family members

The sensing of bacteria with a DAP-PGN cell wall relies on sensing PGN glycan chains or derived metabolites. Several of the PGRP family members enhance or diminish the interactions of PGRP-LC and PGRP-LE with their cognate elicitors. While PGRP-SD had originally been proposed to function in Toll pathway signaling to enhance the detection of Gram-positive bacteria such as *S. aureus* (Bischoff et al. 2004; Wang et al. 2008), this finding was not confirmed upon studying a CRISPR-Cas9 null mutant that revealed instead a positive role in sensing Gram-negative bacteria, in keeping with its three-dimensional structure that revealed features consistent with PGRP-SD binding to DAP- and not LYS-PGN (Iatsenko et al. 2016; Leone et al. 2008). PGRP-SD is induced through IMD pathway activation and its phenotype is not visible at the very early stages of IMD signaling. It may protect PGN from binding to PGRPs that have a

negative impact on IMD pathway signaling (Iatsenko et al. 2016). One is PGRP-LF, which contains two PGRP extracellular domains but a very short cytoplasmic tail unable to initiate downstream canonical IMD signaling (Maillet et al. 2008). While PGRP-LF had originally been proposed to compete with PGRP-LC for DAP-PGN binding, the structural analysis of its two PGRP domains revealed that these domains are unexpectedly unable to bind PGN even though the key amino-acids for binding to DAP-PGN are present in its sequence. However, the PGRP-LF ectodomain is able to bind to PGRP-LCx with an affinity similar to that measured for the PGRP-LCa ectodomain binding to PGRP-LCx in the absence of TCT. Both interactions are increased seven-fold when TCT is added. Thus, by binding to PGRP-LCx, PGRP-LF may prevent the formation of signaling PGRP-LC multimers and hence negatively regulate the initiation of IMD pathway signaling (Basbous et al. 2011). Indeed, the IMD pathway is constitutively active in the absence of PGRP-LF.

PGRP-LA, *PGRP-LC*, and *PGRP-LF* form a cluster of genes on the third chromosome. The sequence of PGRP-LA indicates it is unable to bind to PGN. *PGRP-LA* is poorly expressed in the fat body but is enriched in barrier epithelia. Accordingly, a *PGRP-LA* mutant did not display any phenotype with respect to the systemic immune response triggered by *Ecc15*; yet, the ectopic overexpression of its D splice isoform strongly activated the IMD pathway, likely by seeding amyloid fibers through its cRHIM domain. Interestingly, the *PGRP-LA* loss-of-function mutant displayed a decreased induction of the IMD pathway in larval tracheae and in the adult midgut. Taken together, these results suggest that PGRP-LA promotes IMD pathway activation in local epithelial immune responses (Gendrin et al. 2013).

Two of the three *Drosophila* nonaspannin proteins, which contain nine transmembrane domains have been shown to interact with PGRP-LC (Perrin et al. 2015). TM9SF4 appears to be required for the trafficking or maintenance of PGRP-LC to the surface of S2 cells. Its downregulation, however, did not impair IMD pathway signaling in cultured cells and in flies. It is an open question whether the sensitivity of TM9SF4 mutants to bacterial infections is caused by defects in the cellular immune response since PGRP-LC appears to play at best an ancillary role in this process (Bergeret et al. 2008; Perrin et al. 2015).

PGRP-SBs, PGRP-SCs, and PGRP-LB have been shown to be functional amidases (Bischoff et al. 2006; Charroux et al. 2018; Costechareyre et al. 2016; Garver et al. 2006; Gendrin et al. 2009; Guo et al. 2014; Mellroth et al. 2003; Paredes et al. 2011; Zaidman-Remy et al. 2006, 2011). Some of them play major roles in preventing detrimental IMD pathway activation by degrading polymeric or monomeric PGN into metabolites unable to trigger the IMD pathway. PGRP-SBs have been shown to be secreted in the hemocoel and to be active against DAP-type PGN. However, the deletion of both PGRP-SB1 and PGRP-SB2 together or in combination with other PGRP amidases did not reveal any immune phenotype (Paredes et al. 2011; Zaidman-Remy et al. 2011). Both PGRP-SC2 and PGRP-LB are strongly expressed in the gut (Costechareyre et al. 2016). Yet, only the latter plays a major role in degrading PGN in the gut lumen and in preventing further systemic activation of the IMD pathway in the fat body, even though *PGRP-SC2* mutants display enhanced aging of the gut through increased dysbiosis of the microbiota and concurrent hyperproliferation of intestinal stem cells (Costechareyre et al. 2016; Guo et al. 2014). In the framework of the systemic immune response, it appears that PGRP-SC2 is the major player to degrade immuno-stimulatory PGN. While PGRP-SC1 has been described as being required for phagocytosis and Toll pathway activation independently of its amidase activity, it does not appear to play any regulatory role in the IMD pathway (Costechareyre et al. 2016; Garver et al. 2006; Paredes et al. 2011).

The constitutive activation of the IMD pathway in the gut resulting from the inactivation of some of its negative regulators is detrimental to the life span of flies (Guo et al. 2014; Paredes et al. 2011; Ryu et al. 2008). In the framework of the systemic immune response to infection, one might expect that any detrimental role of enhanced IMD pathway activation would lead to a shortened survival to infection, as has indeed been observed with the injection of *Pseudomonas entomophila* (Costechareyre et al. 2016). Unexpectedly, mutants for PGRP-LB and PIRK/RUDRA lead respectively to no change and increased survival to an *Ecc15* challenge, in keeping with an increased expression of AMPs to which *Ecc15* is at least partially sensitive (Aggarwal et al. 2008; Zaidman-Remi et al. 2006). Thus, the exact level of IMD pathway activation results in differing effects according to the infectious pathogen.

Impact of bacterial infections on the local and systemic immune responses

The digestive system represents one of the major and most extensive interface with the environment. Indeed, flies feed on a microbe-rich diet such as decaying fruits and the gut barrier is not as tight as the exoskeleton cuticle. Digestion occurs in the midgut, a section of the digestive tract not protected by cuticle but by a semi-permeable chitinoproteinacous membrane known as the peritrophic matrix that prevents the direct contact of most bacteria with the underlying intestinal epithelium (Lemaitre and Miguel-Aliaga 2013). The

gut lumen and a digestive tract diverticulum, the crop, are colonized by the microbiota, which strongly influences the biology of the fly at multiple levels (Broderick and Lemaitre 2012; Pais et al. 2018). It is therefore essential to be able to fight off pathogenic bacteria without unnecessarily clearing off potentially beneficial microbiota. The microbiota is not as diverse as in humans and is made of some 20–30 species with a handful of prevailing ones such as *Lactobacillus plantarum* or *Acetobacter pomorum*. Very few species are actually able to truly colonize the digestive tract. PGN derived from the microbiota or ingested bacteria are sensed by enterocytes through PGRP-LE and PGRP-LC, which are distributed in distinct sub-regions of the midgut with PGRP-LC being more prevalent in the anterior part and PGRP-LE in the posterior regions (Bosco-Drayon et al. 2012). As for the systemic immune response, PGRP-LC detects extracellular PGN, whereas PGRP-LE binds to intracellular TCT. PGRP-LE plays a major role in setting up the tolerance level of the microbiota by regulating the expression of IMD pathway negative regulators (Charroux et al. 2018). Interestingly, the level of TCT to which PGRP-LE is exposed is modulated by intracellular forms of PGRP-LB, thereby allowing a fine control of IMD pathway activation within enterocytes independently of systemic IMD pathway activation (Charroux et al. 2018). The extracellular form of PGRP-LB secreted in the lumen limits the passage of PGN through the gut by an unknown mechanism that shields it from the action of the intracellular forms of PGRP-LB in enterocytes. When an intestinal infection occurs, PGN levels increase yet PGRP-LB manages to limit the passage of PGN to the hemocoel. As a result, the induction of the systemic immune response is limited. PGN originating from the gut also reaches octopaminergic neurons where it is sensed by PGRP-LE and activates the IMD pathway to inhibit egg laying (Kurz et al. 2017). As in enterocytes, an intracellular form of PGRP-LB modulates what appears to be a form of behavioral immunity.

Interorgan communications between the gut and the fat body may prime the organism for a potential systemic infection. This is documented in larvae: the ingestion of *Ecc15* induces the activation of the systemic immune response, especially that of the IMD pathway (Basset et al. 2000; Foley and O'Farrell, 2003; Shia et al. 2009; Wu et al. 2012; Yang et al. 2019). In contrast, there is hardly any activation of the systemic innate immune response when *Ecc15* bacteria are ingested by adult flies (Basset et al. 2000), unless the flies are mutant for *PGRP-LB* or *PGRP-SC2* (Neyen et al. 2012; Zaidman-Remy et al. 2006). Reactive oxygen species generated by the dual oxidase (DUOX) enzyme in enterocytes or by the nitric oxide (NO) synthase (NOS) appear to be required for gut to fat body communication in larvae (Foley and O'Farrell, 2003; Wu et al. 2012). Of note, the work on

NO signaling has been performed using chemical inhibitors or NO donors. An ultimate confirmation awaits reproducing these data with a null *NOS* mutant (Yakubovich et al. 2010). Several studies have reported an involvement of hemocytes in relaying the signal from gut to fat body in larvae (Basset et al. 2000; Charroux and Royet 2009; Shia et al. 2009; Yang et al. 2019). In one study, IMD pathway activation is required in enterocytes and leads to the production by hemocytes of aldose reductase by an unknown mechanism. This leads to an increased concentration in the hemolymph of polyols such as sorbitol or galactitol obtained from the catalysis of glucose or galactose by aldose reductase. These polyols in turn activate the metalloproteinase 2 (MMP2) that would cleave off the extracellular domain of PGRP-LC on the surface of fat body cells and thereby initiate IMD pathway activation (Yang et al. 2019). Of note, the ectopic transgenic expression of *mmp2* had earlier been shown to lead to the cleavage of the extracellular part of the PGRP-LC receptor and to the activation of the IMD pathway. The significance of this finding was difficult to assess since IMD pathway activation was normal in *mmp2* mutant larvae after septic injury (Schmidt et al. 2011). Thus, the Yang et al. study provided a physiological context in which to interpret this observation. It will be interesting to determine whether PGN fragments are also involved in the activation of the systemic immune response in larvae, for instance, by testing whether it is enhanced in *PGRP-SC* mutants, since the major PGN degradation function of PGRP-SC takes place in the hemolymph (Costechareyre et al. 2016). Conversely, it remains to be shown that the polyol pathway is also active in adults.

Sensing of Gram-positive bacterial and fungal infections extracellularly triggers the Toll pathway

The Toll pathway is required in the host defense against two strikingly distinct categories of pathogens, prokaryotic Gram-positive bacteria and eukaryotic fungi. In contrast to the IMD pathway where the activation of signaling takes place at the level of the cytoplasmic membrane or intracellularly, the sensing of infections that trigger the Toll pathway takes place extracellularly in the hemocoel (Fig. 2). As noted earlier, Toll does not directly recognize microbial compounds but binds to the Spätzle cytokine, which gets activated by proteolytic cleavage by SPE (Jang et al. 2006). SPE gets activated by two proteolytic cascades, one that involves the upper protease ModSP and the other the Persephone (PSH) protease (Buchon et al. 2009; El Chamy et al. 2008; Gottar et al. 2006). ModSP is activated by sensors
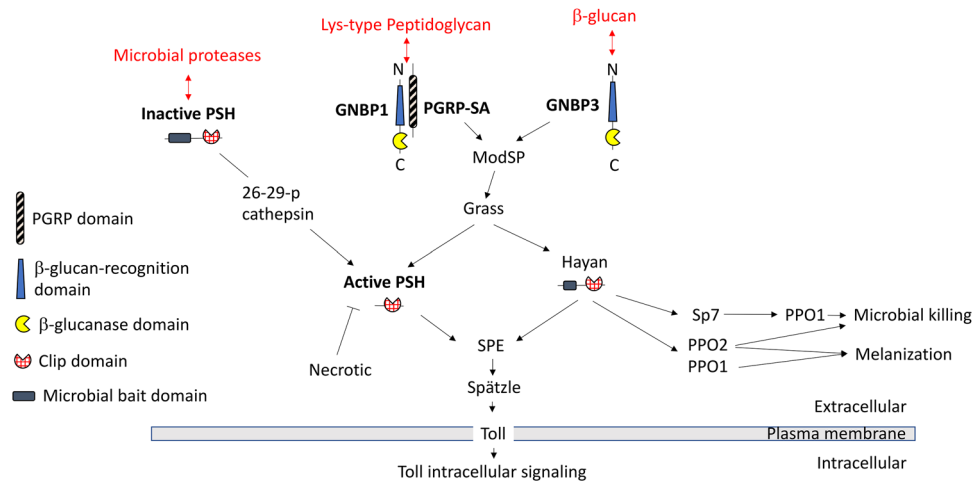
Fig. 2 Sensors of microorganisms activating the Toll pathway. Lys-type peptidoglycans (LYS-PGNs) found at the surface of Gram-positive bacteria are sensed by a complex of GNBP1 and PGRP-SA, the former processing LYS-PGN for detection by PGRP-SA. ß-glucans found at the surface of fungi can be detected by GNBP3. GNBP1 and GNBP3 can activate ModSP through their enzymatically-inactive ß-glucanase domain, that in turn activates Grass. Next, Grass is thought to activate both PSH and Hayan, which function redundantly. The sensing of microbial proteases through Persephone (PSH) bait domain leads to the formation of a cleaved pre-activated inactive PSH that gets further processed by the circulating 26-29-p cathepsin protease to a mature PSH. PSH can also be directly activated by subtilisin (not shown). Active PSH and Hayan can both activate SPE, that processes Spätzle to activate the Toll intracellular signaling pathway. The Necrotic serpin avoids the spontaneous self-activation of PSH. In addition to the activation of Toll intracellular signaling, Hayan activates the Sp7 protease as well as the two prophenoloxidases PPO1 and PPO2. These extracellular enzymes are involved in microbial killing and activation of melanization, which may be separate processes

that detect LYS-PGN or ß-(1–3)-glucans, whereas PSH acts as a bait for the proteases secreted by bacterial or fungal pathogens.

Genetic screens allowed to identify PGRP-SA and GNBP1 as being required in host defense against several Gram-positive bacterial strains and GNBP3 as being involved in fighting off fungal infections (Gobert et al. 2003; Gottar et al. 2006; Michel et al. 2001). While the X-ray structure of PGRP-SA was established early on, that of the N-terminal domain of GNBP3 was determined later on (Mishima et al. 2009; Takahasi et al. 2009), in keeping with the slower identification of GNBP3 that required screens that also tested fungal elicitors of the immune response. The structure of PGRP PGN-binding domains has been discussed above. As for PGRPs, the first GNBPs have been isolated biochemically in larger insects such as the silkworm *Bombyx mori* through their ability to bind to microbial cell wall compounds, namely ß-glucans (Ochiai and Ashida 1988; Yoshida et al. 1986, 1996). A first member of the silkworm GNBP family was purified based on its ability to bind to the cell wall of Gram-negative bacteria, hence the name (Lee et al. 1996). Like other members of the family, GNBP1 contains a N-terminal ß-(1–3)-glucan recognition domain (ßGRP) as well as a C-terminal domain with homology to ß-(1–3) and ß (1–3, 1–4) glucanases, the catalytic site of which has not been conserved. However, even though it has been reported to bind to LPS in vitro, genetic analysis

unambiguously ascribed a role for GNBP1 in sensing Gram-positive and not Gram-negative bacterial infections (Gobert et al. 2003; Kim et al. 2000; Pili-Floury et al. 2004). The PGRP-SA receptor had been identified earlier as also being required for the activation of the Toll pathway by Gram-positive bacteria with LYS-PGN (Michel et al. 2001). The overexpression of both PGRP-SA and GNBP1 is sufficient to ectopically activate the Toll pathway in the absence of infection (Gobert et al. 2003). Mechanistically, GNBP1 appears to have a muramidase activity that hydrolyzes PGN from *Micrococcus luteus* or *Enterococcus faecalis*, but not *S. aureus*. This muramidase activity is enhanced when GNBP1 is bound to PGRP-SA. Interestingly, the produced polymeric muropeptides can trigger the Toll pathway in a PGRP-SA-dependent but GNBP1-independent manner. Thus, GNBP1 processes some LYS-PGN to present it to PGRP-SA (Filipe et al. 2005; Wang et al. 2006). Of note, in the beetle *Tenebrio molitor*, it has been proposed that lysozyme processes PGN and thereby provides a substrate for clustering PGRP-SA that then would recruit GNBP1 and initiate downstream signaling (Park et al. 2007).

In contrast, GNBP3 is required for sensing the fungal cell wall (Gottar et al. 2006; Mishima et al. 2009). The N-terminal ß-GRP domain has been shown to bind to relatively long ß-glucan chains with an optimal length ranging between 20 and 40 glucan units. The structure of the immunoglobulin-fold ßGRP domain has been solved, and

two distinct models have been proposed to account for its binding to ß-glucans (Mishima et al. 2009; Takahasi et al. 2009). One feature possibly accounting for the ability of the ßGRP domain to bind to long and not short polysaccharide chains has been proposed to rely on a loop that covers the putative glucan binding site that would be displaced only by long glucan chains and then stabilized by two hydrophobic residues present in the lid, which is conserved only in ß-glucan-binding members of the GNBP family but not in GNBP1 (Mishima et al. 2009).

It has been proposed that the function of the ß-glucanase-like domain of GNBPs mediates the activation of the downstream proteolytic cascade, namely the modular serine protease ModSP that functions just downstream of both GNBP1 and GNBP3 (Buchon et al. 2009; Roh et al. 2009). Of note, three genes in the *Drosophila* genome encode the ßGRP domain only and would therefore not be expected to be directly involved in triggering the proteolytic cascades that activate the Toll pathway or melanization. One of the corresponding proteins, GNBP-like3, is one of the most strongly induced protein by a fungal immune challenge (Levy et al. 2004). It has been reported to be acting as an AMP against Gram-positive bacteria (Barajas-Azpeleta et al. 2018), findings that are not congruent with our analysis of null mutants that reveals an action against a pathogenic yeast (Huang, Quintin et al. in preparation). Finally, two cryptic GNBP members are found in the *D. melanogaster* genome but are affected by mutations found also in wild fly populations retrieved from diverse locations worldwide and also in closely related *Drosophila* species (Quintin 2009).

Sensing the enzymatic activity of microbial virulence factors elicits the systemic humoral immune response

The activation of the *Drosophila* systemic humoral immune response described so far relies on receptors that sense cell wall components. This view corresponds to the Pathogen (or microbial)-associated molecular pattern–Triggered Immunity (PTI) theorized in plants (Jones and Dangl, 2006) and in animals (Gaidt et al. 2021). It has been proposed that pathogens are selected for their expression of virulence factors, some of which might target PTI components. In response, the host has to develop countermeasures that target these virulence factors, a form of immunity known as effector-triggered immunity (ETI). This would usher cycles of selection for other virulence factors and their host antidotes. While evidence for such cycles remains scarce in *Drosophila*, the sensing of microbial proteases through the Persephone (PSH) protease is evocative of ETI (El Chamy et al. 2008; Gottar et al. 2006). An alternative view is that pathogens may mask the cell wall components that

are detected by the innate immunity basal sensing system (Box 1). The detection of the enzymatic activity of secreted virulence factors would allow the host to still perceive the presence of an infection within the hemocoel. A precursor study suggested that some microbial proteases are required for Dorsal nuclear uptake in cultured fat bodies. This activity was however absent when hemolymph was not added to the cultured fat bodies (Bettencourt et al. 2004). The PSH Clip-domain-containing protease is present in hemolymph and contains a bait domain able to be cleaved by various microbial proteases. This leads to the formation of an inactive PSH that gets further processed by a circulating cathepsin protease known as 26–29-p (Issa et al. 2018). Clip domain proteases are secreted as zymogens that get activated by proteolytic cleavage of the Clip prodomain by the protease upstream in the cascade at a site just upstream of the catalytic domain (Veillard et al. 2016). This site contains residues detected usually by enzymes with a trypsin-like specificity. PSH contains an unusual histidine residue instead of the commonly found arginine or lysine, which can be cleaved only by subtilisin secreted by *Bacillus subtilis* or by the 26–29-p cathepsin, only once the bait domain has been processed. The bait domain is evocative of that found in mammalian α2-macroglobulin, a general suicide inhibitor of all classes of proteases. Formally, the situation is akin to that found in plants in which targets of virulence factors are monitored by guards that detect pathogen-induced modifications of the target: the bait region is a target that allows the 26–29-p guard to trigger the Toll pathway by activating the PSH protease that will activate the downstream SPE protease. Of note, for the PTI part of the pathway elicited by the sensing of cell wall compounds, PSH and another protease known as Hayan appear to function redundantly upstream of SPE (Dudzic et al. 2019). A dedicated serine protease inhibitor, the necrotic serpin (NEC), avoids the spontaneous self-activation of PSH. *nec* mutants are short-lived and display a constitutive activation of the Toll pathway as well as disseminated melanization (Levashina et al. 1999; Ligoxygakis et al. 2002).

As regards the activation of the IMD pathway by bacterial proteases, some evidence has been presented in S2 cells that the *E. coli* cell wall–associated protease OMP is required for the cleavage of PGRP-LC after exposure to live bacteria (Schmidt et al. 2011). This finding awaits however further in vivo validation. The IMD pathway can also be activated by a bacterial virulence factor, this time intracellularly. The cytotoxic necrotizing factor 1 (CNF1) produced by some pathogenic *E. coli* strains catalytically targets the Rho GTPase family member Rac2 for a post-transcriptional modification, the deamidation of a specific glutamine residue that transforms it into a glutamic acid. This transformation abolishes the GTPase activity of Rac2 and locks it into a GTP-bound locked active state. It then activates the IMD

Box 1 Bacterial PGN may not be easily accessible to the sensors that activate the IMD and Toll pathways

The structure of the Gram-negative cell wall with an outer layer made up of LPS precludes a direct access of the thin layer of PGN to PGRP receptors, especially when membrane-bound. It is therefore somewhat surprising that this cell wall compound is the one targeted by evolutionary selection in *Drosophila*. One possibility is that Gram-negative infections are sensed mostly through the small PGN fragments released by bacteria during cell growth and division that involve PGN remodeling since the PGN layer confers its shape and rigidity to the bacterium. This is however not the case in infection models in which microorganisms are introduced within the hemocoel through a wound since there is a rescue of the *PGRP-LC* null phenotype by the PGRP-LCx isoform that senses solely polymeric PGN fragments (Neyen et al. 2012). The rescue is stronger however when both PGRP-LCx and PGRP-LCa are provided. One possibility is that some introduced bacteria are attacked by AMPs produced by the weak immune response triggered by injury itself and thereby expose the PGN layer to receptors, as has been documented in the case of the Eater phagocytic receptor (Chung and Kocks 2011). In a similar way, mutations of the cell wall of *Bacillus thuringiensis* that make them more susceptible to AMP action correlate with an enhanced activation of the systemic immune response (Attieh et al. 2019). Alternatively, hemocytes might be able to process bacteria and release PGN fragments. Interestingly, a lysosomal protein, Psidin, has been shown to be required for the induction of *Defensin* and to some degree of *Diptericin* in larvae (Brennan et al. 2007). These two hypotheses need to be experimentally tested.

We have recently discovered that ingested *Pseudomonas aeruginosa* bacteria are able to colonize the inner tissues of the fly without triggering the IMD pathway at the systemic level and remain silent and nonpathogenic (Chen et al. in preparation). In a few rare cases, the bacteria are "reactivate", which is that they start proliferating exponentially, a phenomenon that correlates with the induction of the systemic immune response (Limmer et al. 2011). Thus, the "choice" of PGN as a targeted cell wall compound may enable the fly to trigger an energetically costly immune response only when necessary, which is when bacteria are active and not dormant, at least as regards Gram-negative bacterial infections.

The situation is different as regards Gram-positive bacteria for which the PGN is present in the outer cell wall and thus should be more accessible in as much as the sensors are circulating proteins. However, PGN is not the sole component of the cell wall. Teichoic acids can be either attached to the cell membrane (lipoteichoic acid: LTA) or to PGN (wall teichoic acids: WTA) and contribute to masking it, for example, in *S. aureus* or some bacilli such as *B. thuringiensis* (Atilano et al. 2011; Kamar et al. 2017). One mechanism contributing to *S. aureus* near escape of detection by PGRP-SA is the secretion of autolysin enzymes that "trim" PGN chains exposed on the surface of the bacterial cell wall. As a result, there is no easily accessible binding site for PGRP-SA, in keeping with WTA being required to mask PGN chains (Atilano et al. 2014). These masking strategies contribute to the virulence of *S. aureus*. A similar role can be ascribed to the D-alanylation of WTA, for both *S. aureus* and *B. thuringiensis* (Kamar et al. 2017; Tabuchi et al. 2010). This modification decreases the negative charges on the surface of the cell wall and therefore diminishes the attraction of AMPs, most of which are cationic, resulting in enhanced resistance to the action of secreted AMPs. The link between sensitivity to AMPs and higher activation of the IMD pathway is however not essential. While both *fliK* and *fla* mutants that both affect the formation of the flagella are able to induce the IMD pathway stronger than wild-type *B. thuringiensis* bacteria, only the former is more sensitive to AMPs in a process that is independent of the formation of the flagellum (Attieh et al. 2019, 2020).

It appears that *Drosophila* enterocytes are able to sense the D-alanylation of teichoic acids from *L. plantarum* independently of the IMD pathway to promote the expression of digestive enzymes and thereby participates in the dialogue between the microbiota and the larval host that leads to enhanced growth under nutrition-poor conditions (Matos et al. 2017). It remains to be determined whether the sensing of D-alanylated teichoic acids would also elicit or inhibit additional host defenses against intestinal infections.

ß-(1,3)-glucans are also not easily accessible for GNBP3 detection. In *Candida albicans* or *C. glabrata*, GNBP3 binds only at the site of the bud scar, the remainder of the fungal cell wall being recovered by layers of mannans and other polysaccharides (Mishima et al. 2009). *Aspergillus fumigatus* conidia are poor inducers of the Toll pathway (Xu et. al., submitted), likely because their cell wall is masked by a layer of hydrophobins (Aimanianda et al. 2009). The PSH branch of Toll pathway activation may have been selected for as it counteracts the immune detection evasion strategies of microorganisms.

pathway independently of its action on the cytoskeleton by directly binding to IMD itself. The activation of the IMD pathway occurs downstream of PGRP receptors and is protective against the pathogen. Interestingly, a similar scheme of NF-κB signaling activation at the level of the IMD-homologs Rip1 and Rip2 occurs also in mammals (Boyer et al. 2011, 2012).

Besides PTI and ETI, the innate immune response has been proposed to respond to so-called danger signals. In this respect, it is interesting to note that the Toll pathway is triggered through PSH in larvae mutant for the caspase *Dronc*, suggesting that it gets activated when apoptosis is prevented, presumably through the release in circulation of some unidentified host protease during the ensuing necrosis. The beneficial aspects of the immune response in this particular setting remain unclear (Ming et al. 2014) but may become relevant in the course of an infection when cells get damaged. As regards the IMD pathway, it does get activated in a PGRP-independent manner in *DNaseII* mutants (Mukae et al. 2002). DNaseII is required to digest DNA from apoptotic bodies in the lysosomes of macrophages. How intracellular DNA is sensed in *Drosophila* remains unknown at present. As described further below, the recently identified *Drosophila* cGAS/STING pathway does not appear to be activated by DNA but by double-stranded RNA (dsRNA) and thus is not a prime candidate. One component involved in this evolutionary conserved response is Eyes absent, which is found in a complex with IKKß (Liu et al. 2012).

The other major humoral response is melanization. It is closely intertwined with the extracellular part of the Toll pathway down to the step just prior to SPE activation (Dudzic et al. 2019; Matskevich et al. 2010). For instance, the overexpression of GNBP3 or GNBP1/PGRP-SA can lead to PPO cleavage even in *spätzle* mutants (Matskevich et al. 2010). Thus, the GNBP1/PGRP-SA and GNBP3 sensors of microbial cell wall compounds trigger the ModSP/Grass proteolytic cascade, which leads to SPE activation through the redundant proteases PSH and Hayan that appear to have been evolutionarily duplicated rather recently in the Melanogaster group of *Drosophila* flies; they also likely act redundantly in parallel for the activation of PPOs. Interestingly, the bait region of PSH is also found in Hayan albeit slightly truncated, suggesting that Hayan may also be activated by microbial proteases as is the case for PSH (Dudzic et al. 2019). The finding that GNBP3 can be found in biochemical complexes together with PPOs further suggests that the melanization enzymes might be brought in close proximity to invading fungi, except if this association is disrupted as observed when the flies are infected by the entomopathogenic fungus *Beauveria bassiana* through the cuticle (Matskevich et al. 2010).

Many septic injury models produce by definition a wound, which is not neutral for the host as it triggers several responses that lead to wound healing and the maintenance of homeostasis (Box 2). The response to wound such as the production of ROS or AMPs may also tell prospective pathogens that they have entered an unfriendly territory and coax them to express virulence programs that may not be used upon intestinal infections and subsequent escape from the digestive tract (Limmer et al. 2011; Nehme et al. 2007).

In summary, the detection of infections that leads to the activation of humoral responses depends both on binding to specific microbial cell wall components and the detection of the enzymatic activity of virulence factors that are secreted either extracellularly such as proteases or intracellularly such as CNF1. It is not established at present that the activation of the immune responses triggered in *Dronc* mutants are relevant to microbial infections, whereas EYA is not required for IMD pathway activation induced by a microbial challenge (Liu et al. 2012).

Influence of metabolites released by the microbiota or pathogens in the gut on *Drosophila* biology.

The gut barrier represents a special frontier between the environment and the internal milieu of the host. The ingested food is rich in microorganisms, some of which are potentially beneficial or detrimental. It is thus important to adjust immune responses so that the microbiota is tolerated under normal conditions but that a strong response against ingested pathogens can be appropriately triggered when needed. In the current paradigm, intestinal host defenses rely on the production of microbe-killing ROS and the production of AMPs by enterocytes. We shall not focus on the different impacts of the microbiota on the gut and more generally on host physiology as these have been recently reviewed (Ferguson and Foley 2021; Lesperance and Broderick 2020). Here, we discuss how specific microbial metabolites modulate immune responses and the homeostasis of the intestinal epithelium (Ferrandon, 2013; Kim and Lee 2014).

A major constituent of the microbiota found in laboratory-raised *Drosophila* is *Acetobacter pomorum*. It has been shown to release acetate that is required for its beneficial action on growth of larvae raised on nutrient-poor food (Shin et al. 2011). In the adult gut, the short-chain fatty acid acetate acts on lipid homeostasis in enterocytes of the anterior midgut through the IMD pathway (Kamareddine et al. 2018). Unexpectedly, the critical cell type in which the IMD pathway is activated is not the enterocyte but entero-endocrine cells able to express the endocrine peptide tachykinin. Secreted tachykinin acts on neighboring enterocytes and represses triglyceride synthesis (Song et al. 2014). Acetate promotes IMD

Box 2 Sensing wounds

The topic of wound healing in *Drosophila* has been extensively studied and revealed some additional mechanisms participating in eliciting innate immune responses, even though some experimental systems rely on sterile injury (Niethammer 2016). Wound healing involves both epithelial responses with extensive cytoskeletal rearrangements in the cells close to the injury site and the recruitment of inflammatory cells. Model systems have been developed for all developmental stages, from embryo to adults, and also include imaginal discs as well as developmental processes such as the dorsal closure that occurs during embryogenesis (Kiehart et al. 2017; Lee and Miura, 2014; Smith-Bolton 2016; Stramer and Dionne 2014; Tsai et al. 2018; Weavers and Martin 2020). In the context of the study of the systemic innate immune response in adults, "clean" injuries trigger a mild, short-lived stimulation of the IMD and Toll pathways, as well as some other signaling pathways such as TGFß (Clark et al. 2011; Lemaitre et al. 1997). Dawdle is an activin-like protein, whereas Decapentaplegic is similar to bone morphogenetic proteins, both of which belong to the TGFß superfamily. The former is induced by the Toll pathway and limits the melanization reaction. The latter is mildly induced by wounding or infection and participates in the resolution of the antimicrobial response (Clark et al. 2011).

A universal signal released at the wounding site is H2O2 produced by DUOX in epidermal cells. At low concentrations, it recruits hemocytes, which are themselves able to amplify the signal and potentially produce ROS in large enough quantities to attack microorganisms that enter the hemocoel through the wound and may at the same time further damage host cells (Chakrabarti and Visweswariah 2020; Razzell et al. 2013). The epithelium then engages resilience mechanisms to protect itself that include the JNK and Nrf2 pathways as well as other components (Weavers et al. 2019). The recruitment of hemocytes to the wound involves the Draper receptor that gets activated by the Src42A adapter that functions as a ROS sensor and phosphorylates the Shark SYK kinase (Evans et al. 2015). There is also a transcriptional response occurring in embryos that relies on the Grainy head transcription factor. This response involves the Stitcher tyrosine kinase receptor that triggers a signaling cascade involving Src42A (Tsarouhas et al. 2014; Wang et al. 2009). Again, the Src42A adapter appears to play a central role in mediating protective responses against wounding. Furthermore, its ROS sensing function has been shown to be required to promote the polarization of junctions and of the cytoskeleton in the cells surrounding the wound (Hunter et al. 2018). Of note, the transcriptional response to wounds in early embryos also involves the Toll pathway that appears to be induced through a third protease cascade that remains unidentified to date (Capilla et al. 2017).

Wounds like cell lysis resulting from viral infections release cytosolic content in the extracellular medium. It had initially been reported that actin would be sensed by an unidentified receptor and lead to intracellular signaling that would activate NOX to produce H2O2 that would in turn be sensed by SRC42A and use Shark to induce the production of the UPD3 cytokine (Srinivasan et al. 2016). The actual inducer appears to be α-actinin, which is much more potent than actin (Gordon et al. 2018). In the pupae, endogenous proteases are also released upon wounding and activate tissue-repair through the activation of Growth-blocking peptides (GBP1 and GBP2) that act through the Mtlh10 receptor to trigger an intracellular calcium wave (O'Connor et al. 2021). It will be interesting to determine whether the proteases that process pro-GBPs into GBPs are also able to activate PSH or Hayan, which potentially mediate this wound response in pupae.

Large wounds also elicit a protective response to trauma that involves the Hayan protease as well as ROS generated by POs that act on neurons (Nam et al. 2012). Unexpectedly, cuticular injuries also influence the homeostasis of the intestinal epithelium (Chakrabarti et al. 2016; Chakrabarti and Visweswariah, 2020; Lee and Miura 2014).

Is wounding necessary to trigger a full-blown systemic immune response? The finding that non-proliferating bacteria that have escaped from the digestive tract into the hemocoel do not appear to trigger the immune response suggests that injury in itself may represent a potent cofactor (Braun et al. 1998; Haller et al. 2018; Limmer et al. 2011). It might do so by triggering a mild short-lived immune response that may nevertheless allow the production of AMPs that would locally attack and lyse bacteria thereby releasing peptidoglycan fragments that constitute elicitors of a sustained immune response. Alternatively, the ROS produced upon wounding may contribute to this process. Clearly, more investigations are required to resolve this point.

pathway signaling in enteroendocrine cells and favors the expression of AMPs albeit weakly and of tachykinin. Acetate does not directly activate the IMD pathway: it is transported inside enteroendocrine cells by the monocarboxylic acid transporter Tarag and increases the acetyl-CoA pool. It has been proposed that it would influence the activity of the acetyltransferase Tip60 complex that in turn would increase the expression of PGRP-LC indirectly through acetylation of the histone H2Aν. This acetylated histone would then promote the transcription of ecdysone-regulated genes including PGRP-LC (Jugder et al. 2021). It remains unclear whether the increased levels of PGRP-LC are sufficient on their own to promote tachykinin transcription through the IMD pathway or whether the enhanced PGRP-LC levels would allow it to react in a more sensitive manner to PGN released by the microbiota. Of note, IMD pathway activation paradoxically promotes *Vibrio cholerae* infection partially by wasting host metabolic reserves. The susceptibility to infection was decreased when components of the acetate response pathway were silenced by RNA interference in enteroendocrine cells.

The phenomenon described above may not occur under all conditions as the constitution of the microbiota is influenced by the composition and pH of the food. In some laboratories, *Acetobacter* species are not major components of the microbiota or are absent and *Lactobacillus* species predominate. A major metabolite produced by *Lactobacillus* is lactate, which is transported within enterocytes by monocarboxylic acid transporters. It produces NADH by lactate dehydrogenase and triggers the production of ROS through the Nox1 NADPH oxidase (Iatsenko et al. 2018; Jones et al. 2013). This leads to an enhanced proliferation of intestinal stem cells. It also triggers the Nrf2-dependent antioxidant response and leads to an enhanced protection against oxidative injury (Jones et al. 2015). However, in dysbiosis conditions in which the number of *Lactobacillus plantarum* is increasing such as when IMD signaling is impaired in *PGRP-SD* mutants, the production of ROS becomes detrimental and leads to a shortened lifespan through premature dysplasia of the intestinal epithelium (Fast et al. 2018; Iatsenko et al. 2018).

ROS have been proposed to represent a major host defense against intestinal infections in *Drosophila* based on multiple lines of evidence. The most important one is provided by knocking down DUOX expression, a NADPH oxidase with two domains, an intracellular NADPH domain, and an extracellular peroxidase domain thought to generate HOCl (Ha et al. 2005a). The silencing of *Duox* by RNAi silencing using a ubiquitous driver or a *caudal*-GAL4 driver expressed only in the posterior midgut leads to an enhanced susceptibility to ingested *Ecc15, Salmonella typhimurium*, *E. coli*, and unexpectedly *Saccharomyces cerevisiae*, an excellent source of food for flies (Ha et al. 2005a, 2009a). Proteins extracted from bacteria retrieved from wild-type infected guts are carbonylated, whereas lipids are peroxidized. A dye

developed to detect HOCl strongly stains the anterior midgut but surprisingly not the posterior part of the midgut (given the silencing experiment with the *caudal* driver) of flies that have ingested *Ecc15* (Lee et al. 2013). The heme-containing peroxidase known as Immune-regulated catalase (IRC) is thought to limit the self-damages inflicted by DUOX. Indeed, *IRC* mutants succumb to the ingestion of killed *Ecc15*, presumably from the ROS generated by DUOX (Ha et al. 2005b). The DUOX-dependent intestinal defense may be prevalent and only ROS-resistant microorganisms seem to be susceptible to IMD pathway intestinal defenses that appears to act as a second line of defense (Ryu et al. 2006).

The question that arises is how this defense system is elicited by pathogens and not by commensals. Biochemical purification studies from *Ecc15* culture supernatant suggest that the bacterial metabolite uracil is secreted by pathobionts and not by commensal microorganisms (Lee et al. 2013). In keeping with these results, a URA⁻ *Ecc15* strain unable to secrete uracil no longer leads to a positive R19S staining and persist longer in the gut. However, the identification of a putative uracil receptor able to activate DUOX within enterocytes remains elusive to this date. Uracil would also trigger the hedgehog (HH) pathway, again in undefined manner. This receptor is thought to be endocytosed upon uracil binding together with the HH-dependent Cad99C to form a GαQ-PLCß signaling endosome that leads through the release of intracellular calcium stores to the biochemical activation of DUOX enzymatic activity (Lee et al. 2015). In conjunction with the upper part of the IMD pathway (PGRP-LC and IMD but not Relish) that diverges onto the p38 pathway, the GαQ-Plcß axis also leads to the induction of the *Duox* gene at the transcriptional level through the ATF2 transcription factor (Ha et al. 2009b). Interestingly, PLCß appears to be required for the repression of the p38 pathway when only commensal bacteria are present in the gut lumen.

In conclusion, metabolites released by gut bacteria modulate or trigger immune responses that are independent of classical AMP expression regulation with important impact on the homeostasis of the gut epithelium.

Role of hemocytes in the host defense: phagocytosis and links with the systemic immune response

Much emphasis has been initially placed on the characterization of the systemic immune response due to the predominant role of AMPs in host defense. As regards Gram-negative bacteria, most AMP families regulated by the IMD pathway are active on them (Diptericins, Drosocins, Attacins, and Cecropins) (Bulet and Stocklin, 2005). Thus, the ancillary role of phagocytosis can be revealed only when

the IMD pathway is inactivated at least to some degree (Elrod-Erickson et al. 2000; Nehme et al. 2011). Alternatively, a predominant role for phagocytosis has been revealed in intestinal infection models with Gram-negative bacteria that are able to escape from the gut compartment into the hemocoel such as *Serratia marcescens* or *Pseudomonas aeruginosa* (Haller et al. 2018; Limmer et al. 2011; Nehme et al. 2007). Hemocytes prevent the proliferation of bacteria that have crossed the intestinal barrier, at least initially in the case of *P. aeruginosa.* Interestingly, phagocytosis is powerless when these bacteria are injected in the hemocoel in a systemic infection model.

As regards Gram-positive bacteria, the function of phagocytosis in host defense against these pathogens was more easily established (Charroux and Royet 2009; Defaye et al. 2009; Nehme et al. 2011). This may reflect the relative paucity of *bona fide* AMPs active against Gram-positive bacteria identified so far. Indeed, Defensin is active on Gram-positive bacteria in vitro, but unexpectedly is no longer expressed in IMD pathway mutants, which resist like wild-type flies to Gram-positive bacterial infections (Rutschmann et al. 2000). A locus of ten Bomanin genes has been identified, the deletion of which phenocopies Toll pathway mutants (Clemmons et al. 2015). However, no secreted Bomanin active against Gram-positive bacteria have been identified to date. Some Gram-positive bacteria such as *Staphylococcus aureus* are not sensitive to the action of the core Toll pathway but are limited by phagocytosis and melanization (Defaye et al. 2009;

Dudzic et al. 2019; Nehme et al. 2011). This latter defense is activated through GNBP1 and PGRP-SA (Matskevich et al. 2010). When only a few bacteria are introduced in the hemocoel, then melanization, and not phagocytosis, becomes the relevant defense (Dudzic et al. 2019).

As regards fungi, the cellular immune response may partially mediate the host defense against yeast forms of pathogens. For instance, phagocytosis acts only in a second line after the Toll pathway against injected *C. glabrata* (Quintin et al. 2013). We have recently identified a role for phagocytosis against injected conidia of *Metarhizium robertsii*, which form yeast-like hyphal bodies and not when the fungus invades the fly through the cuticle and forms hyphae (Wang et al. in preparation).

Mechanistically, invading microorganisms need to be sensed by dedicated receptors present on the surface of macrophage-like plasmatocytes present as sessile or circulating hemocytes in the hemocoel (Fig. 3). As in mammals, opsonization appears to be also involved in insects: opsonins are soluble molecules that bind to various microbes and promote their engulfment by acting as adapters that provide a conserved molecular interface to phagocytes. These molecules are briefly presented below.

Nimrods

Nimrods are a group of 12 *Drosophila* phagocytic receptors characterized by a subtype of epidermal growth factor
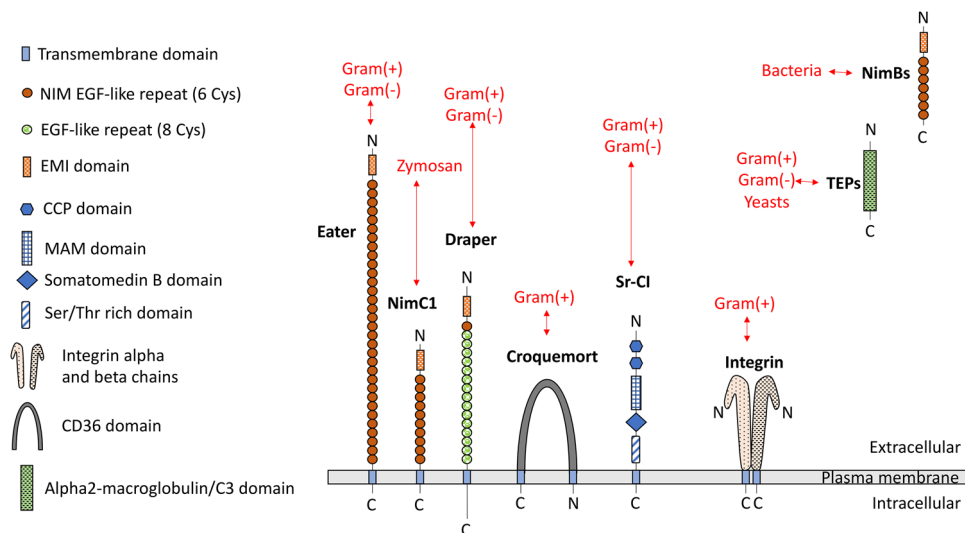
Fig. 3 *Drosophila* phagocytosis receptors and opsonins. Eater, NimC1 and Draper are three members of the Nimrod family, that are characterized by a subtype of epidermal growth factor (EGF) repeat called Nimrod (NIM) repeat. Eater and Draper can detect bacteria, although NimC1 can recognize yeast zymosan particles. Croquemort and Sr-C1 are two members of the scavenger receptor family that sense bacteria. The integrin made by αPS3 and βν was shown to be involved in the phagocytosis of *S. aureus*. Some thioester-containing proteins (TEPs) and some members of the Nimrod B-type subfamily (NimBs) are soluble molecules that bind to various microbes and promote their engulfment by phagocytes (opsonins). NimBs can bind bacteria and TEPs had been shown to recognize both bacteria and yeasts. (Adapted from Melcarne et al. 2019a)

(EGF) repeat called Nimrod (NIM) repeat (Kurucz et al. 2007; Somogyi et al. 2008). Ten genes encoding these receptors are clustered on the second chromosome and two (*eater* and *draper*) are clustered on the third chromosome. Two subgroups of the Nimrod receptor family members containing a transmembrane domain can be described: the C-type (NimC1-NimC4 and Eater) and the Draper-type (NimA and Draper) receptors. In contrast, the NimB family members are secreted and circulate in the hemolymph.

The members of the Nimrod C-type subfamily contain multiple NIM domains, whereas the Draper type is characterized by only one such repeat. NimC4, also called Six-Microns-Under (SIMU), is involved in apoptotic cell phagocytosis upstream of Draper through phosphatidylserine recognition (Kurant et al. 2008). Eater and NimC1 mediate the engulfment of bacteria (Bretscher et al. 2015; Kocks et al. 2005; Kurucz et al. 2007; Melcarne et al. 2019b). Both receptors carry a cytosolic tail with predicted phosphorylation sites.

eater is a target of the transcription factor Serpent, a major transcription factor expressed in most hemocytes. *eater* is expressed in S2 cells and plasmatocytes from both larvae and adult flies. *eater* knockdown and deficiencies uncovering the *eater* gene region and neighboring genes allowed to show its important role in Gram-negative and Gram-positive bacteria phagocytosis (Kocks et al. 2005; Nehme et al. 2011). A soluble Fc-tagged receptor variant of Eater directly binds to Gram-positive bacteria and Cecropin A-pretreated Gram-negative bacteria, although the specific ligand remains unknown (Chung and Kocks 2011). The role of *eater* in the phagocytosis of Gram-positive, but not Gram-negative bacteria, was confirmed in an *eater* null knock-out mutant (Bretscher et al. 2015). Nevertheless, the host defense against the ingested Gram-negative bacteria *Serratia marcescens* or *Pseudomonas aeruginosa* remains impaired also in the KO mutant, possibly because the cell surface properties of bacteria that have crossed the gut barrier are altered (DF and coworkers, unpublished) (Limmer et al. 2011; Nehme et al. 2007). This mutant also led to the identification of a role for Eater in the adhesion of hemocytes to tissues, at the expense of its role in phagocytosis (Bretscher et al. 2015).

NimC1 was initially identified as a target of the plasmatocyte-specific P1 antibody. Knockdown experiments showed that NimC1 is required for *S. aureus* uptake (Kurucz et al. 2007). NimC1 binds to bacteria in vitro. In contrast to Eater, a role for NimC1 in the phagocytosis of latex beads and yeast zymosan particles, but not bacteria, was established using a *NimC1* null KO mutant (Melcarne et al. 2019b). The role of NimC1 in phagocytosis of latex beads was confirmed in an in vivo knockdown experiment (Hao et al. 2018). The contribution of NimC1 to bacterial engulfment is somewhat overshadowed by that of

Eater. The two receptors are also complementary to some extent as *NimC1*; *eater* double mutants are deficient for the phagocytosis of all types of bacteria tested (Melcarne et al. 2019b).

Draper carries only one NIM domain, several EGF repeats, and one Emilin (EMI) domain. Draper is expressed on plasmatocytes, glial cells, and epithelial cells. Its expression is regulated by the glial transcription factor glial cell missing (Gcm) (Freeman et al. 2003). Draper had initially been shown to recognize apoptotic cells and this function in evolutionarily conserved by its homologues CED-1 in *Caenorhabditis elegans* or MEGF10 in humans and JEDI in mouse (Hamon et al. 2006; Mangahas and Zhou 2005). More recently, Draper was shown to mediate *S. aureus* and *E. coli* phagocytosis (Cuttell et al. 2008; Hashimoto et al. 2009; Shiratsuchi et al. 2012). The Draper ligand on the surface of *S. aureus* is lipoteichoic acid (Hashimoto et al. 2009).

Scavenger receptors

Scavenger receptors had first been described in mammalian macrophages, where they recognize modified low-density lipoproteins (mLDL) (Brown and Goldstein 1983). These receptors can recognize polyanionic ligands on microbes and dying cells in many species.

Twelve class B scavenger receptors are found in *Drosophila*. They belong to the mammalian CD36 family, initially discovered for its role in apoptotic cell clearance (Savill et al. 1992). Many of the class B receptors are expressed in the gut where their function is not characterized. One of them, Croquemort (CRQ), is specifically expressed in plasmatocytes and is a receptor for apoptotic cells (Franc et al. 1996, 1999) and for *S. aureus* (Stuart et al. 2005). Several recent studies revealed a contribution of CRQ in phagosome maturation rather than in particle recognition (Guillou et al. 2016; Han et al. 2014; Meehan et al. 2016). Silencing of *crq* expression specifically in plasmatocytes allowed to show that it may be involved in lipid uptake as for other CD36 family members (Woodcock et al. 2015). Therefore, it is an open possibility that CRQ is indirectly involved in phagocytosis, as lipid uptake may affect the phagocytic process. Another class B receptor, Peste, is required for phagocytosis of *Mycobacterium fortuitum* and *Listeria monocytogenes*, but not *E. coli* or *S. aureus*, as shown in RNAi screens made in S2 cells. These results remain to be confirmed in flies (Agaisse et al. 2005; Philips et al. 2005).

Four receptors belong to the class C scavenger receptor family in *Drosophila*, Sr-CI, Sr-CII, Sr-CIII, and Sr-CIV. Sr-CI is specifically expressed on the surface of plasmatocytes and can recognize a large variety of ligands like the mammalian class A scavenger receptor (Pearson et al. 1995). In vitro studies delineated its capacity to bind to Gram-negative and Gram-positive bacteria, but not yeasts

(Pearson et al. 1995; Ramet et al. 2002). Its transcription is upregulated after bacterial challenge (Irving et al. 2005). The Sr-CI locus shows a high degree of polymorphism, likely due to positive selection (Lazzaro 2005). However, the function of Sr-CI in bacterial recognition has never been confirmed in vivo by using null mutants.

Integrins

In addition to their function in adhesion, integrins have been shown to mediate phagocytosis in various organisms. The *Drosophila* genome encodes five α- and two β- integrin subunits. The βν integrin was shown to be involved in the phagocytosis of both apoptotic cells and *S. aureus*. PGN is likely to be the integrin ligand as shown using cell wall–deficient bacteria (Shiratsuchi et al. 2012). Furthermore, βν was shown to form a heterodimer with αPS3 for the phagocytosis of both apoptotic cells and *S. aureus* (Nonaka et al. 2013).

Opsonins

The complement factor C3b and antibodies are classical opsonins in mammals. In *Drosophila*, six genes encode proteins that carry a domain structurally related to the mammalian complement factor C3 family, named the thioester-containing proteins (TEPs).

The *Tep1*, *Tep2*, *Tep3*, and *Tep4* genes contain a signal peptide, indicating that they are secreted proteins and could function as opsonins. It was shown that these four genes are upregulated in adults upon bacterial challenge (Bou Aoun et al. 2011; Irving et al. 2005; Lagueux et al. 2000). *Tep5* is thought to be a pseudogene (Bou Aoun et al. 2011; De Gregorio et al. 2002b), while *Tep6* (also called *Mcr*) plays an unexpected role in the establishment of septate junctions in the intestinal epithelium (Batz et al. 2014; Hall et al. 2014). TEP6 is therefore unlikely to play any role in phagocytosis, at least in this context. The expression of *Tep* genes had been studied by in situ hybridization and by using reporter gene constructs (Bou Aoun et al. 2011; Dostalova et al. 2017). Thus, it was shown that TEP1, TEP2, TEP3, TEP4, and TEP6 are all expressed in plasmatocytes. *Tep1*, *Tep2*, and *Tep4* transcripts are also detected at low levels in the fat body and some barrier epithelia. They show a strong upregulation after bacterial challenge, since they are regulated by the stress-responsive JAK-STAT pathway (Bou Aoun et al. 2011; Lagueux et al. 2000). In addition, *Tep2* and *Tep4* appear to be regulated by the systemic immune response (De Gregorio et al. 2002b).

The importance of the TEPs in phagocytosis of bacteria was first demonstrated in the mosquito *Anopheles gambiae* (Levashina et al. 2001). A RNAi study in *Drosophila* S2 cells suggested that TEPs can bind and enhance phagocytosis of *E. coli* (*Tep2*), *S. aureus* (*Tep3*), and *Candida albicans*

(*Tep6*) (Stroschein-Stevenson et al. 2006). The specificity of these associations remains to be established in vivo. Rather, additional evidence in support of opsonization in insects comes from work using a compound mutant lacking *Tep1*, *Tep2*, *Tep3* and *Tep4* (Dostalova et al. 2017). These four TEPs operate in both the humoral and cellular response of *Drosophila* immunity, by promoting Toll pathway activation and phagocytosis of Gram-positive bacteria (Dostalova et al. 2017). It was recently shown that TEP4 acts as an opsonin that favors the phagocytosis of *P. aeruginosa* in an oral infection assay (Haller et al. 2018). In conclusion, some TEPs may indeed bind to microbes and promote phagocytosis, although the possibility is open that they present additional functions. The receptors mediating the uptake of TEPs remain unidentified.

Another class of potential opsonins are the Nimrod B–type proteins, the function of which remains poorly characterized. The five members, NimB1-B5, contain multiple NIM domains that are conserved in other insect species (Estevez-Lao and Hillyer, 2014; Ju et al. 2006; Matsunaga and Fujiwara 2002). *Drosophila* NimB1 and NimB2 can bind bacteria in vitro (Zsámboki et al. 2013). A recent study has shown that NimB5 is not involved in phagocytosis, but it is produced by the fat body to regulate plasmatocyte adhesion and proliferation rate. NimB5 is induced upon starvation and adjusts plasmatocyte numbers to the metabolic state of the host (Ramond et al. 2020).

Finally, PGRP-SC1A was reported to act as an opsonin for *S. aureus* and Toll pathway activation (Garver et al. 2006). However, null mutations of *PGRP-SC1* did not reveal any role in Toll activation, although an effect modest in comparison to that detected for PGRP-SC2 mutants has been reported after a challenge with *E. faecalis* (Paredes et al. 2011). This was further corroborated by exposing S2 cells overexpressing either of these two *PGRP-SC* genes to killed *E. faecalis*, which led to the stimulation of *Drosomycin* expression (Costechareyre et al. 2016). As the Toll pathway can be activated in S2 cells solely by providing the mature Spätzle ligand, the expression of *Drosomycin* likely results from IMD pathway activation. As PGRP-SC degrades peptidoglycan, it might affect bacterial cell wall structure and indirectly promote phagocytosis.

DSCAM1

Down syndrome cell adhesion molecule 1 (Dscam1) belongs to the immunoglobulin family, and its gene organization consists in clusters of variable exons, flanked by constant exons, leading to about 38,000 potential isoforms by alternative splicing (Schmucker et al. 2000). Dscam1 has initially been implicated in the development of the nervous system and neuron wiring, where Dscam1 isoform interactions shape the dendritic pattern (Wojtowicz et al. 2004; Zhan et al. 2004). In 2000, it was

shown that infection leads to the production of specific secreted isoforms that can be detected in the *Drosophila* hemolymph (Watson et al. 2005). However, a study did not reveal any change of Dscam1 splicing upon infection (Armitage et al. 2014). A knockdown experiment in S2 cells showed that Dscam1 is involved in binding and uptake of *E. coli* by hemocytes and suggested that this protein might act as an opsonin (Watson et al. 2005), a function not yet demonstrated in vivo.

PGRP-LC

RNAi experiments initially showed that PGRP-LC is involved in the phagocytosis of Gram-negative but not Gram-positive bacteria (Ramet et al. 2002). However, it was shown later that PGRP-LC hardly contributes to Gram-negative bacteria phagocytosis in S2 cells (Kocks et al. 2005).

Links between the cellular immune response and the activation of the systemic immune response

As regards adults, the genetic ablation of plasmatocytes using hemocyte-specific drivers failed to reveal any impact on the inducibility of the tested AMP genes (Charroux and Royet 2009; Defaye et al. 2009). However, a later study revealed an unexpected requirement for hemocytes in the case of *Drosocin* expression in the fat body upon an immune challenge. The IMD pathway is activated through PGRP-LC in hemocytes and leads to the release of the Unpaired3 cytokine, which in turn triggers

the expression of *Drosocin* in the neighboring fat body tissue and the tracheal epithelium (Sanchez Bosch et al. 2019). Thus, hemocytes appear to function as sentinels not only to phagocytose microorganisms that have escaped from the digestive tract but also in the case of systemic infections. Why this system functions solely to induce *Drosocin* and not other AMP genes remains to be addressed.

With respect to larvae, we have already described the requirement for hemocytes to trigger a systemic activation of the IMD pathway upon the ingestion of *Ecc15* (Basset et al. 2000; Yang et al. 2019). The genetic ablation of hemocytes in larvae led to a decreased activation of the systemic immune responses mediated by the Toll and the IMD pathways (Shia et al. 2009). In the case of Toll, it has been proposed that hemocytes contribute to systemic activation of its pathway by providing the Spätzle cytokine whereas the case of the IMD pathway has not been further investigated (Shia et al. 2009). However, the *domino* mutant, which lacks hemocytes among other defects, nevertheless exhibited a normal induction of the Toll and IMD-mediated systemic immune responses (Braun et al. 1998).

Sensing viral infections

Viral nucleic acids elicit the innate immune response in both protostomes and deuterostomes (Fig. 4). In insects, the sensing of virus-derived dsRNA elicits several antiviral responses.
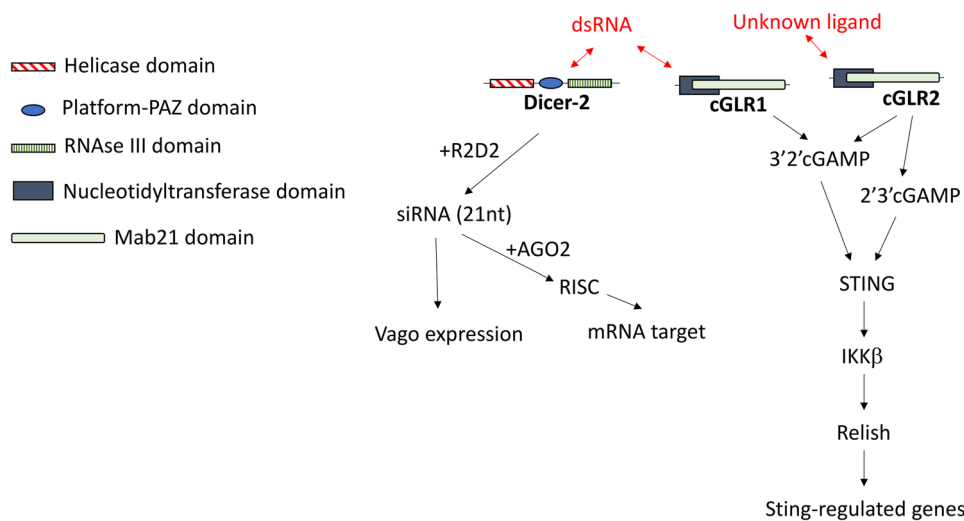
Fig. 4 Sensing viral infections in *Drosophila*. Double-stranded (ds) RNAs are sensed by Dicer-2, that together with R2D2 process them into siRNAs, one strand of which is loaded on AGO2. The involvement of AGO2 allows the formation of the RISC complex that targets the mRNA corresponding to the complementary siRNA sequence. In parallel, siRNAs induce the expression of Vago. dsRNAs are also sensed by the cGAS-like receptor 1 (cGLR1) that synthesizes 3'2'cGAMP cyclic dinucleotides. Another sensor, cGLR2, is acti-

vated by an unidentified ligand, and synthesizes 3'2'cGAMP and 2'3'cGAMP. These two cGAMPs then activates *Drosophila melanogaster* STING. The latter activates the terminal part of the IMD pathway by interacting with IKKß independently of IKKγ. This STING-Relish axis controls the production of specific genes referred to as STING-regulated genes. The JAK-STAT pathway is likely induced indirectly by cytosolic components such as α-actinin that are released from infected cells lysed by viral infections (not shown)

The DEAD-box RNA helicase DCR-2 is the major sensor of the vsiRNA pathway that acts upon long dsRNAs and is related to mammalian RIG-I (Guo et al. 2019; Schneider and Imler 2021). It contains two domains potentially involved in sensing dsRNAs, a platform-PAZ domain and a N-terminal helicase domain that belongs to the mammalian RIG-I family of cytosolic RNA sensors. RIG-I discriminates between capped cellular transcripts and the phosphorylated termini of viral RNAs. Likewise, DCR-2 does not use cellular transcripts as templates. It uses its helicase domain to initiate the processive cleavage of blunt termini viral dsRNAs, in keeping with a potential evolutionarily ancient role in the detection of non-self RNA. Interestingly, there is a second mode of cleavage, this time of RNAs with 3′OH overhang termini that involves the platform-PAZ domain and is required for processing RNAs of the endo-siRNA pathway, an activity that may involve accessory dsRNA-binding proteins such as the PD isoform of Loquacious (Marques et al. 2013; Sinha et al. 2015, 2018).

As noted earlier, JAK-STAT pathway activation may result from the production of the Unpaired cytokines by damaged cells. In the case of IIV-6, the production of Upd3 depends on the production of ROS by NOX that in turn activates the p38b pathway. It has been proposed that the activation of NOX is somehow mediated by cellular debris of lysed cells as actin-containing fractions trigger a rather similar intracellular signaling pathway in "receiver" cells (see also Box 2) (West and Silverman 2018).

The noncanonical IMD pathway that partially mediates host defense against DCV, CrPV, vesicular stomatitis virus, and Kallithea virus is evocative of the mammalian cGAS-STING pathway that triggers the interferon response upon sensing DNAs in the cytosol, a compartment in which DNA is normally absent (Ablasser and Chen 2019; Goto et al. 2020; Wu and Chen 2014). cGAS senses dsDNA and generates a cyclic dinucleotide known as 2′3′cyclic GMP-AMP (cGAMP). cGAMP then activates STING that in turn acts through TBK1 to stimulate interferon regulatory factors. Interestingly, STING can also be activated by cyclic dinucleotides produce by invasive bacteria such as Listeria (Hansen et al. 2014). In contrast, DmelSTING activates the terminal part of the IMD pathway by interacting with IKKß independently of IKKγ that appears to be solely required for IMD canonical signaling. This STING-Relish axis controls the production of specific genes referred to as STING-regulated genes (Cai et al. 2020). The hunt for *Drosophila* cGAS homologs has been fruitful. Two cGAS-like receptors (cGLR) required for host defense against viral infections have been identified (Holleufer et al. 2021; Slavik et al.

2021). Insect cGLRs present a distinct nucleic binding site that lacks a Zn-finger motif as well as a secondary binding site. Accordingly, the substrate of cGLR1 is dsRNA longer than 30 bp and not DNA. cGLR2 is activated by an unidentified ligand. Single *cGLR* mutants are more sensitive than wild-type flies but are less susceptible than the *cGLR1/cGLR2* double mutant to viral infections (DCV, Kallithea virus but not VSV nor IIV-6). Surprisingly, cGLR1 predominantly synthesizes 3′2′cGAMP instead of 2′3′cGAMP, a phenomenon that appears to be widespread in Diptera. This may reflect a selective pressure exerted by viruses able to counteract 2′3′cGAMP signaling. Indeed, a family of viral enzymes, poxins made by poxviruses, and some Lepidopteran viruses specifically cleave 2′3′cGAMP and thereby allow the virus to escape detection (Eaglesham et al. 2019). The selection of 3′2′cGAMP signaling would have allowed Dipterans to elude the action of these virulence factors.

A recent study based on a comparative affinity proteomics approach on human, mouse, and *Drosophila* cells led to the identification of 128 proteins with an impact on viral growth. TAO kinases were identified to bind specifically to dsRNA and to display antiviral activity (Pennemann et al., Nature communications, *in press*). In mammals, TAO kinases appear to be required for full induction of IRF3-dependent cytokines such as Interferon-ß and IP-10. In flies, the sole TAO kinase is required for the induction of Sting-regulated gene 1.

Our present understanding of the host defense against viral infections is that it relies on sensing viral dsRNAs or potentially on detecting host molecules released from cells lysed by the virus. Future studies will reveal whether evolution has also selected for ETI against specific viral virulence factors, the synthesis of 3′2′cGAMP to potentially avoid poxin action being a step in that direction. Another example is provided by a long noncoding RNA (lncRNA), the VSR-interacting RNA, VINR, which accumulates in the nucleus of DCV-infected S2 cells. This accumulation also occurs upon the ectopic expression of the DCV viral suppressor of RNAi (VSR) protein 1A (DCV-1A). DCV-1A suppresses RNAi by binding to dsRNAs and masking them from the innate immunity sensing system. It likely stabilizes VINR steady-state expression by protecting this lncRNA from degradation. VINR in turn activates a noncanonical IMD pathway involved in host defense against DCV and against bacteria. Thus, VINR is part of a host counter-defense strategy against a viral effector that suppresses the canonical RNAi antiviral pathway, a counter counter-defense (Zhang et al. 2020).

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