CHAPTER 17

INSECT IMMUNITY: FROM SYSTEMIC TO CHEMOSENSORY ORGANS

Insect immunity: from systemic to chemosensory organs protection

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Abstract

Insects are confronted to a wide range of infectious microorganisms. Tissues in direct contact with the environment, such as olfactory organs, are particularly exposed to pathogens. We review here the immune mechanisms operating in insects to control infections. Experiments conducted on the model organism *Drosophila melanogaster* (fruit fly) have provided genetic evidence that insects rely on both cellular and humoral mechanisms to control infections. Once epithelial barriers have been breached, circulating or membrane-associated innate immunity receptors trigger signaling in the fat body and lead to secretion of high concentrations of antimicrobial peptides active on fungi and bacteria in the hemolymph. This induced response involves the evolutionarily conserved Toll and immune deficiency (IMD) signaling pathways, which promote nuclear translocation of transcription factors of the NF-κB family. In addition, different subsets of differentiated blood cells or hemocytes can neutralize bacteria, fungi or parasites by phagocytosis, production of microbicidal compounds, or encapsulation. An alternative to mount costly immune responses is to sense pathogens through chemosensory cues and avoid them. Interestingly, some families of molecules, including the Toll receptors, participate in both olfaction and immunity.

1.Introduction

Insects represent by far the largest class of multicellular organisms, both in terms of number of species (corresponding to more than half of the animal species documented) and number of individuals (Misof et al. 2014). They also exhibit a fantastic variation of morphologies, which make them a fascinating group to study.

Insects have colonized all terrestrial biotopes and are exposed to all kind of infectious agents, raising the question of how they defend themselves (see Chapters 1-6). There are several specific reasons to be interested in host-pathogen interactions in insects. First, infection of insects can cause important economic losses (e.g. flacherie or pébrine disease of silkworms; contribution to colony-collapse disorder in honey-bees) (also see Chapters 1-3). Second, hematophagous insects such as *Aedes* or *Anopheles* mosquitoes can transmit viral (caused by so-called arthropod-borne viruses or arboviruses, e.g. dengue, yellow fever, West-Nile virus) or parasitic (e.g. malaria) diseases to mammalian hosts (see Chapter 1). Third, microbial pathogens (e.g. baculoviruses) can be used as biological control agents against insect pests, which necessitates some knowledge of the host response to these microorganisms (see Chapter 4).

To these, we may add a fourth and last reason, which is that the fruit fly *Drosophila melanogaster* is a valuable model organism used in the biomedical field to decipher complex issues in biology, including immunology (Fernández-Hernández et al. 2016).

Whereas the immune system of vertebrates is composed of two arms, innate and adaptive immunity, invertebrates and insects in particular only rely on innate immunity to counter infections. Innate immunity, which senses infection through preformed receptors, rapidly reacts to the invasion of microorganisms and triggers the production of antimicrobial compounds (Figure 17.1) (Hoffmann et al. 1999). We present below the main mechanisms involved in insect immunity, which bear many similarities with mammalian innate immunity, betraying common phylogenetic origins (Hoffmann, 2003).

Figure 17.1

Emphasis is placed on the *Drosophila* model, which has provided strong genetic evidence for involvement of several pathways in insect immunity. We first present the systemic humoral

response and its regulation, which bears several interesting similarities with the induction of inflammation in vertebrates. We then highlight how two cell intrinsic mechanisms, RNA interference and apoptosis, participate in the control of viral infections. The insect immune system also encompasses a cellular arm, and we present the different population of hemocytes present in the blood and associated with tissues, and discuss their contribution to host defense. Finally, we turn to immunity at barrier epithelia, before closing the chapter with the particular case of chemosensory organs.

2. The systemic humoral response to infection by bacteria, fungi and protozoa

2.1 A battery of secreted antimicrobial peptides contribute to humoral immunity

One hallmark of the immune response of insects is the secretion in the hemolymph of a cocktail of small (<10kDa for most of them), cationic antimicrobial peptides (AMPs).

These peptides, which participate in host defense in all metazoa, but also in plants, were initially discovered in an insect, the moth *Hyalophora cecropia* (Steiner et al., 1981). AMPs belong to different structural families and are active in micromolar concentrations against a broad range of microbes, with some specificity. They act on the microbial cell wall, although some have intracellular targets (Imler and Bulet 2005) (see Figure 17.1).

The most commonly found AMPs in insects are the α-helical Cecropins, which are mainly active against a range of Gram-negative bacteria, and the disulphide-bridged Defensins, mostly active against Gram-positive bacteria (Boman et al. 1991; Hoffmann and Hetru 1992; Vilmos and Kurucz 1998). Besides these, each insect expresses a specific repertoire of AMPs (Koehbach, 2017). One AMP specific to *D. melanogaster* is the antifungal peptide Drosomycin, which turned out to be an important marker of the immune response for the deciphering of the pathways leading to AMP expression (Zhang and Zhu, 2009).

2.2 Evolutionarily conserved signaling pathways control AMP expression

AMPs are induced upon infection, and are secreted in the hemolymph, where their combined concentration can reach 300 μM in the fruit fly *D. melanogaster*. The transcriptional induction of the genes encoding AMPs is mediated by factors of the NF-κB family, which in mammals play important roles in the induction of inflammation and immunity (Hoffmann et al. 1999). These transcription factors are retained in the cytosol of non-infected cells by ankyrin-repeat containing

inhibitors of the IκB family. In response to an immune challenge, they rapidly translocate to the nucleus. Two pathways, named Toll and IMD, regulate AMP expression in insects, through activation of different members of the NF-κB family (Ferrandon et al. 2007) (Figure 17.2).

Figure 17.2

The Toll pathway, named after the transmembrane receptor Toll, is involved in the humoral response to fungi and Gram-positive bacteria (Valanne et al. 2011). It is activated by a neurotrophin-like cytokine, Spaetzle, which is expressed as an inactive precursor unable to bind Toll (Weber et al. 2003). Infection by fungi or Gram-positive bacteria triggers a proteolytic cascade that culminates in the activation of the serine protease Spaetzle Processing Enzyme (SPE) and generates an active Toll ligand (Jang et al. 2006). Activation of Toll triggers intracytoplasmic signaling through the death domain (DD) adapter proteins DmMyD88 and Tube. This leads to activation of the serine/threonine kinase Pelle through specific homotypic DD-DD interaction (Sun et al. 2004). Pelle phosphorylates the IκB-like molecule Cactus, triggering its degradation by the proteasome. This releases the NF-κB transcription factor DIF, which translocates to the nucleus and activates expression of genes encoding AMPs such as antifungal peptide *drosomycin* (Rutschmann et al. 2000; Daigneault et al. 2013).

The IMD pathway, named after the *Drosophila* gene *immune deficiency* (*imd*), mediates inducible expression of antibacterial peptides (e.g. Diptericin, Drosocin) in response to infection by Gram-negative bacteria (Georgel et al. 2001). The pathway is activated by receptors of the PGRP family (see below) and involves the DD adapter proteins IMD and dFADD, and the caspase DREDD (Naitza et al. 2002). One target of DREDD is IMD itself. The new N-terminus of IMD then recruits the complex TAB2/TAK1, promoting the activation of the serine/threonine kinase TAK1 (Paquette et al. 2010). TAK1, together with the E3 ubiquitin ligase Uev1A/Ubc13, then activates the IκB kinase (IKK) complex formed by the kinase DmIKKβ and its regulatory subunit DmIKKγ. DmIKKβ subsequently phosphorylates the NF-κB protein Relish, thus promoting its cleavage by DREDD (Stoven et al. 2003). The 110kDa Relish contains an N-terminal inhibitory ankyrin-repeat domain, which restrains the transcription factor to the cytosol. Proteolytic cleavage by DREDD releases the active domain of Relish, which is then free to

translocate to the nucleus, to trigger expression of antibacterial peptides genes, e.g. *diptericin* and/or *drosocin* (Stoven et al. 2003).

Interestingly, both the Toll and IMD pathways are evolutionarily ancient, and bear striking similarities with the mammalian Toll-like receptor (TLR)/interleukin-1 receptor (IL-1R) and Tumor necrosis factor receptor (TNF-R) pathways, respectively. The genetic characterization of these pathways in the *Drosophila* model organism was instrumental in revealing the interplay of molecules involved, and in particular played a decisive role in revealing the importance of TLRs in mammalian innate immunity (Hoffmann 2003).

2.3 Non-self and danger signals trigger humoral immunity

In spite of the similarities mentioned above, the sensing of microorganisms upstream of the Toll and IMD pathways exhibit some differences with mammals.

Activation of innate immunity involves receptors commonly known as pattern-recognition receptors (PRRs), which recognize molecular motifs, or "patterns", shared by large groups of microorganisms and essential for their biology (e.g. lipopolysaccharide found in the cell wall of all Gram-negative bacteria, double-stranded RNA generated during viral replication), but absent from the host (Medzhitov and Janeway 2002). Whereas TLRs represent a major family of PRRs in vertebrates, Tolls in insects function as cytokine receptors or adhesion molecules (McIlroy et al. 2013; Paré et al. 2014). Recognition of microbial infection is mediated by receptors that belong to two other families of PRRs, the peptidoglycan (PGN) recognition proteins (PGRPs) and the β-glucan binding proteins (GNBPs) (Ferrandon et al. 2007). Initially identified in the silkworm moth *Bombyx mori*, these molecules were genetically characterized in *Drosophila* (Yoshida et al. 1986; Choe et al. 2002; Gottar et al. 2002, 2006).

The IMD pathway in flies is activated by a characteristic feature of PGN from Gram-negative bacteria (shared by Gram-positive bacilli), namely the presence of a diaminopimelic acid (DAP) residue at the third position in the four amino-acid peptide bridge between the chains of glycans (Kaneko et al. 2006). Two negatively charged groups of DAP form a strong electrostatic interaction with an Arginine residue in a specific pocket in the transmembrane receptor PGRP-LC, thus triggering signaling (Chang et al. 2005). A second member of the PGRP

family in flies, PGRP-LE, exists as either a secreted isoform that facilitates interaction between PGN and PGRP-LC, or an intracellular isoform that senses PGN from intracellular bacteria (Steiner 2004; Royet et al. 2011). Of note, this cytosolic receptor also triggers a cell intrinsic response to infection, namely autophagy (Yano et al. 2008). Therefore, the IMD pathway is activated by PRRs that directly sense PGN from infecting bacteria.

By contrast, in the Toll pathway, recognition occurs upstream of Toll, and is mediated by circulating PRRs. In the case of many important Gram-positive bacteria, a Lysine residue replaces the DAP in the peptide stem of PGN, and this difference is sensed by PGRP-SA, with a contribution from PGRP-SD. Thus, a subtle difference in the structure of PGN between Gram-negative and Gram-positive bacteria is responsible for the activation of either the IMD or the Toll pathway, respectively (Steiner 2004; Royet et al. 2011). Recognition of fungi, on the other hand, involves a PRR from a different family, GNBP3, which binds β1-3-glucans (Gottar et al. 2006).

A second member of the GNBP family, GNBP1, participates to the sensing of Gram-positive bacteria, together with PGRP-SA (Gobert et al. 2003). Thus, GNBPs are not restricted to the sensing of fungal components. The PRRs acting upstream of Toll activate proteolytic cascades that culminate in the production of an endogenous ligand for the Toll receptor (Ferrandon et al. 2007).

Besides PGRPs and GNBPs, the Toll pathway can also be activated by the sensing of abnormal proteolytic activity in the hemolymph. This is mediated by the circulating zymogene Persephone, which can be activated by fungal or bacterial proteases (El Chamy et al. 2008; Gottar et al. 2006 ; Issa et al. 2018). These proteases are often used to penetrate the insect cuticle, and as such are important virulence factors, which can be sensed by Persephone. Upon activation, this serine protease activates a proteolytic cascade leading to processing of the Spaetzle precursor (Levashina et al. 1999; Ligoxygakis et al. 2002).

2.4 Complement factors contribute to anti-parasitic immunity

A family of secreted complement-like factors known as TEPs (thioester containing proteins) participate in host-defense in insects.

In vertebrates, the TEP family includes not only the protease inhibitors α_2-macroglobulins, but also the complement factors C3, C4 and C5 (Nonaka, 2000; Shokal and Eleftherianos, 2017).

Initially identified as immune-induced genes in *Drosophila*, the TEPs have been best characterized in the mosquito *Anopheles*, which transmit the malaria parasite to mammals (Levashina et al. 2001; Blandin and Levashina, 2004). Out of the 19 TEPs identified in the genome of *Anopheles gambiae*, TEP1 is an extremely polymorphic gene that encodes for an acute phase secreted protein, which binds to the surface of bacteria and parasites. It promotes phagocytosis of bacteria and killing of parasites at the ookinete stage (Blandin et al. 2004; Obbard et al. 2008). Polymorphisms in the gene encoding TEP1 explains a substantial part of the variability in transmission of *Plasmodium* parasites observed between *A. gambiae* individuals (Blandin et al. 2009). Interestingly, TEP1 bears similarities to the mammalian complement factor C3, both at the structural and functional levels (Baxter et al. 2007; Fraiture et al. 2009). In particular, two leucine-rich repeat proteins, APL1 and LRIM1, function like complement control proteins, preventing TEP1 from binding to self-tissues in the absence of infection.

2.5 RNAi and apoptosis: two cell intrinsic mechanisms of antiviral immunity

Viruses represent a special challenge for multicellular organisms. Indeed, their small size, their great diverse shape, very robust replicable structure and simplicity of organization offer limited options for sensing and neutralization by the immune system. In addition, they replicate in the cytoplasm or nucleus on infected cells, which blurs the distinction between self and non-self. Finally, their error prone polymerases allow them to evolve rapidly, facilitating evasion from antiviral immune defenses (see Chapter 18 about evolution of chemosensory proteins).

RNA interference (RNAi) provides insects with a powerful mechanism of antiviral defense. It involves RNA targeting enzymes from two families, the Dicers and the Argonautes (AGOs) (Figure 17.3).

Figure 17.3

RNAi was first identified as a potent antiviral defense mechanism in plants (Ding 2010).

More recently, RNAi was also found to play an important role in the control of viral infection in insects, eventually providing a new area of research for insect control (see also Chapter 5).

Central to the RNAi mechanism are the enzymes of the AGO family, which mediate highly specific neutralization of target RNA molecules (Herzog and Ameres 2015). The specificity of AGO enzymes is achieved by their association with small RNAs, which guide them towards RNA molecules with complementary sequences. Three RNAi pathways, involving different members of the AGO family, have been defined in insects: (i) the small interfering (si)RNA pathway involves AGO-2, and is activated by double stranded (ds)RNA. siRNAs are produced by the RNaseIII enzyme Dicer-2, which forms a complex with the dsRNA binding protein (dsRBP) protein R2D2 (Paro et al. 2015); (ii) the micro (mi)RNA pathway involves AGO-1, Dicer-1 and its dsRBD co-factor Loquacious, and modulates expression of insect genes, in particular during tissue development (Carthew et al. 2016); (iii) Finally, the piRNA pathway involves the other AGO proteins (Piwi, Aubergine and AGO3 in *Drosophila*).

Piwi-associated RNAs (piRNAs) are involved in the control of mobile genetic elements, including the retrovirus gypsy, in the germ line of *Drosophila* (Siomi et al. 2010). Virus-derived piRNAs are also generated in infected insect cell lines as found in *Drosophila* and mosquitoes. However, the piRNA pathway does not appear to participate in antiviral host defense at least in the fruit fly *Drosophila* (Petit et al. 2016). It is still unclear whether it participates in the control of viral infections in mosquitoes (Morazzani et al. 2012; Vodovar et al. 2012; Léger et al. 2013).

Demonstration of the critical role of the siRNA pathway in antiviral immunity in insects relies on three lines of evidence: first, genetic data indicating that siRNA pathway mutants (*Dicer-2*, *r2d2* or *AGO2*) are hypersensitive to RNA virus infections, and contain increased viral load (Galiana-Arnoux et al. 2006; van Rij et al. 2006; Wang et al. 2006); second, siRNAs of viral origin can be detected in infected cells or insects from a variety of species (Aliyari et al. 2008; Aguiar et al. 2015); and third, viral suppressors of RNAi (VSRs), which counteract the immune defense of the fly, have been identified in several insect viruses (van Rij et al. 2006).

RNA interference provides a highly specific and even adaptive mechanism to degrade

viral nucleic acids in infected cells. Indeed, the sensing by Dicer-2 of a molecular pattern characteristic of viral infection, double stranded RNA, trigger the production of guide siRNAs for AGO2, thus programming it to specifically neutralize viral RNAs (Kemp and Imler 2009). RNAi is a cell autonomous process in *Drosophila* (Roignant et al. 2003), although in the context of infectious viral dsRNA released upon cell lysis can be taken up by non infected cells and trigger the antiviral siRNA pathway (Saleh et al. 2009). In addition, viral dsRNA and siRNAs can be shared between cells by cytoplasmic bridges (Karlikow et al. 2016). Besides RNAi, apoptosis is another cell intrinsic mechanism of antiviral defense operating in insects (Clem 2015; Nainu et al. 2015) (see also Chapter 5).

3. Cellular responses to infections

3.1 Differentiation of hemocytes in insects

Hematopoiesis in insects occurs during embryogenesis and in larvae. It originates from the head mesoderm in embryos, and from a specific hematopoietic organ, the lymph gland, in larvae.

This gland is composed of two primary lobes, located on the sides of the aorta, which grow and form more posterior secondary lobes. While the secondary lobes contain only frequently dividing precursors called prohemocytes (small rounded cells [4-6 μm diameter] with a high nucleocytoplasmic ratio), the primary lobe can be differentiated in three zones. The medullatory zone composed of prohemocytes; the cortical zone composed of differentiating hemocytes; and a group of 20-30 cells at the posterior end of each lobe forming the posterior signaling center (PSC). The PSC plays a key regulatory role in third instar larvae, maintaining the balance between multipotent prohemocytes in the medullary zone and controlling hemocyte differentiation (Krzemień et al. 2007; Mandal et al. 2007). Interestingly, odorant receptors activation contributes to blood progenitors maintenance in *Drosophila* (Shim et al. 2013).

Several kinds of hemocytes have been described in insects and, because of the great diversity of insects and the important variability of histological features of the cells within one insect, it is difficult to provide a unified view of the different types of blood cells. Nevertheless, based on morphological and functional characteristics, it appears that insects contain a few blood cell types, which bear resemblance to cells of the myeloid lineage in vertebrates. They include

the macrophage-like plasmatocytes and other non-phagocytic cells, which seem to be characteristic of particular groups of insects (e.g. crystal cells and lamellocytes in *Drosophila*) (Figure 17.4).

Figure 17.4

Regulation of hemocyte differentiation involves evolutionarily conserved transcription factors also participating in hematopoiesis in mammals (e.g. Runx, GATA, STAT families). In addition to the larval lymph gland, hemocytes are found in circulation or in sessile patches of cells accumulating in various locations of the body (Lanot et al. 2001; Hillyer 2016).

3.2 Plasmatocytes phagocytose microbes and dead cells

Plasmatocytes form the majority of differentiated blood cells (90 to 95% of hemocytes in *Drosophila* larvae). They are relatively round cells, with diameter of 8-10 μm, which display phagocytic activity. Accordingly, their cytoplasm is rich in endoplasmic reticulum and lysosomes.

In some conditions, these cells exhibit pseudopod-like extensions (e.g. at the onset of metamorphosis), suggesting that the podocytes described in some insects may represent a specialized subtype of plasmatocytes. Commonly referred to as macrophages, plasmatocytes engulf and degrade dead cells, debris and invading pathogens. They also produce antimicrobial peptides in response to infection and participate to tissue remodeling through the secretion of extracellular matrix proteins (see Figure 15.4). Phagocytosis involves several types of receptors, including the CD36 scavenger receptor homologue Croquemort for the removal of apoptotic cells and the EGF domain protein Eater (Franc et al. 1999; Kocks et al. 2005).

3.3 Phenoloxydases catalyze the production of melanin and toxic microbicidal by-products

Crystal cells (5% of larval hemocytes in *Drosophila*) are round cells with a 10-12 μm diameter and characteristic paracrystalline cytoplasmic inclusions. They are not involved in phagocytosis, but play a key role in melanization, participating to host defense and wound healing.

The crystal cell inclusions consist of massive amounts of components of the melanization

enzymatic cascade, such as the prophenolxydase enzyme (PPO; Lu et al. 2014). A poorly characterized serine protease cascade converts the prophenoloxydase zymogen into active phenoloxydase. This oxydo-reductase then catalyzes the oxidation of phenols to quinones, which then polymerize into melanin. In the process, microbicidal reactive oxygen species such as hydrogen peroxide and nitric oxyde are generated. Insects express a varying number of PPOs. In *Drosophila*, two of the three PPOs, PPO1 and PPO2 are expressed in crystal cells (the third member of the family, PPO3, is expressed in lamellocytes). They contribute to the resistance to bacterial and fungal infections, but also to large parasites, in conjunction with lamellocytes (Binggeli et al. 2014).

3.4 Lamellocytes encapsulate large parasites

Lamellocytes are large (15-40 μm diameter) flat adherent cells, which encapsulate and neutralize objects too large to be phagocytosed by plasmatocytes (see Figure 17.4).

Interestingly, these cells can only be seen in parasitized larvae, indicating that their differentiation represents a dedicated immune response. Parasitization occurs frequently in nature when Hymenopteran wasps such as *Leptopilina* lay their eggs in larvae (Colinet et al. 2013). Detection of the egg, or of any object, even artificial, too large to be phagocytosed by plasmatocytes generates a signal that triggers a differentiation program in the PSC of the lymph gland, leading to the production and release of lamellocytes. These cells then adhere tightly to the foreign object and surround it to form a capsule. Crystal cells are subsequently recruited and melanization of the capsule participates in the containment and killing of the parasite.

In addition to these cell-mediated immune functions, hemocytes also participate in humoral response through production of AMPs and secretion in the hemolymph.

4. Immunity at barrier epithelia

In insects, as in many other organisms, surface epithelia represent a first barrier to infection. Surface epithelia are sites of major physiological functions involving exchange with the external environment, such as nutrient absorption, gas exchange, water conservation and reproduction. As a consequence, these cells are largely exposed to microorganisms (see Chapter 4).

Host-defense in epithelia is mediated by both physical and chemical barriers.

4.1 Secreted chitin-based matrices form an efficient physical barrier

One hallmark of insects is the presence of an exoskeleton, the cuticle, which forms an efficient physical barrier against infection but also as insecticide (Balabanidou et al. 2018) (see Chapter 3).

The cuticle is composed of layers of chitin, a long chain polymer of N-acetyl-glucosamine secreted by the ectodermal epithelium, which can be cross-linked with proteins to increase rigidity and resistance. This protective layer also covers the tracheal tubes, which allow oxygen to diffuse to tissues. Only the thinner sub-branches, the tracheolae, which ultimately bring oxygen directly to cells, are devoid of cuticle. In the digestive tract, the foregut and hindgut are lined by impermeable cuticle, but not the midgut, where food absorption occurs. However, the epithelial cells of the midgut are protected from the external environment by a peritrophic matrix, lining the gut epithelium and isolating it from the food bolus. Composed of chitin and glycoproteins, the peritrophic matrix is produced continuously or upon ingestion of a meal (depending on the type of insects) by a specialized organ at the entry of the midgut, the cardia. It prevents direct contact of microbes with midgut epithelial cells and limits the action of microbial toxins produced in the gut, in a manner similar to mucous secretions in the mammalian gut (Kuraishi et al. 2011; Xuan et al. 2015; Liu et al. 2017).

These chitin-based barriers can be breached by injuries or pathogen-secreted enzymes. This leads to activation of chemical barriers, a second layer of defense in epithelia.

4.2 Chemical barriers to infection in epithelia

The *Drosophila* digestive tract produces two different types of antimicrobial effectors, reactive oxygen species (ROS) and AMPs, which function in a complementary and probably synergistic manner to control infections.

In the digestive tract of dipteran insects microbicidal ROS are produced by the dual oxidase (DUOX) enzyme, which is also known to be essential to avoid eggshell dessication

(Dias et al. 2013). This member of the NADPH oxidase family is expressed at a basal level in the gut, killing dietary yeasts but sparing the microbiota (Bae et al. 2010). Upon gut infection, it is strongly induced, leading to the destruction of many microbes. Induction of DUOX is mediated by the nucleotide uracil released by infecting bacteria. Uracil is thought to be sensed by an unidentified G-protein coupled receptor, signaling through the protein Gαq and the phospholipase C (PLC) β (Lee et al. 2013).

Another chemical barrier to infection operating in the digestive tract, but also in other epithelia is the production of AMPs. Initially characterized for their role in the systemic humoral response (see above), AMPs are also expressed in epithelia from the digestive tract, the respiratory tract, the excretory system or the reproductive tract. AMPs are expressed in a tissue-specific manner in epithelia, either constitutively or in response to local activation of the IMD signaling pathway (Tzou et al. 2000).

5. Immunity in chemosensory organs

5.1 Chemosensory organs are permanently in contact with the environment

In insects, sensing of the environment occurs through specialized organs like antennae and maxillary palps (see Chapters 9-20). An opening in the cuticule at the level of these structures allows permanent exchanges with the environment. Therefore, chemosensory organs may represent a crucial portal of entry through which specific micro-organisms and/or pathogens enter a susceptible insect host to cause disease or infection (see Chapter 4).

Olfactory sensilla possess a discontinuous cuticule containing pores that range from 10 nm to 25 nm, followed by a pore kettle of 10 nm to 20 nm diameter (Cribb and Merritt 2013). Hence, even the smallest viruses, which have a diameter of *ca.* 30nm, cannot pass through these pores. Below the cuticle, intercellular septate junctions form a second barrier compromising the spreading of infectious microorganisms (e.g. Bonnay et al. 2013). These natural barriers, however, may not be sufficient to prevent infection by bacterial microorganisms that can secrete specific chitinolytic enzymes (or chitinases; Patil et al. 2000). In addition, pathogens can access chemosensory organs through other routes, as suggested by the recent identification of a viral nucleocapsid in the antenna of *Rhodnius prolixus* (Oliveira et al. 2017).

Just like the rest of the insect body, antennae contain hemolymph within an antennal vessel. In Cockroaches (*blattidae*), this vessel contains hemocytes (Pass 1985), which may participate in host defense in the antennae. This is, however, not relevant for all insects, since the diameter of the antennal vessel is too small to allow circulation of hemocytes (e.g. 1-2 μm), as found for instance in mosquitoes (Boppana and Hillyer 2014).

5.2 Constitutive expression of AMPs in chemosensory organs

Expression of AMPs largely contributes to the protection of chemosensory organs against microbial infection. In the fall armyworm *Spodoptera frugiperda*, antennae and maxillary palps strongly express AMPs. Most strikingly, Cecropin and Defensin peptides are expressed at higher levels in unchallenged antennae and maxillary palps than in the fat body after bacterial infection (Legeai et al. 2014). In the silkworm moth *B. mori*, antennae also express immune-related genes such as phenoloxidase, a member of the Toll receptor family and the immune-induced gene Hdd13 (Zhao et al. 2015). Of note, this expression of AMP might be regulated, as *Drosophila* antennae express Dnr1, a negative regulator of the IMD pathway that blocks Dredd activity (Foley and O'Farrell 2004; Anholt and Williams 2010).

Interestingly, the expression of immune genes in chemosensory organs appears to differ between social casts in the leaf-cutting ant *Atta vollenweideri*. Indeed, analysis of the antennal transcriptome of different social casts revealed a higher expression of most immune genes in antennae from queens compared to males and workers. In addition, differences in the sets of immune genes expressed were observed between workers and queens (e.g. hymenoptaecin expressed in the former and defensin in the latter) (Koch et al. 2013).

5.3 Induction of pherokines (CSP-like) upon microbial challenge

Besides AMPs, many other protein families are up-regulated in the insect body in response to bacterial infection (Irving et al. 2001; De Gregorio et al. 2002). Interestingly, these include molecules related to the insect chemosensory protein family (see Chapters 14 and 18). The most striking examples are pherokine-2 and -3, which are related to OS-D/A10, a secreted factor highly expressed not only in olfactory organs but throughout the whole insect body (Figure 17.5)

(Picimbon et al. 2000a,b, 2001; Picimbon, 2003). Secretion of the 13kDa Phk-2 protein in the hemolymph is induced specifically in response to infection with DCV, whereas expression of the mRNA encoding Phk3 is induced by bacterial and fungal infection (Sabatier et al. 2003). Unfortunately, the function of these *Drosophila* molecules remains unknown. However, studies in other insects such as the whitefly *Bemisia tabaci,* the silkworm moth *B. mori* and the red flour beetle *Tribolium castaneum* strongly point to the involvement of the chemosensory protein family in insect defense (Xuan et al. 2015; Liu et al. 2014, 2016, 2017).

Other "olfactory" binding protein families are possibly involved in the insect response to chemical/viral infection (Xuan et al. 2015). For example, a transcriptomic analysis revealed an enrichment of pheromone binding proteins in hemocytes from *Aedes aegypti*, the mosquito vector of dengue, chikungunya or zika viruses (Bartholomay et al. 2004). In addition, two putative odor binding proteins (OBPs), OBP10 and OBP22 are up-regulated in the salivary glands of *A. aegypti* upon infection by dengue virus (Sim et al. 2012). Interestingly, silencing of these two genes impaired the efficiency of blood feeding. Regulation of chemosensory proteins by infection was also reported in another mosquito, the malaria vector *A. gambiae,* Indeed, the genes annotated as *obp4*, *7* and *obpd-2 (obp domain 2)* are upregulated following infection with the fungus *Beauveria bassiana*, whereas *obpd-1* (obp *domain 1)* is downregulated upon infection with *Salmonella typhimurium* (Aguilar et al. 2005).

In the silkworm *Bombyx mori*, CSP11 was among the 50 genes induced by *B. bassiana* infection (Hou et al. 2013). It was also among the 17 CSP genes upregulated by insecticide (Xuan et al. 2015). Finally, studies in yet another insect, honeybees, point to a role for olfaction in induction of a hygienic behavior leading to a better ability to remove brood infested with *Varroa destructor.* Indeed, transcriptomic analysis of honeybees exposed to this ectoparasite showed that tolerant bees mainly differ from the others by a higher expression of genes regulating olfaction (Navajas et al. 2008) (see Chapter 6).

5.4 Biological significance of the immune induction of OBP/CSP molecules

Induction of chemosensory proteins (CSPs) and odor binding proteins (OBPs) in response to infection could reflect behavioral modifications participating in the resistance to infection.

Indeed, although immunity represents a crucial aspect of resistance to infection, induction of an immune response is metabolically costly (Lazzaro 2015) and can have deleterious side effects (Cao et al. 2013). Avoiding infection by sensing contaminated food sources represents an interesting alternative for all animals, including insects. This is particularly relevant for insects like the fruit fly *D. melanogaster*, which is known to feed on decaying/fermenting microbe-rich organic matter (Petkau et al. 2016).

Recent studies exploiting the *Drosophila* genetics toolbox emphasize the importance of gustatory and olfactory circuits in pathogen avoidance. For example, flies can taste the presence of bacterial LPS through gustatory neurons expressing Gr66a receptors. The subsequent activation of the chemosensory cation channel DTRP1 triggers a strong aversive response monitored in both feeding and egg laying assays (Soldano et al. 2016). Fruit flies can also distinguish food sources covered with toxic microbes. This is mediated by the microbial odorant geosmin (trans-1,10-dimethyl-trans-9-decalol), which alert sensory neurons expressing the olfactory receptor OR56a and averts the fly to unsuitable feeding and breeding sites (Stensmyr et al. 2012) (see Chapter 12). Also in support for a role of CSPs/OBPs in behavioral modifications associated with infection, flies are more sexually attracted to individual fed on the same diet and this appears to depend on the composition of the gut microbiota. In particular, the symbiontic bacteria *Lactobacillus plantarum* affects the levels of cuticular hydrocarbon sex pheromones (Sharon et al. 2010; Venu et al. 2014). The mechanism by which this occurs is unknown, but could involve transmission of information through a cytokine participating in the innate immune response (Ringo et al. 2011). Indeed, a connection between immune stimulation and modulation of social interaction was reported in honeybee (Richard et al. 2008).

Alternatively, upregulation of OBP/CSP-related proteins may reflect functions other than olfaction as strongly emphasized by Xuan et al. (2015). Indeed, many chemosensory or odorant binding related proteins were annotated on the sole basis of their tissue specific expression in olfactory tissues or sequence homology to known proteins, and their names may not reflect their real function (Picimbon, 2014). Indeed, a shared characteristic of the olfactory and immune systems is the need for soluble carriers of hydrophobic moieties to be delivered at plasma membrane receptors (e.g. pattern recognition receptors or odorant receptors) (Figure 15.5). As an

example, the protein RYA3, which is expressed exclusively in the rat olfactory mucosae, exhibits significant sequence homology to the LPS-binding protein, which transports LPS in the serum (Dear et al. 1991). Thus, an intriguing possibility is that some odorant-like proteins participate in tissue-specific defense mechanisms in olfactory organs, rather than olfaction *per se*. This is strongly supported by the existence of CSPs (and OBPs) in bacterial species such as *Acinetobacter baumannii* (Liu and Picimbon 2017). A second intriguing example was recently provided by the demonstration that OBP6 from tsetse flies activates a transcriptional program leading to differentiation of crystal cells and increased melanotic immune response. Of note, Obp28a, the orthologue of OBP6 in *Drosophila*, induces a similar response in fruit flies. Therefore, it appears that one OBP regulated by gut microbiota plays a conserved role in cellular immunity in dipteran insects (Benoit et al., 2017). This is reminiscent of the connection between olfactory stimulation and maintenance of a pool of progenitor blood cells in *Drosophila* larvae (Shim et al. 2013).

Figure 17.5

5.5 Receptors shared between immunity and chemosensory pathways

Interestingly, some receptors activating innate immunity also have a role in olfaction. For example, CD36-related receptor participate in immunity and pheromone detection in *Drosophila* (Benton et al. 2007).

Several proteins from this family function in the immune system of animals as scavenger receptors recognizing lipids derived from bacteria. In flies, one family member, Peste, mediates uptake of mycobacteria by phagocytes (Philips et al. 2005). In addition, three other CD36-like receptors, CG10345, CG31741 and Croquemort mediate phagocytosis of Leishmania parasites, resulting in decreased parasite burden in flies (Okuda et al. 2016).

Of note, the latter of these receptors, Croquemort, also participates in the uptake and disposal of apoptotic cells (Franc et al. 1999). Sensory neurone membrane protein 1 (SNMP1) also belongs to the CD36 family. It is expressed in a population of olfactory sensory neurons where it is required for activation of odorant receptors by lipid-derived pheromone ligands

(Rogers et al. 1997; see Chapter 12). Thus, CD36 receptors appear to participate in a common function in olfaction and innate immunity, coupling sensing of hydrophobic, lipid-derived ligands to activation of signaling transmembrane receptors (Benton et al. 2007; Gomez-Diaz et al. 2016) (see Figure 17.5).

Toll receptors represent another example of a family whose members function in both immune system and nervous system, in particular olfaction. Toll receptors participate in host defense in both mammals and insects, albeit by different means. Indeed, whereas mammalian TLRs function as PRRs, directly interacting with microbial ligands (Lu and Sun 2012). *Drosophila* Toll function as receptor for the cytokine Spaetzle (Weber et al. 2003) (see figure 17.5). This dichotomy is supported by phylogenetic analysis, which reveals that insect Tolls and mammalian TLRs evolved independently (Imler and Zheng 2004).

Interestingly, it is becoming apparent that several *Drosophila* Toll receptors function in the nervous system and can affect olfaction. The *Drosophila* genome encodes a family of six neurotrophin-like ligands including Spaetzle. Some of them have been shown to promote cell survival in the central nervous system and to influence motor exon targeting (Zhu et al. 2008). Toll 6/7/8 function as receptors for these neurotrophins (McIlroy et al. 2013; Ballard et al. 2014) (see Figure 17.5). Thus, a subset of Toll receptors actively participates in the development of the nervous system in *Drosophila*.

Interestingly, Toll6 and Toll7 also instruct wiring specificity in the *Drosophila* olfactory circuit (see Chapters 9-11). Toll2 and Toll8 could participate in the establishment of wiring specificities in other parts of the nervous system (Ward et al. 2015). Interestingly, a cell adhesion role for Toll receptors has also been reported during embryogenesis in the fly (Paré et al. 2014). In addition, Toll6 and Toll7 are known to promote microtubule dynamics in motoneurons through a non-canonical pathway, thus enabling rapid activity-dependent structural plasticity. There is no indication at this stage that the involvement of Toll receptors in these neuronal functions impacts the resistance to infections. Interestingly, however, in another model organism, the nematode *Caenorhabditis elegans*, the only Toll receptor encoded by the genome, Tol-1, promotes the development of neurons that are required for sensory detection on bacterial

microbes (Pradel et al. 2007; Brandt and Ringstad 2015), providing a connection between the function of Toll receptor in chemosensory neurons and infection.

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Figure legends

Figure 17.1 The insect humoral response to microbial infections.
Representative examples of antimicrobial peptides are shown with their microbial targets. Their concentration in the hemolymph of immune challenged *Drosophila* is indicated in parenthesis. These peptides are secreted by the fat body, but also plasmatocytes and surface epithelia.

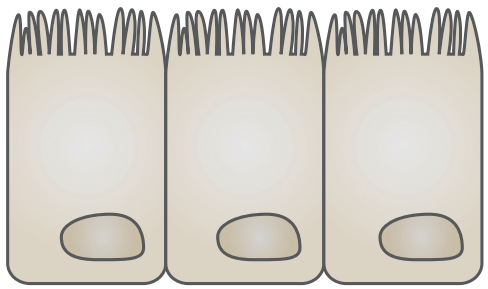
Figure 17.2 Overview of the *Drosophila* Toll and IMD pathways.
The Toll pathway is activated by a proteolytically processed form of the cytokine Spaetzle. Proteolytic cascades upstream of Spaetzle are induced upon sensing of ß-glucans or lysine-type peptidoglycan (derived respectively from the cell wall of fungi and most Gram positive bacteria) by circulating PRRs, or upon detection of abnormal proteolytic activity associated with infection. Activation of Toll leads to nuclear translocation of the NF-κB transcription factor Dif and/or

Dorsal, which promote expression of AMPs such as Drosomycin. The IMD pathway is activated by members of the PGRP family that sense DAP-type peptidoglycan found in the cell wall of Gram-negative bacteria. It activates the NF-κB transcription factor Relish, which controls genes encoding AMPs (e.g. Diptericin) or negative regulators of the pathway (e.g. Pirk, PGRP-SC and PGRP-LB).

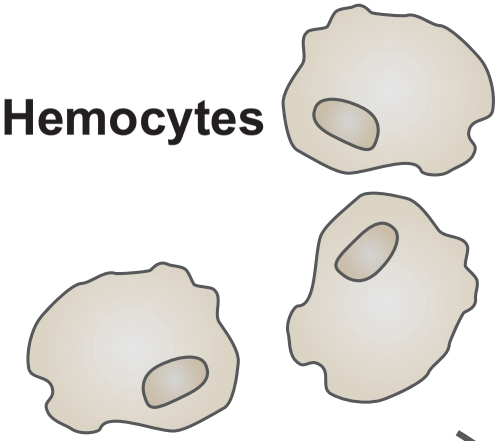
Figure 17.3 The antiviral small interfering RNA (siRNA) pathway.
Upon infection by a positive strand RNA virus, translation of the viral RNA dependent-RNA polymerase (RdRP) leads to initiation of replication. The resulting double-strand RNA (dsRNA) is sensed by the RNAseIII enzyme Dicer-2 (Dcr-2) and cleaved into virus-derived siRNAs duplexes. Upon loading onto Argonaute 2 (AGO2), one strand of the duplex is discarded and the remaining strand will guide the nuclease AGO2 and the RNA induced silencing complex (RISC) towards viral RNAs. Many insect viruses express suppressors that antagonize the siRNA pathway at different levels.

Figure 17.4 Cellular immunity in the fruit fly *Drosophila melanogaster*.
Differentiation of prohemocytes generates three types of hemocytes, which contribute by different mechanisms to host defense (See paragraph 3.1)

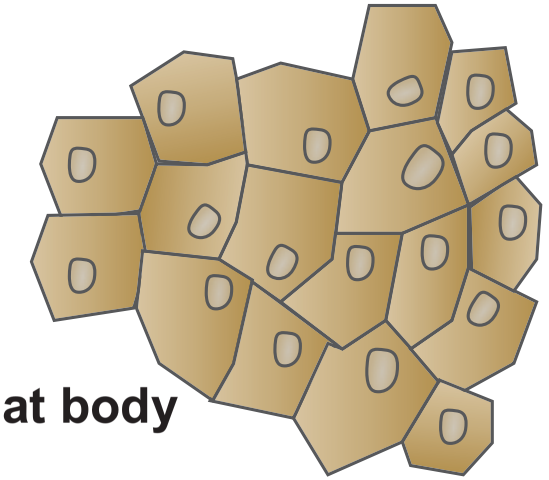
Figure 17.5 Shared molecular mechanisms of immunity and olfaction.
(A) Sequence alignment of *D. melanogaster* OS-D/A10, and its paralogs Pherokine-2 and -3, which are induced by infection. A model for the 3D structure of OS-D/A10 is shown at the bottom (Swissmodel; see Chapters 14 and 18). The hydrophobic binding pocket (arrowhead) is highlighted in blue. (B) Involvement of CD36-related receptors in the transfer of hydrophobic ligands to signaling transmembrane receptors in the olfactory system and in the immunological system (adapted from Benton et al. 2007). (C) Function of Toll receptors in the immune and nervous systems of insects. Whereas mammalian TLRs directly sense microbial ligands, as shown for TLR4 recognizing lipopolysaccharides (LPS) bound to the lipid-binding protein MD2, Toll receptors from insects are activated by neurotrophin-related cytokines.



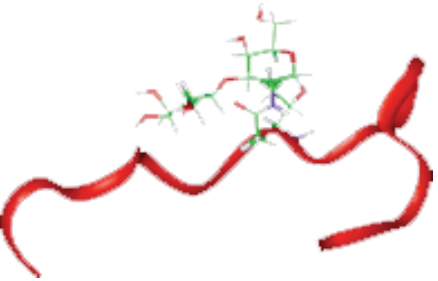
Epithelia



Hemocytes

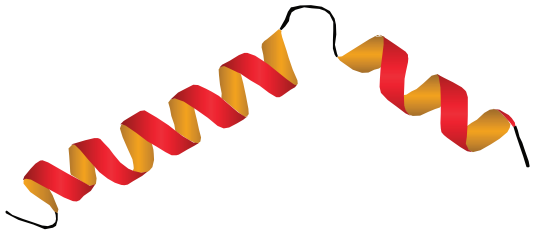


Fat body



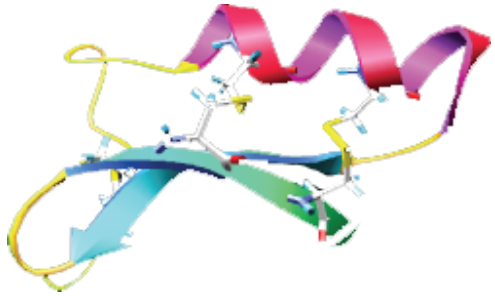
Drosocin
(40μM)

Gram negative bacteria



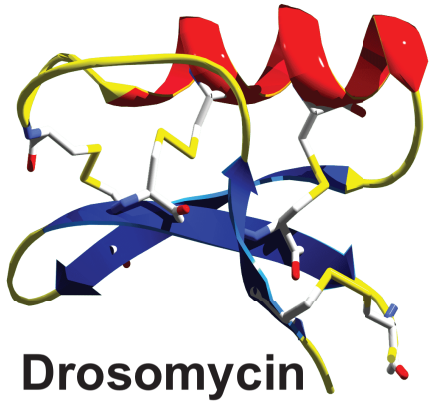
Cecropin
(20μM)

Gram negative bacteria
(and some fungi and yeast)



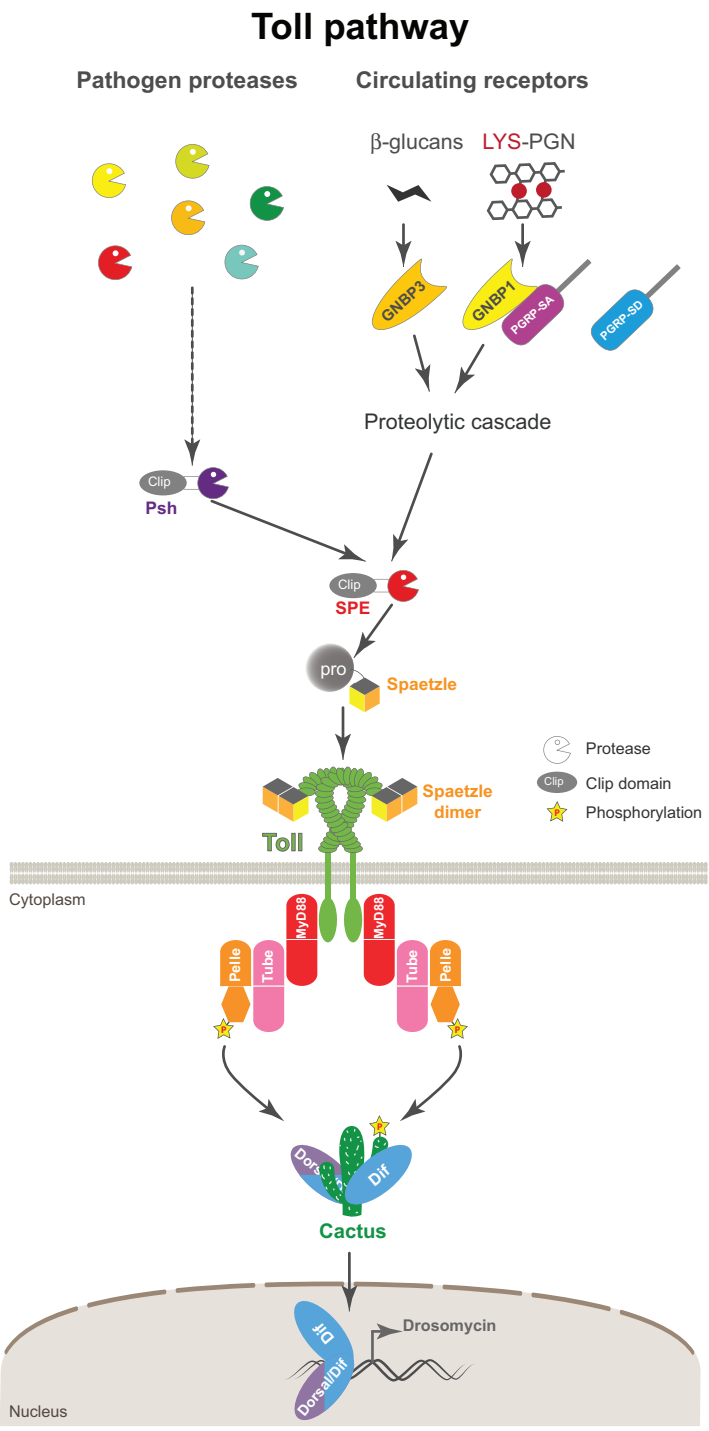
Defensin
(1μM)

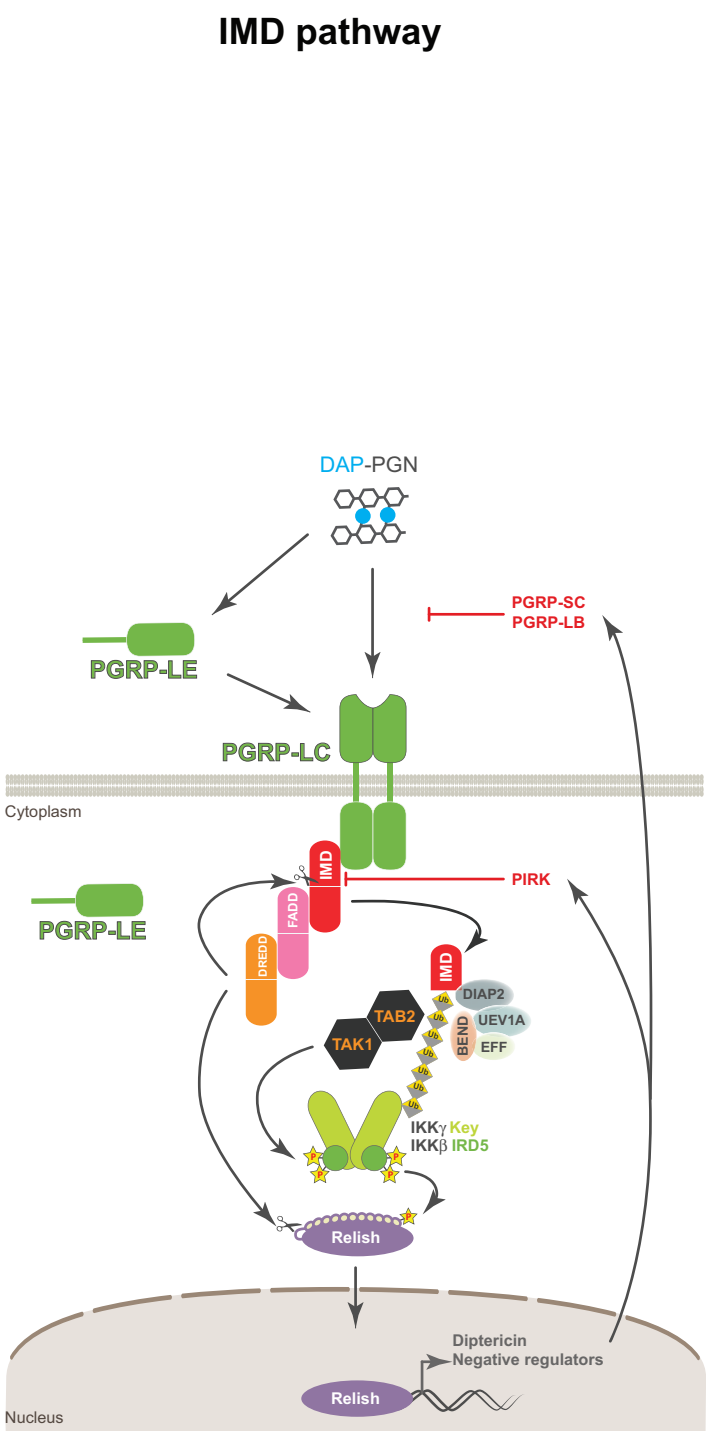
Gram positive bacteria
(and some fungi and yeast)

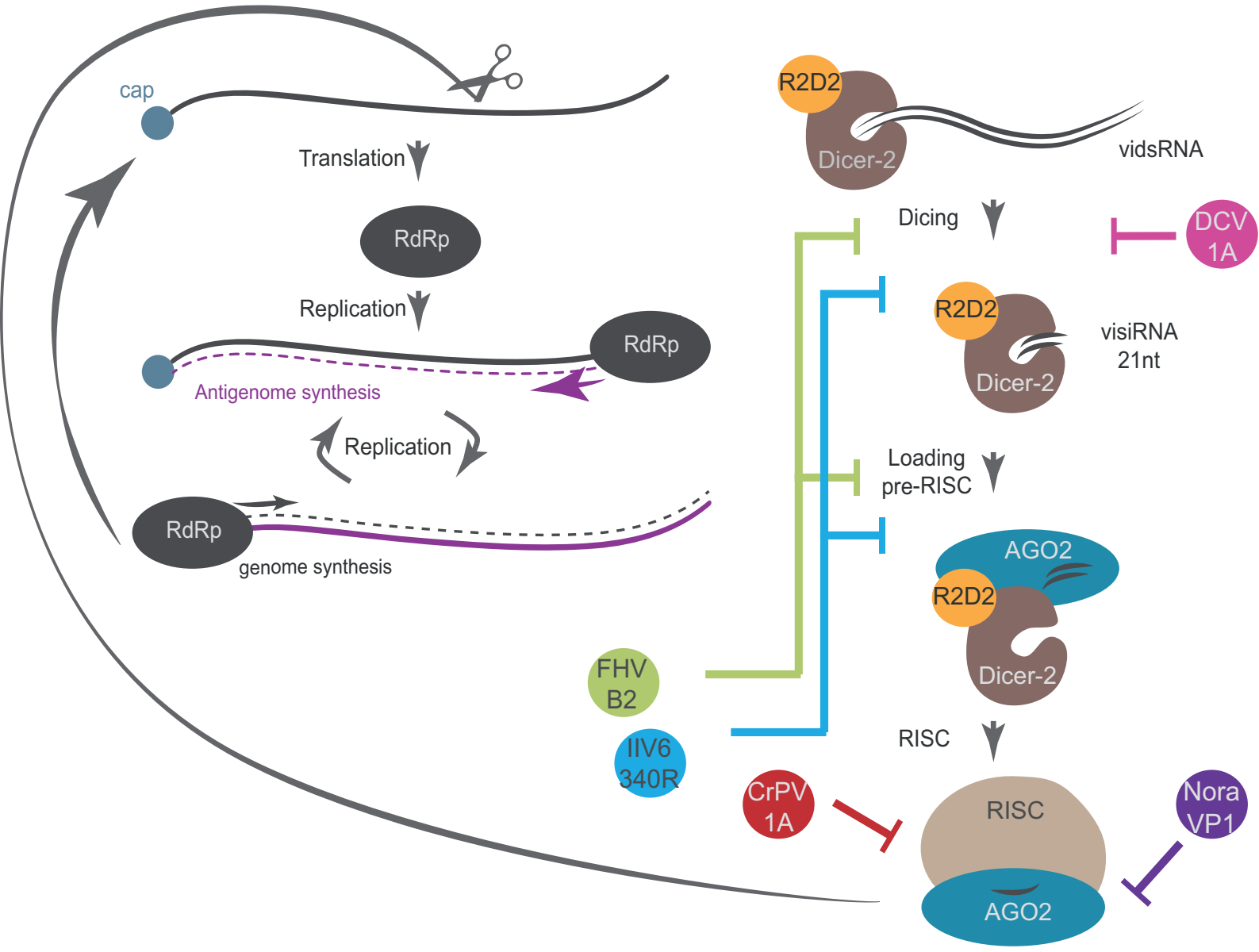


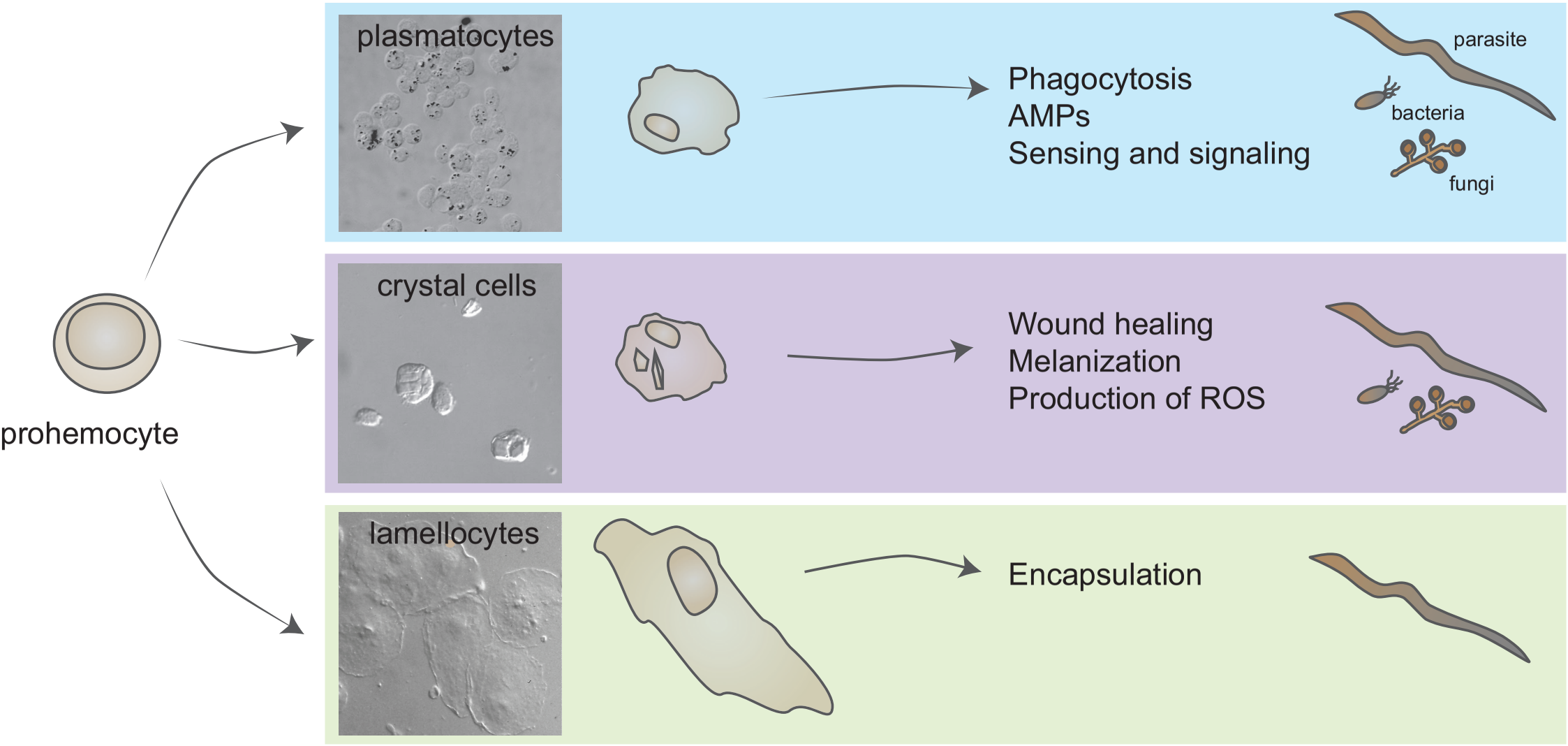
Drosomycin
(100μM)

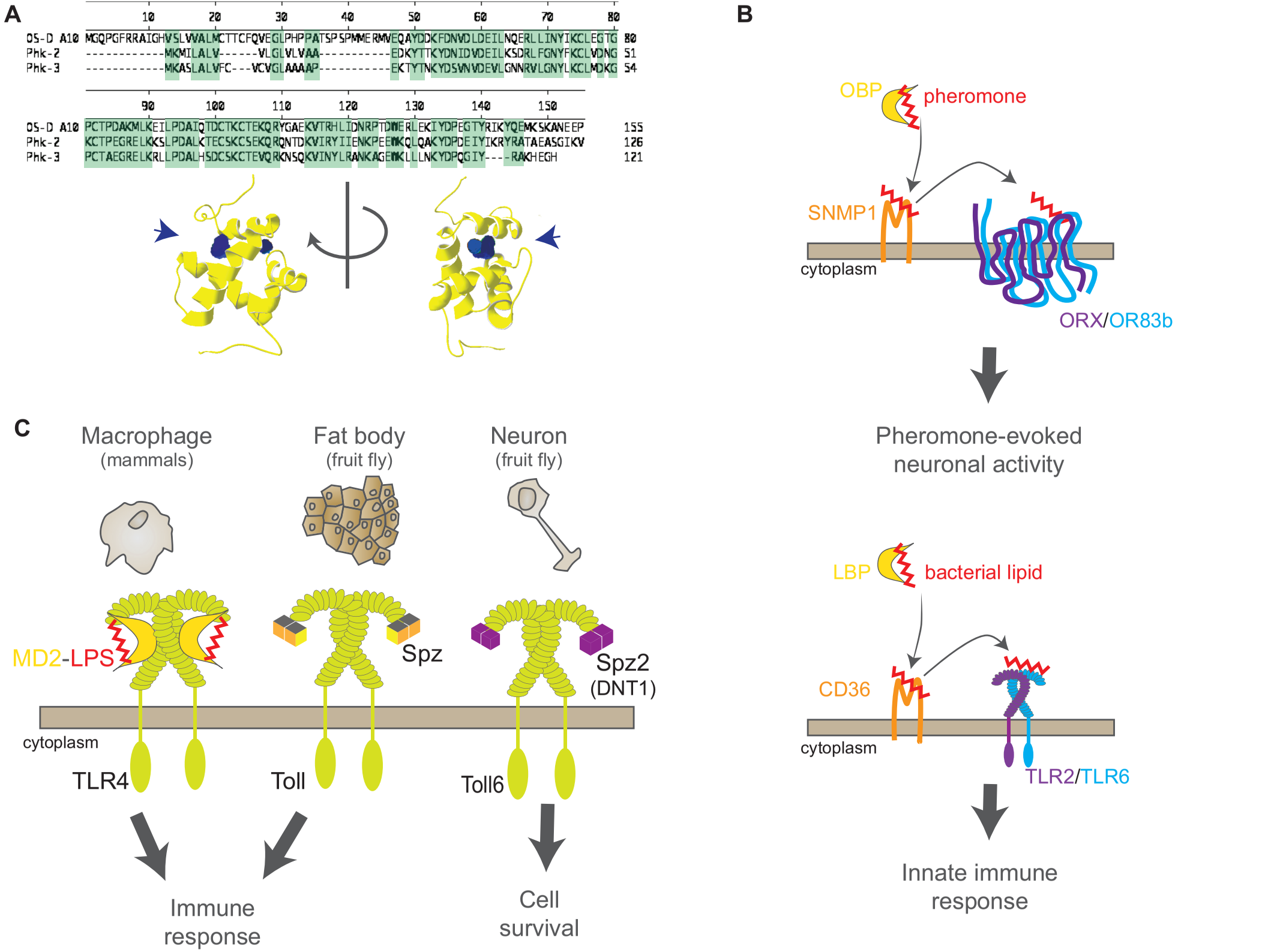
Fungi


Toll pathway
Pathogen proteases
Circulating receptors
β-glucans
LYS-PGN
GNBP3
GNBP1
PGRP-SA
PGRP-SD
Proteolytic cascade
Clip
Psh
Clip
SPE
pro
Spaetzle
Protease
Clip domain
Phosphorylation
Spaetzle dimer
Toll
Cytoplasm
MyD88
Pelle
Tube
MyD88
Tube
Pelle
Dif
Cactus
Drosomycin
Dif
Dorsal/Dif
Nucleus


IMD pathway
DAP-PGN
PGRP-SC
PGRP-LB
PGRP-LE
PGRP-LC
Cytoplasm
IMD
PIRK
PGRP-LE
FADD
DREDD
IMD
DIAP2
TAB2
TAK1
BEND
UEV1A
EFF
IKKγ Key
IKKβ IRD5
Relish
Diptericin
Negative regulators
Relish
Nucleus


cap
Translation
RdRp
Replication
RdRp
Antigenome synthesis
Replication
RdRp
genome synthesis
R2D2
Dicer-2
vidsRNA
Dicing
DCV
1A
R2D2
Dicer-2
visiRNA
21nt
Loading
pre-RISC
AGO2
R2D2
Dicer-2
FHV
B2
IIV6
340R
RISC
CrPV
1A
RISC
Nora
VP1
AGO2


plasmatocytes
crystal cells
lamellocytes
prohemocyte
Phagocytosis
AMPs
Sensing and signaling
Wound healing
Melanization
Production of ROS
Encapsulation
parasite
bacteria
fungi


A
10 20 30 40 50 60 70 80
OS-D A10 MGQPGFRRAIGHVSLVWALMCTTCFQVEGLPHPPATSPSPMMERMVEQAYDDKFDNVDLDEILNQERLLINYIKCLEGTG 80
Phk-2 ----------MKMILALV------VLGLVLVAA----------EDKYTTKYDNIDVDEILKSDRLFGNYFKCLVDNG 51
Phk-3 ----------MKASLALVFC---VCVGLAAAAP----------EKTYTNKYDSVNVDEVLGNNRVLGNYLKCLMDKG 54
90 100 110 120 130 140 150
OS-D A10 PCTPDAKMLKEILPDAIQTDCTKCTEKQRYGAEKVTRHLIDNRPTDNERLEKIYDPEGTYRIKYQEMKSKANEEP 155
Phk-2 KCTPEGRELKKSLPDALKTECSKCSEKQRQNTDKVIRYIIENKPEENKQLQAKYDPDEIYIKRYRATAEASGIKV 126
Phk-3 PCTAEGRELKRLLPDALHSDCSKCTEVQRKNSQKVINYLRANKAGENKLLLNKYDPQGIY----RAKHEGH 121
B
OBP
pheromone
SNMP1
cytoplasm
ORX/OR83b
Pheromone-evoked
neuronal activity
C
Macrophage
(mammals)
Fat body
(fruit fly)
Neuron
(fruit fly)
MD2-LPS
Spz
Spz2
(DNT1)
cytoplasm
TLR4
Toll
Toll6
Immune
response
Cell
survival
LBP
bacterial lipid
CD36
cytoplasm
TLR2/TLR6
Innate immune
response