# The Multilayered Innate Immune Defense of the Gut

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In the wild, the fruit fly *Drosophila melanogaster* thrives on rotten fruit. The digestive tract maintains a powerful gut immune barrier to regulate the ingested microbiota, including entomopathogenic bacteria. This gut immune barrier includes a chitinous peritrophic matrix that isolates the gut contents from the epithelial cells. In addition, the epithelial cells are tightly sealed by septate junctions and can mount an inducible immune response. This local response can be activated by invasive bacteria, or triggered by commensal bacteria in the gut lumen. As with chronic inflammation in mammals, constitutive activation of the gut innate immune response is detrimental to the health of flies. Accordingly, the *Drosophila* gut innate immune response is tightly regulated to maintain the endogenous microbiota, while preventing infections by pathogenic microorganisms. (*Biomed J 2015;38:276-284*)

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The optimal functioning of the intestinal immune response requires discrimination between the indigenous, symbiotic gut microbiota and potential microbial pathogens. Understanding of the antimicrobial responses in the D*rosophila* gut has been advanced by the development of oral infection models,[1-8] studies of gut structure,[3,9-20] characterization of commensal microbiota,[6,21-26] and use of axenic flies.[27-30] These studies reveal that the basal immune response is weakly induced by the commensal gut microbes and strongly enhanced upon pathogen immune challenge.[23] Regulatory mechanisms which dampen the basal immune response are essential to avoid chronic lethal reactions,[31] but the system must remain responsive to acute infectious challenges.[29,32] Ingestion of pathogenic bacteria causes physiopathological alterations in epithelial barriers, both from bacterial toxins and by damage resulting from the host's own immune response.[3,5,31,33-36] Subsequent repair mechanisms promote the regeneration of stem cells to re-establish gut integrity and a homeostatic balance at the intestinal barrier. These repair mechanisms involve several host signaling pathways.[29,32,37-40] In addition, regeneration of intestinal cells is also modulated indirectly by the commensal microbiota, which complements the host physiology in several ways.[29,34,41,42] Notably, the commensal microbiota seem to be involved in the regulation of larval growth and have been linked to age-related diseases, associated with gut dysplasia and loss of intestinal integrity.[43-47] Finally, as in mammals, commensal microbes seem to contribute to protective reactions against pathogen challenge.[8,48] Study of gastrointestinal infections by the yeast *Candida albicans* in *Drosophila* larvae indicate that the commensal microbes enhance host survival during infection, both in wild-type and immunodeficient flies. Thus, the microbiota may affect pathogens either through direct competition or by altering the gut environment.[8,17] In this review, we provide the latest updates on the anatomy of the *Drosophila* gut and the regulation of the induced immune response in the intestinal mucosa. The repair mechanisms of the injured intestine are beyond the scope of this article, but recent reviews covering this topic are presented. The role of the commensal microbiota in fly physiology is covered by Ma *et al.* in this issue.

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## Hold it tight: The *Drosophila* gut anatomy

The gut is subdivided into foregut, midgut, and hindgut regions [Figure 1], each consisting of a monolayered epithelium surrounded by visceral muscles, nerves, and tracheae, with the nervous system playing a key role in the regulation of nutrient intake, and fluid and ion balance.[49] Ingested food passes the pharynx and is either stored to the crop or sent directly to the midgut where the digestion starts.[50] The crop itself is a specialized region of the foregut where a variety of immune proteins are secreted, including thioester containing proteins (TEPs).[51] The nature and arrangement of the gut epithelial cells differs depending on their position along the anteroposterior axis and their developmental origin.

The foregut and hindgut epithelia are of ectodermal origin, are lined on the apical side by an impermeable cuticle, and have pleated septate junctions which seal their paracellular spaces. In contrast, the midgut epithelium is of endodermal origin, is not covered by cuticle, and its integrity relies on smooth septate junctions. The midgut is protected by the peritrophic matrix (PM), a semi-permeable membrane allowing the passage of monomeric enzymes and nutrients, but not bacteria: It is here that food absorption occurs. The midgut can be subdivided into an anterior midgut region, the acidified copper cell region, and the posterior midgut, with the copper cell region perhaps functioning as a stomach [Figure 1]. Recently, the adult midgut was further subdivided into six anatomical regions (R0–R5) separated by narrow epithelial boundaries and with distinct metabolic and digestive functions.[40] In the ileum and rectum of the hindgut, water and salt re-absorption takes place from the food bolus. The gut harbors a simple bacterial community, of four or five major phylotypes, from *Lactobacillus* and *Acetobacter* genera.[22]

In larvae, processing of complex substrates starts before ingestion itself, with clusters of larvae regurgitating digestive enzymes and possibly antimicrobial peptides (AMPs).[52] Four gastric cecae in the anterior midgut represent a specific larval feature, and are major sites of digestion and absorption in other insects.[53]

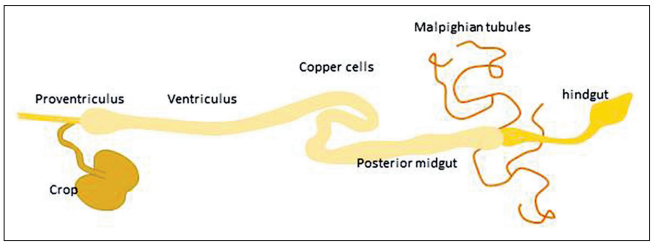


**Figure 1:** The *Drosophila* gut anatomy. The *Drosophila* gut includes three main parts: the foregut (crop), the midgut, and the hindgut. The midgut is divided in four sections: the proventriculus, the ventriculus, the copper cells, and the posterior midgut, respectively, along the anterio-posterior axis of the insect body. The malpighian tubules which ensure the osmoregulatory and excretory functions connect to the gut at the junction between the midgut and the hindgut.

The functioning of the digestive tract, with its resident commensal bacteria, is directly threatened by exogenous microbial pathogens. Consequently, gut epithelia are heavily shielded to resist microbial aggression by means of specialized junctions that seal the single cell layer, a thin cuticle that covers the foregut and the hindgut, and the PM which isolates the bolus from the midgut epithelial cells.[17] As revealed by electron microscopy, four different layers of chitin fibrils and glycoproteins (e.g. peritrophins)[54,55] are secreted at the beginning of the anterior midgut by the proventriculus[56] and self-assemble to form the PM.[56] Peritrophin genes are also expressed in more distal parts of the midgut, suggesting that this barrier is remodeled along the length of the gut.[40]

Studies using corrosive agents reveal a role for the PM in the defense against enteric bacteria both in *Aedes* and *Drosophila*.[57,58] The chitin-binding protein drosocrystallin is strongly expressed upon oral infection, and drosocrystallin mutants show reduced PM width and a shorter lifespan. These mutants show increased susceptibility to oral infections by *Serratia marsescens* and *Pseudomonas entomophila*, or to ingestion of the *Pseudomonas* pore-forming toxin monalysin. These results demonstrate that the PM plays an important role in the host defense by protecting the gut cells from intestinal pathogens and/or their toxins.[59,60] Additional studies also suggest that the PM protects insects from xenobiotics such as dichloro-diphenyl-trichloroethane (DDT[61]). In addition to the PM, a mucin layer covers the midgut enterocytes in *Drosophila*, but its role in the host defense has yet to be investigated.[17]

Physical barriers also play a critical role in preventing the deleterious effect of chronic activation of the immune response by the commensal microbes.[62] Indeed, mutants for the big bang gene, which encodes multiple membrane-associated postsynaptic density protein 95 (PSD-95), discs large, zonula occludens-1 (ZO-1) (PDZ) domain containing protein isoforms, displayed loose septate junctions on the apical side of the enterocytes and a constitutive activation of the anterior midgut immune response. This phenotype correlates with a shortened lifespan of *bbg* mutants, which can be restored by clearing resident bacteria using antibiotic treatment. These observations are reminiscent of the chronic inflammation characteristic of mammalian bowel diseases and indicate that intact intestinal cell junctions are required for immune tolerance towards the endogenous gut microbes. The role of epithelial integrity in the host defense is further supported by the phenotype of *MyoIB* mutants. The myosin IB protein is required to maintain the highly

ordered structure and composition of the brush border layer in the larval midgut epithelium. Both *MyoIB* and *bbg* mutant flies are hypersensitive to enteric infections by pathogenic bacteria.[62,63]

## The active immune response of the gut

### *AMP production*

A major aspect of *Drosophila* host defense is the pathogen-induced expression of AMPs through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor family members. Two signaling cascades, Toll and immune deficiency (IMD), regulate the systemic immune response, characterized by secretion of AMPs from the fat body cells into the hemocoel cavity of the insect.[64] These pathways are activated via the detection of microbial elicitors by cognate pattern recognition receptors (PRRs) of the peptidoglycan (PGN) recognition protein (PGRP) and the Gram-negative binding protein (GNBP) families.[65-67] The Lys-type PGNs of Gram-positive bacteria and β-glucans from fungal cell walls trigger the Toll pathway upon binding to a PRR complex including PGRP-SA, GNBP1, and PGRP-SD and the GNBP3 receptor, respectively.[68-75] The meso-diaminopimelic acid (DAP)-type PGNs of Gram-negative bacteria and bacilli activate the IMD pathway through transmembrane PGRP-LC and/or secreted/cytosolic PGRP-LE receptors.[76-85] AMP expression is also elicited by the sensing of danger signals such as secreted microbial proteases upstream of the Toll pathway.[68,86] This dual sensing mechanism allows a graded Toll pathway activation threshold, matched to the severity of the infection. Danger sensing also avoids the mechanisms deployed by some pathogens to impede the detection of microbial moieties by PRRs.[68]

In the gut epithelium, the expression of AMP-encoding genes is typically regulated by the IMD pathway and bacterial PGN represents the only identified elicitor to date [Figure 2].[69,77,87] Bacterial recognition is regionalized along the length of the gut. PGRP-LE is the predominant intracellular receptor for monomeric PGN in the midgut. PGRP-LC acts mostly in the proventriculus and the hindgut and concomitantly with PGRP-LE in the ventriculus as a detector of extracellular monomeric and polymeric PGN.[15,85] The dominant members of the *Drosophila* gut bacteria, *Acetobacter* and *Lactobacilli* spp., carry DAP-type PGNs and activate the IMD pathway at a basal level, which allows homeostatic relationship between this microbiota and the host immune response.[15]

Any dysregulation of the IMD pathway alters this balance. For example, upregulation of AMP gene expression induces intestinal dysbiosis, marked by the overgrowth of *Gluconobacter morbifer,* which is normally only a minor component of the natural gut microbial community. The resultant gut pathology is associated with a reduced lifespan.[23,31] Several layers of control ensure a tight regulation of the IMD pathway in order to tolerate the commensal microbiota and fight pathogen infection when required [Figure 2]. The first layer involves negative regulators of the IMD pathway, some of which alter the initial steps of sensing and signaling processes by targeting either the bacterial elicitor or the host receptor. Catalytic members of the PGRP family have an amidase activity, related to the bacteriophage T7 lysozyme, that degrades PGN into non-immunostimulatory molecules.[88] Among these catalytic PGRPs, PGRP-LB has a predominant role in the modulation of the IMD pathway in the gut.[31,89] *PGRP-LB* expression is activated by the microbiota and further enhanced upon infection.[15,23] In the absence of infection, *PGRP-LB* mutant flies express high levels of AMP, compared to wild-type flies. This increased expression is suppressed under axenic conditions, demonstrating that PGRP-LB is required to maintain the low basal immune response to microbiota.[31] A non-catalytic, membrane-bound signaling-deficient PGRP, PGRP-LF, also acts as a negative regulator of the IMD pathway.[90,91] PGRP-LF has two PGN recognition domains and a high affinity for PGRP-LC when bound to the monomeric PGN of Gram-negative bacteria, tracheal cytotoxin (TCT). Thus, PGRP-LF hinders the assembly of the receptor complex upstream of IMD.[92] At the intracellular level, the accessibility of both PGRP-LE and PGRP-LC receptors to the downstream components of the cascade is antagonized by Pirk (Poor IMD Response upon Knock in), also referred to as PIMS (PGRP-LC–interacting inhibitor of IMD signaling) or Rudra.[28,93,94] Indeed, Aggarwal *et al.*, showed that the Pirk inhibitor interacts with PGRP-LC, PGRP-LE, and IMD, thus disrupting the signaling complex. Like PGRP-LB, Pirk is a critical component in the negative feedback loop that maintains a balanced IMD response following bacterial infection.[28] Interestingly, flies lacking PGRP-LF have developmental defects that are attenuated when flies are reared on antibiotics.[90] Similarly, *PGRP-LB*, *Pirk* double mutants have reduced lifespan that is improved when flies are maintained in axenic conditions.[31] Clearly, these negative regulators prevent the spurious activation of the immune response by the fly indigenous microbes. Additional negative regulators of the IMD pathway have been identified, mainly acting via ubiquitylation and the degradation of intracellular components of the cascade.[17,95-102] Altogether, these regulatory components downregulate the IMD cascade following immune stimuli, allowing a balanced AMP response to be re-established once the infection is cleared.[103,104]

Another layer of control is provided by the functional compartmentalization of the *Drosophila* gut.[15,23,105] Although the IMD pathway responds to the endogenous

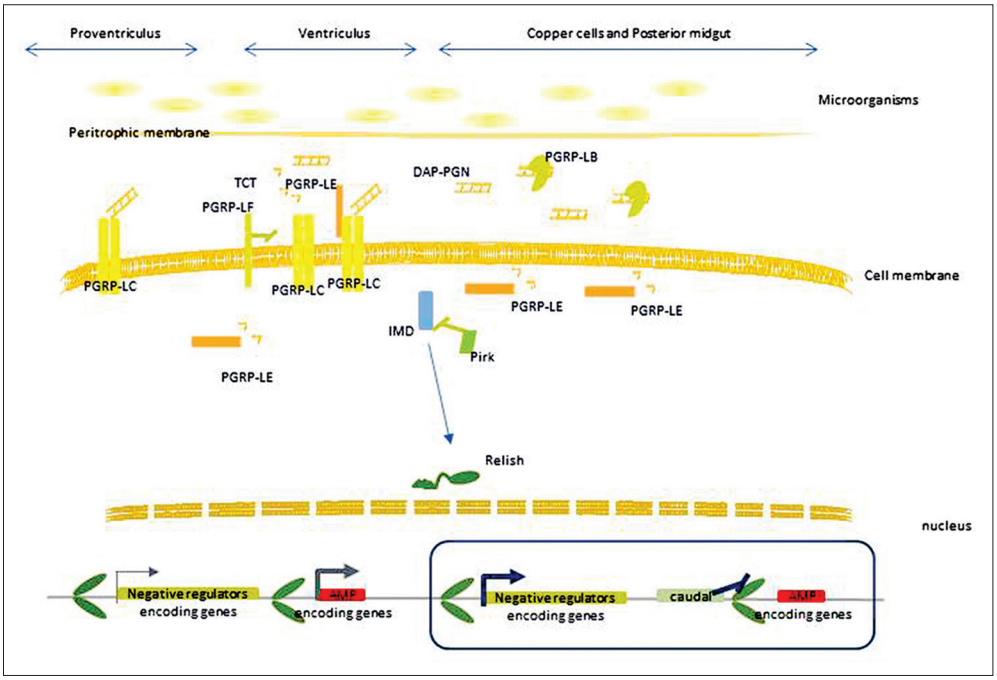


**Figure 2:** Regulation of the IMD pathway in the *Drosophila* midgut. The *Drosophila* gut is regionalized according to the major roles of PGRP receptors inducing the IMD pathway and to the nature of the concomitant induced responses. Monomeric and polymeric PGNs trigger the polymerization of PGRP-LC receptor isoforms which have a predominant role in the proventriculus. PGRP-LE exclusively controls the IMD pathway activity in the copper cells and the posterior midgut, where it acts as an intracellular receptor of TCT. Both receptors are required in the ventriculus. Following PGN binding, oligomerized receptors initiate an intracellular cascade involving the IMD adaptor protein, which leads to the nuclear translocation of a NF-κB transcription factor, Relish. Relish controls the expression of multiple genes including those encoding AMPs or negative regulators of the IMD pathway. Relish activity is inhibited by the homeobox gene *caudal* in the posterior section of the midgut. Signaling through the IMD pathway is modulated by the amidase activity of PGRP-LB which degrades PGN into non-stimulatory entities. The signaling complex of the IMD pathway is monitored by PGRP-LF which hinders the multimerization of the TCT–PGRP-LC complex and Pirk which limits the accessibility of the PGRP-LE and PGRP-LC receptors to the downstream components of the cascade. Filled arrows indicate the predominant response. Abbreviations used: AMPs: Antimicrobial peptides; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PGN: Peptidoglycan; PGRP: Peptidoglycan recognition protein; Pirk: Poor Imd Response upon Knock in; TCT: Tracheal cytotoxin.

microbes all along the gut, the expression of NF-κB–dependent AMPs and immunosuppressor genes follows different patterns. In the midgut, *PGRP-LB* and *Pirk* are constitutively expressed, whereas many AMP-encoding genes are repressed by the homeobox gene *caudal* [Figure 2].[15,23] Strikingly, the production of negative regulators, induced by the commensal bacterium *Lactobacillus plantarum*, is shifted toward PGRP-LE–mediated AMP production, in response to infection by *Erwina carotovora*. In 2012, Bosco-Drayon *et al.*, proposed that AMP production might require stimulation by a higher bacterial load compared to negative regulator expression. This hypothesis is supported by the observation that IMD pathway activation increases with elevated microbiota density during aging. However, it is also tempting to speculate that a dual mechanism integrating the detection of PAMPs and danger signals would apply to the regulation of mucosal gut immunity, allowing the tolerance of autochthonous bacteria and directing the antimicrobial response toward allochthonous bacteria. Although such a mechanism has not been described for the IMD pathway to date, a transcriptomic analysis of the immune response in the gut of infected flies has revealed the induction of antimicrobial encoding genes, the drosomycin-like peptides, independently of the IMD pathway. These AMPs, proposed to be antifungal, are induced under stress conditions and intestinal damage through the activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway.[29,34]

### *Reactive oxygen species production*

In addition to the orderly AMP production, a balanced redox system is essential for the host defense and maintenance of gut homeostasis in *Drosophila*.[106-109] Oral infection of adult flies is associated with the rapid production of reactive oxygen species (ROS)[106,107] generated by dual oxidase (DUOX), a member of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family [Figure 3].[106] This ROS production is required to control dietary yeast, which would otherwise proliferate in the gut and kill the fly.[109] Furthermore, RNA interference (RNAi)-mediated knock-down of *duox* in the intestinal epithelium leads to enhanced susceptibility of the flies to

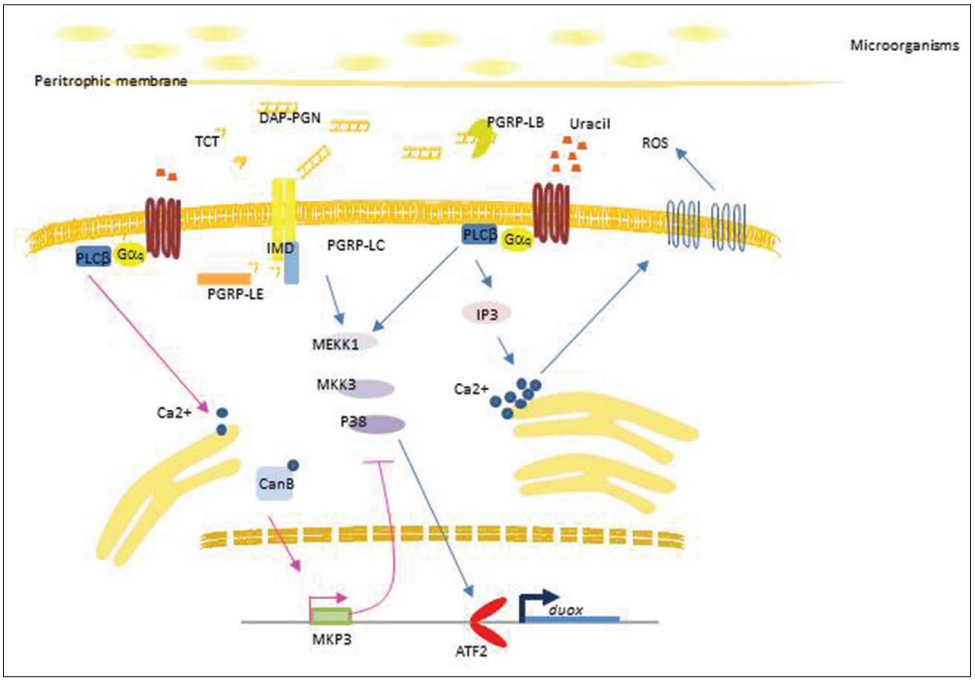


**Figure 3:** Regulation of ROS production in the *Drosophila* midgut. ROS are produced in the *Drosophila* gut lumen though the activity of DUOX. DUOX activity is triggered to a basal level by the gut microbiota and the ingested microorganisms, and is further enhanced upon infection. Uracil released by microorganisms is likely to trigger a G-protein-coupled receptor that activates the Gαq-phospholipase Cβ, which in turn mobilizes intracellular Ca2 + through IP3 generation for DUOX-dependent ROS production. DUOX activity is also regulated at the transcriptional level through the activation of the p38-MAPK pathway leading to the activation of the transcription factor Atf2. Transcription of the *duox* gene also integrates inputs from the Imd pathway. Under basal conditions, *duox* transcription is negatively controlled by the calcium-dependent phosphatase calcineurin B through the induction of mitogen kinase phosphatase 3. Blue and purple arrows indicate the enhancer and inhibitory pathways of DUOX activation, respectively. Abbreviations used: Duox: Dual oxidase; IP3: Inositol triphosphate; MAPK: Mitogen activated protein kinase; MEKK1: MAPKinase and ERK kinase kinase 1; MKK3: Mitogen activated protein kinase 3; ROS: Reactive oxygen species.

minor oral infections, demonstrating that DUOX is part of an essential defense system of the gut mucosa.[106] However, the oxidative burst is deleterious to the host cells and excess ROS are eliminated by the immune-regulated catalase (IRC), which is constitutively expressed in the gut.[107,109,110] Both expression and activity of DUOX are enhanced upon infection [Figure 3]. The DUOX enzyme is calcium-dependent and is regulated by the Gαq-phospholipase Cβ that mobilizes Ca2+ from the endoplasmic reticulum (RE) upon generation of inositol triphosphate.[106,109] DUOX expression is regulated through the activation of the mitogen-activated protein (MAP) kinase pathway, integrating inputs from the PGRP-LC receptor but also from the sensing of microbial activities through a so far unidentified receptor acting upstream of PLC-β [Figure 3].[30,108] The current model proposes that uracil produced by allochthonous bacteria is the determining agonist activating PLC-β, since uracil ingestion leads to ROS generation in a dose-dependent manner. This model is supported by the fact that oral infection with an uracil auxotrophic strain of *E. carotovora* shows increased virulence, while the auxotroph fails to trigger ROS production in the gut.[30] However, the relevance of this observation remains uncertain, since the ability of the major components of the indigenous microbiota to produce uracil has not been demonstrated.

## Conclusion

During the last 5 years, our comprehension of the mechanisms underlying the gut immune response has considerably expanded. The setting up of several infection models combined with genome-wide analyses revealed that the innate immune response in the gut is multilayered and tightly controlled to prevent immune reactions against the endogenous gut microbes. Similarly, the mechanisms involved in epithelial repair after exposure to microbial harm (i.e., resilience mechanisms) are now quite well understood.[50]

### *From PAMP recognition to bacterial pathogenicity*

Several questions remain open

As both the subcellular distribution of PGRPs and the molecular nature of the uracil receptor are unknown, how the microbial PAMPs are sensed in the *Drosophila*

gut remains unclear. In mice, the bacterial flagellin sensor Toll-like receptor 5 (TLR5) is located at the basal surface of enterocytes, but this sensor cannot detect flagellin from the gut luminal flora. Invasive bacteria, however, reach the basal enterocyte surface, where TLR5 is then able to trigger an inflammatory response.[111] Septate junction deficient flies have an enhanced paracellular space that facilitates access of the endogenous flora to the latero-basal side of enterocytes. Similar to TLR5 distribution in mouse enterocytes, a basal cellular distribution of PGRP-LC may explain the potent innate immune response observed in these mutant flies.[62]

How the damage inflicted to the gut by pore-forming toxins, such as the Cry-toxins from Bacillus thuringiensis or monalysin from *Pseudomonas entomophila*, causes lethality in *Drosophila* is unclear. In mammals, any breach of the gut mucosal barrier results in basal exposure of TLR5 to flagellin from the endogenous flora, leading to TLR5 activation and subsequent chronic gut inflammation.[112] A similar mechanism in the fly would predict that if pore-forming toxins lead to internal exposure to the gut flora, then this exposure would result in enhanced IMD pathway activation and subsequent lethality.[113] Gut damage and food uptake blockage may also contribute to pore-forming toxin pathogenicity.[35]

### *A chicken and egg conundrum, the aging gut phenotype*

The increased titer of the microbial community associated with enhanced IMD pathway activation during aging of the fly remains mysterious.[24] Possibly, this paradoxical correlation could reflect a long-term selection of the bacteria for resistance, leading to a gradual increase in the basal response. Alternatively, increased IMD pathway activation could be linked to an age-related deterioration in gut repair mechanisms, leading to increased exposure to bacterial PAMPs and selection of resistant bacterial strains.

Despite the differences between the mammalian intestine and the fly gut, many parallels between the two establish *Drosophila* as a powerful and fascinating model to decipher fundamental aspects of gut biology. Such knowledge will have implications for understanding the dynamic host/microbiota interactions in the healthy human gut and for the complex mechanisms of intestinal disease syndromes such as Crohn's.[62]

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