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Article

Peptidoglycan Sensing by the Receptor PGRP-LE in the *Drosophila* Gut Induces Immune Responses to Infectious Bacteria and Tolerance to Microbiota

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SUMMARY

Gut epithelial cells contact both commensal and pathogenic bacteria, and proper responses to these bacteria require a balance of positive and negative regulatory signals. In the *Drosophila* intestine, peptidoglycan-recognition proteins (PGRPs), including PGRP-LE, play central roles in bacterial recognition and activation of immune responses, including induction of the IMD-NF-κB pathway. We show that bacteria recognition is regionalized in the *Drosophila* gut with various functional regions requiring different PGRPs. Specifically, peptidoglycan recognition by PGRP-LE in the gut induces NF-κB-dependent responses to infectious bacteria but also immune tolerance to microbiota through upregulation of pirk and PGRP-LB, which negatively regulate IMD pathway activation. Loss of PGRP-LE-mediated detection of bacteria in the gut results in systemic immune activation, which can be rescued by overexpressing PGRP-LB in the gut. Together these data indicate that PGRP-LE functions as a master gut bacterial sensor that induces balanced responses to infectious bacteria and tolerance to microbiota.

INTRODUCTION

Human mucosal surfaces are colonized by heterogeneous communities of hundreds of bacterial species called microbiota. These gut-associated bacteria have coevolved with the host and confer beneficial effects, including help in metabolizing nutrients, modulation of immune responses, and defense against pathogens. Recent findings indicated that the intestinal epithelium should not be seen as a simple physical barrier confining these bacteria to the gut lumen, but as a well-evolved mucosal immune tissue populated by cells poised to defend against pathogenic incursions and curtail inflammatory responses toward commensal bacteria (Garrett et al., 2010). If it is well accepted that the intestinal epithelium displays a dual response toward infectious and commensal bacteria, we do not really understand the molecular mechanisms allowing the simultaneous balanced activation of these somewhat antagonistic responses in a single epithelium.

Drosophila is a very powerful model to study developmental processes and immune mechanisms (Ferrandon et al., 2007). Its relatively simple tissue organization and sophisticated genetics are some of the advantages of using it as an experimental model to dissect gut-microbe interactions. As for the mammalian intestinal tract, the *Drosophila* gut is equipped with a battery of physical and chemical tools that control luminal bacterial proliferation and prevent bacteria-induced damage to the gut epithelia. Two types of molecular effectors act synergistically to restrict growth and proliferation of gut-invading microorganisms: antimicrobial peptides (AMP) and ROS (Charroux and Royet, 2012; Ha et al., 2005, 2009a, 2009b). Whereas AMP gene transcription is under the control of both the Toll and IMD signaling pathways in the systemic antimicrobial response, it is solely dependent on the IMD cascade in gut epithelial cells (Lemaitre et al., 1995, 1996; Liehl et al., 2006). Recognition of diaminopimelic (DAP)-type PGN derived from most Gram-negative bacteria and some *Bacillales* initiates IMD signaling, ultimately leading to nuclear translocation of an NF-κB family member, Relish (Kaneko et al., 2004; Leulier et al., 2003). Activated Relish subsequently induces the expression of a wide variety of immune effectors such as AMP that, in turn, neutralize the invading pathogens. It is now accepted that *Drosophila* harbors a bona fide microbiota composed of a maximum of 30 phylotypes with 3–4 dominant *Lactobacillale* and *Acetobacteraceae*, some of them having impact on larval growth and on adult mating preference (Chandler et al., 2011; Sharon et al., 2010; Shin et al., 2011; Storelli et al., 2011; Wong et al., 2011). The constant contact of these resident bacteria with the mucosal epithelium raises the question of how the epithelium deals with the continuous input of the immune-activating signals produced by endogenous flora. Several lines of evidence suggest that the host deploys immune tolerance programs that intervene at different levels to regulate IMD pathway activation. This is the case namely at the level of the promoters of some IMD target genes that contain binding sites for the homeobox-containing transcription factor Caudal (Ryu et al., 2008). By binding to these sites, Caudal blocks this

IMD-dependent transcription. At the level of the microbe-derived immune elicitors, the PGN degrading enzymes PGRP-SC1 and PGRP-LB synergize to maintain a low basal level of PGN, which prevents spontaneous overactivation of IMD to the gut microflora (Bischoff et al., 2006; Mellroth et al., 2003; Paredes et al., 2011; Zaidman-Rémy et al., 2006). Yet another mechanism which flies use to make endogenous microflora invisible to their immune system is the modulation of IMD receptor localization to the plasma membrane by the pirk protein (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). Altogether, these negative regulators cooperate to produce an adequate level of constitutive NF-κB activity in gut cells, which is not harmful to the flies under nonpathogenic conditions (Paredes et al., 2011).

Bacterial PGN is the only known IMD pathway elicitor in *Drosophila*. Genetic and biochemical experiments have uncovered its mode of detection during *Drosophila* systemic immune responses. DAP-type PGN is sensed by the membrane-associated PGRP-LC receptor (Choe et al., 2005, 2002; Gottar et al., 2002; Rämet et al., 2002). Through alternative splicing, the PGRP-LC locus can generate three transmembrane proteins (PGRP-LC a, x, and y) that share an N-terminal cytoplasmic signaling domain but have each a different PGN-recognition domain (Werner et al., 2003). RNAi-mediated inactivation of specific PGRP-LC isoforms in S2 cells together with the crystal structure of PGRP-LC ectodomains gave a fairly clear picture of the IMD pathway receptor complex activation (Chang et al., 2006, 2005; Kaneko et al., 2006; Lim et al., 2006; Mellroth et al., 2005; Werner et al., 2003). While recognition of polymeric PGN requires oligomerization of PGRP-LCx, detection of monomeric form of PGN, such as tracheal-cytotoxin (TCT), implicates both PGRP-LCa and PGRP-LCx. In vitro cell culture experiments suggested that another PGRP family member, PGRP-LE, is also involved in DAP-type PGN recognition (Kaneko et al., 2006; Takehana et al., 2004; Yano et al., 2008). While full-length PGRP-LE acts as an intracellular receptor for monomeric PGN, a second form of PGRP-LE with the PGRP domain alone functions non-cell-autonomously to facilitate recognition of polymeric PGN by PGRP-LCx at the plasma membrane (Kaneko et al., 2006).

We provide here a functional analysis of the receptors that act in the *Drosophila* gut to sense bacteria. We show that in contrast to the fat body in which IMD activation depends on PGRP-LC, local gut immune activation is mediated by PGRP-LE. We demonstrate that by recognizing monomeric PGN in the intestinal epithelium, PGRP-LE is not only controlling the intensity of the immune response to infectious bacteria locally but is also preventing spreading of the immune reaction into the whole animal. Our data also demonstrate that by inducing the local production of negative regulators of the IMD pathway such as amidases and by genetically interacting with the immunomodulator pirk, PGRP-LE orchestrates the establishment of the immune tolerance program toward endogenous microbiota. We therefore propose that in the *Drosophila* gut, a unique bacteria sensor, PGRP-LE, is simultaneously required to balance the intensity of the local immune response to infectious bacteria, to prevent the dissemination of this immune reaction to other tissues and finally to establish an immune tolerance to endogenous microbiota.

RESULTS

PGRP-LE and LC Are Both Required to Mediate Bacteria Recognition in the *Drosophila* Midgut

The PGRP-LC protein serves as the main IMD pathway signaling receptor during systemic immune response in the fat body and in the anteriormost part of the midgut, the Proventriculus (Pv) (Basset et al., 2000; Gottar et al., 2002; Zaidman-Rémy et al., 2006). To test whether IMD pathway activation is PGRP-LC dependent in the whole midgut, we monitored the transcription of its target genes in guts of larvae orally infected with an entomopathogenic bacteria, *Erwinia carotovora carotovora* (*Ecc*) (Basset et al., 2000). To avoid unwanted interactions with endogenous flora that may vary in loads and species in between experimental conditions, all infections were performed on axenic individuals orally infected with calibrated doses of single identified bacteria species. Although transcription of AMP genes such as *AttacinD* (*AttD*) and *Diptericin* (*Dpt*) in the larval gut was induced by *Ecc* feeding, this activation was completely PGRP-LC independent in the larval gut (Figure 1A). This prompted us to analyze the role of PGRP-LE, another DAP-type PGN interacting protein which had not yet been implicated in bacteria sensing by the gut. *AttD* and *Dipt* inducibility was respectively abolished and decreased in *PGRP-LE*-null mutants. Since these differences in IMD activation could reflect variation in *Ecc* loads in the alimentary tract of the different mutants, we quantified them. Four hours after ingestion, bacterial loads were similar in the different mutant backgrounds and therefore could not account for the observed differences in IMD pathway activation (Figure S1A). Similar results for *AttD* and *Dipt* transcription (data not shown) and bacterial numeration (Figure S1B) were obtained in adult midguts. Since the apparent contradiction between published results (Zaidman-Rémy et al., 2006) and our data could be explained by a functional compartmentalization of the gut, we tested the competence of different midgut regions to respond to *Ecc* and analyzed the respective roles of PGRP-LE and PGRP-LC in each domain.

Larval and Adult Midgut Immune Responses Are Highly Regionalized

The *Drosophila* larval and adult midguts can be subdivided into 4 morphological domains along the antero-posterior axis: the Pv, Ventriculus (Vtr), Copper cell (Cc) and Posterior midgut (Pmg) (Figure 1B). Although the respective function of theses different domains is not yet totally elucidated, they clearly participate in food mechanical breakdown (Pv), in digestion by creating regions with low pH (Cc), and in nutrient absorption. Using a mixture of Bromophenol blue (BPB) and *Ecc-GFP*, we monitored food uptake and bacteria localization in these different domains. Whereas 4 hr after ingestion, BPB was detected along most of the larval midgut, bacteria were mostly concentrated to the Vtr and Cc regions but were undetectable in the Pmg (Figure 1B). These results were confirmed by bacterial plating of gut content on selective medium (Figure S1C). In contrast, *Ecc-GFP* bacteria fed to adult flies were evenly distributed along the entire midgut (Figure 1B). We then tested IMD pathway activation parameters in the different gut domains using a *Dpt-Cherry* reporter and qRT-PCR. Whereas IMD pathway activation was mainly PGRP-LC-dependent in the Pv, it was

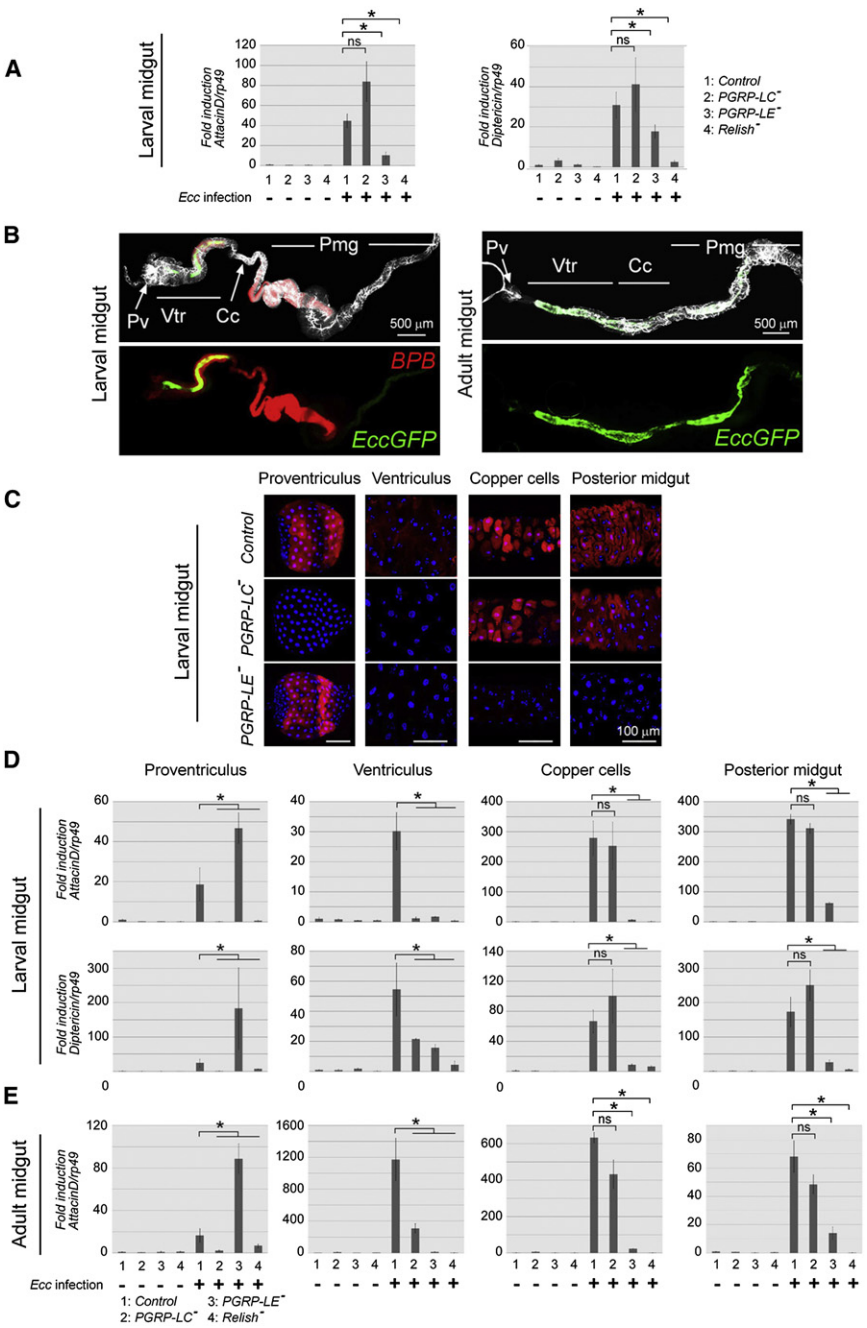


Figure 1. Larval and Adult Midgut Immune Responses Are Regionalized

(A) *Ecc*-mediated AMP gene induction (after 4 hr) in larval midgut requires PGRP-LE.

(B) *Ecc-GFP* bacteria localization in larval (left) and adult (right) guts is shown. *Ecc-GFP* (green) accumulate preferentially in the larval Vtr and Cc regions while food uptake (visualized here with Bromophenol blue [BPB]) reaches the Pmg. In adults, *Ecc-GFP* is found uniformly along the entire midgut. Pictures were taken 4 hr post infection. Pv: proventriculus, Vtr: ventriculus, Cc: copper cells, Pmg: posterior midgut. Scale bar is 500 µm.

(C) PGRP-LC and PGRP-LE are differently required along the anteroposterior axis of the larval gut. Confocal pictures of *Ecc*-infected larval gut regions of the following genotypes: Control (yw;;*Dpt-Cherry*), *PGRP-LC*$^{-}$ (*yw;;PGRP-LC*$^{\Delta E12}$, *Dpt-Cherry*), and *PGRP-LE*$^{-}$ (*yw PGRP-LE*^{112};; *Dpt-Cherry*). Scale bar is 100 µm.

(D and E) AMP gene expression in larval (D) and adult (E) gut domains of *PGRP-LC* and *PGRP-LE* mutants 4 hr post feeding with *Ecc* is shown. For (A), (D), and (E), mRNA levels in axenic control flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histograms correspond to the mean value ± SD of three experiments. Values indicated by symbols (*) are statistically significant ($p < 0.05$). ns: not significantly different. See also Figure S1.

clearly dependent on PGRP-LE in more posterior domains (Figures 1C and 1D). In the Pv, AMP gene induction was positively regulated by PGRP-LC and antagonized by PGRP-LE (Figure 1D). In contrast, transcriptional activation of AMP required both PGRP-LC and PGRP-LE in the Vtr and was fully and exclusively PGRP-LE dependent in Cc and Pmg (Figure 1D). Although bacteria distribution along the midgut was different in larvae and adults, the respective requirements for PGRP-LE and PGRP-LC in the different domains were very similar at both developmental stages (Figure 1E). These results indicate that each gut domain responds specifically to *Ecc* and that the responses are differentially transduced by PGRP-LE or PGRP-LC depending on the domain. While PGRP-LC seems essential in anterior domains, it becomes progressively dispensable in more posterior regions. In contrast, PGRP-LE that is acting as a negative regulator in the anteriormost domain is a required bacteria sensor in the posteriormost part of the midgut.

PGRP-LE Is a Putative Intracellular TCT Sensor in the *Drosophila* Midgut

The *Drosophila* IMD pathway can be activated by both monomeric and polymeric forms of PGN that are recognized by different combinations of PGRP-LC isoforms and of PGRP-LE (Kaneko et al., 2004, 2006; Mengin-Lecreulx and Lemaitre, 2005). Since the relevance of this dual recognition strategy has not been evaluated in vivo, we tested whether we could mimic bacteria mediated gut IMD activation by feeding adults with the monomeric muropeptide called TCT. As shown Figure 2A, AMP gene activation in the different gut domains following TCT feeding perfectly mimicked that observed with *Ecc*. Whereas *AttD* induction was PGRP-LC dependent and antagonized by PGRP-LE in the Pv, it was PGRP-LC and PGRP-LE dependent in the Vtr and fully PGRP-LE dependent in Cc and Pmg domains. These results, which highlight the role of monomeric PGN as an elicitor and of PGRP-LE as a sensor in the midgut were confirmed using other

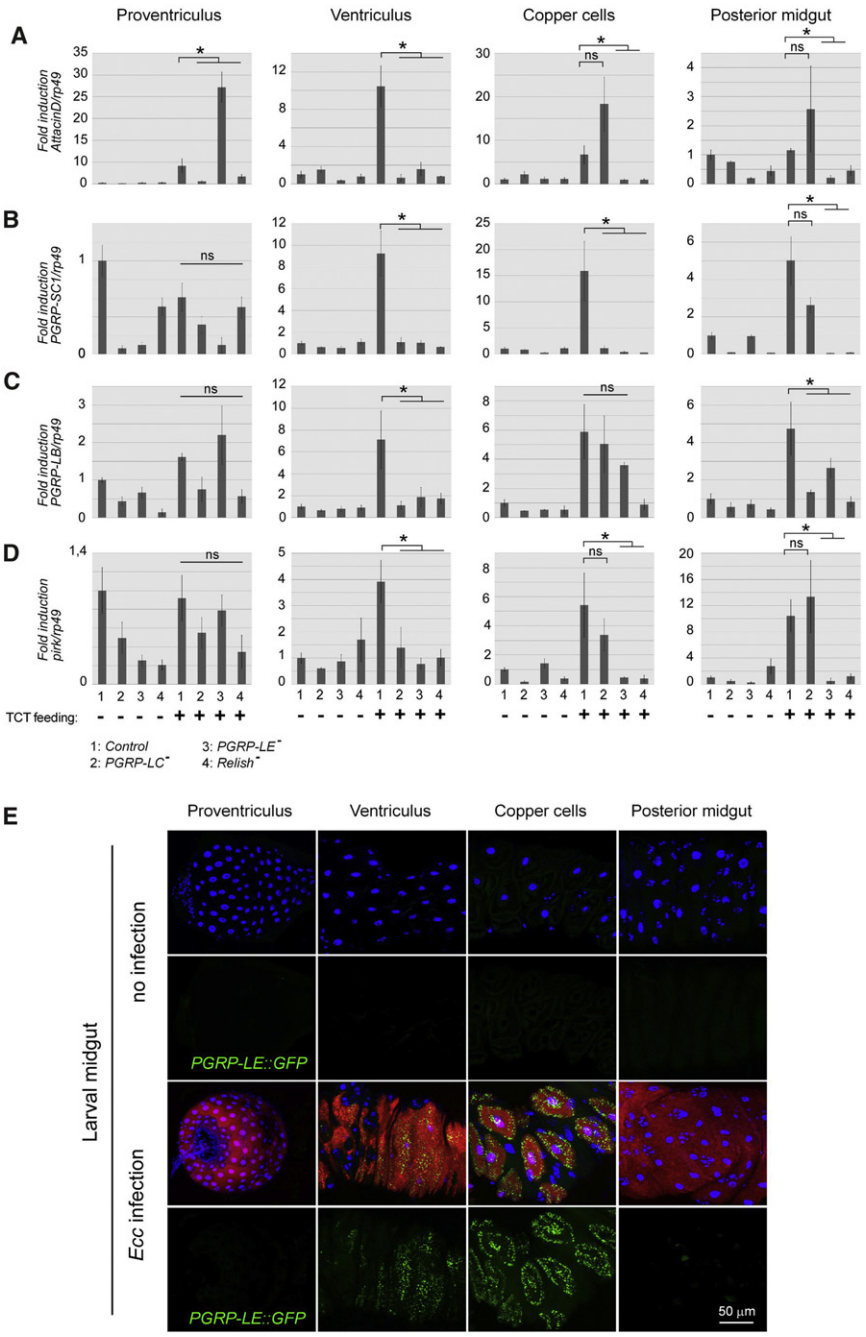


Figure 2. PGRP-LE Detects TCT in the *Drosophila* Gut

(A–D) Transcriptional activation of various IMD target genes in adult midgut domains 24 hr post feeding with TCT. mRNA levels in axenic control flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histogram corresponds to the mean value ± SD of three experiments. Values indicated by symbols (*) are statistically significant ($p < 0.05$). ns: not significantly different.

(E) Confocal pictures of uninfected or *Ecc*-infected *pgrp-PGRP-LE::GFP;Dpt-Cherry* larval guts. PGRP-LE::GFP (green) accumulates in intracytoplasmic vesicles in *Ecc* infected guts. These cells also express the *Dpt-Cherry* transgene (red). Images were taken 24 hr post infection. Scale bar is 50 µm.

IMD targets (Figures 2B, 2C, and 2D). Since cell culture data suggested that TCT recognition by PGRP-LE takes place intracellularly, we analyzed PGRP-LE expression in the gut using a functional GFP-tagged version of the protein regulated by its own promoter. Whereas PGRP-LE::GFP fusion protein was undetectable in nonstimulated guts, it accumulated in intracellular vesicles in the Vtr and Cc regions *of Ecc* infected guts. These vesicles were only detectable in cells that activate IMD signaling since PGRP-LE::GFP positive cells systemically coexpressed the *Dpt-Cherry* transgene (Figure 2E). These data suggest that monomeric PGN is probably the main activator of the gut immune response and that its recognition is mainly transduced via intracellular PGRP-LE but also by PGRP-LC in the Pv and Vtr.

Induction of IMD Negative Regulators by Commensal Bacteria Depends on PGRP-LE

The above results demonstrate that PGRP-LE is the main sensor for bacteria such as *Ecc* that are unable to persist in the gut. We asked whether PGRP-LE would also be implicated in sensing of commensal bacteria. To avoid discrepancies due to variations in microbiota composition, we used an assay allowing recolonization of axenic individuals with physiological quantities of a single commensal bacteria species (see Experimental Procedures and Storelli et al. [2011]). *Lactobacillus plantarum* (*L. plantarum*) which shares all the characteristics of a bona fide commensal was used as a colonizer (Storelli et al., 2011). After recolonization *L. plantarum* was, as for *Ecc*, preferentially localized in anterior gut domains (Figure S2A). However, in contrast to the strong AMP induction observed following *Ecc* ingestion, AMP response was very mild in *L. plantarum* recolonized guts (Figures 3A and 3D). Strikingly, *L. plantarum* gut recolonization induced a very potent transcription of negative regulators of the IMD pathway, such as *PGRP-SC1* and *PGRP-LB*, especially in Cc and Pmg domains (Figures 3B, 3E, and 3F). This upregulation of amidase mRNAs was suppressed in PGRP-LE mutants, a phenotype that could be mimicked by in vivo RNA interference to eliminate PGRP-LE specifically in the gut (Figures S2B and S2C). Consistently, lack of *PGRP-SC1* induction following *L. plantarum* monoassociation in PGRP-LE mutant could be rescued with wild-type PGRP-LE or PGRP-LE::GFP transgenes

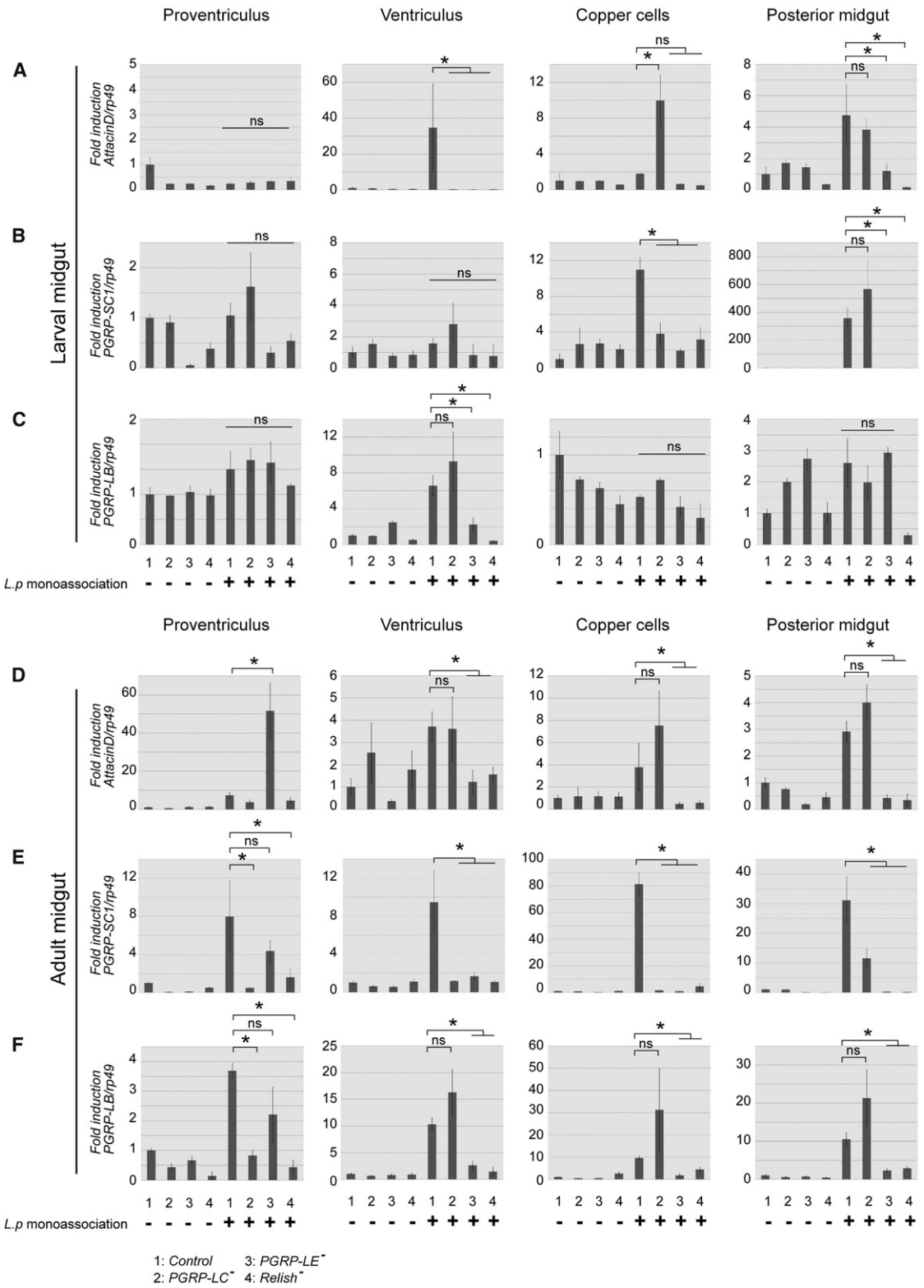


Figure 3. IMD Target Gene Activation by *L. plantarum* Requires PGRP-LE

Transcriptional activation of various IMD targets in the gut of *L. plantarum* recolonized flies. mRNA levels in axenic control flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histograms correspond to the mean value ± SD of three experiments. Values indicated by symbols (*) are statistically significant ($p < 0.05$). ns: not significantly different. See also Figure S2.

(Figure S2D). Altogether, these results show that gut recolonization by the commensal bacteria *L. plantarum* triggers a weak AMP gene induction but a very potent stimulation of transcription of PGRPs with amidase activity which requires the PGRP-LE receptor.

PGRP-LE Cooperates with Pirk to Mediate Immune Tolerance to Commensal Flora in the Gut

To further investigate the role of PGRP-LE in sensing gut flora, we analyzed its genetic interactions with the immune tolerance regulator, pirk. Reducing *pirk* mRNA levels was shown to trigger a rupture of immune tolerance to flora that results in excessive IMD pathway activation (Lhocine et al., 2008). Using our colonization assay, we showed that pirk function is also required to establish tolerance to *L. plantarum* in the larval gut. Indeed, IMD pathway activation was much higher in *pirk* mutant *L. plantarum* recolonized guts than in controls (Figure 4A). This overresponse was completely suppressed in *PGRP-LE;pirk* double mutants but not in *pirk*;*PGRP-LC* mutants (Figure 4A). By using q-RT-PCR and imaging, we showed that the effects of removing *pirk* were strongest in the Cc region (Figures 4B and 4C), suggesting that pirk is more specifically required in this region to prevent IMD pathway activation by *L. plantarum*. Consistently, *pirk* transcripts were enriched in the Cc region compared to other gut domains (Figure S3). In addition to being an IMD pathway attenuator, *pirk* is also an IMD target gene (Kallio et al., 2005; Kleino et al., 2008; Lhocine et al., 2008). Given the importance of PGRP-LE in controlling amidase gene transcription, we asked whether *pirk* mRNA levels could also be regulated by PGRP-LE. As shown Figure 4D, *pirk* was highly induced in the Vtr and Pmg in a fully PGRP-LE-dependent manner, whereas it remained at similar levels in the Cc region. These results indicate that pirk-mediated tolerance to *L. plantarum* is constitutive in the larval Cc domain and inducible in Vtr and Pmg. At the adult stage, *pirk* is also strongly induced by *L. plantarum* in a fully PGRP-LE-dependent manner (Figure 4D). The cooperation between PGRP-LE and pirk was further confirmed by showing colocalization of pirk and PGRP-LE proteins in Copper cell vesicles (Figure 4E). Altogether, these data highlight the intricate and mutual dependence of pirk and PGRP-LE, but not of PGRP-LC, in regulating the immune tolerance to *L. plantarum.* They confirmed that PGRP-LE plays an essential role in sensing commensal bacteria in the gut not only under normal physiological conditions but also in a context of rupture of immune tolerance.

PGRP-LE-Dependent Amidase Expression Prevents IMD Overactivation in *L. plantarum*-Colonized Gut

PGRP-LE function is required to adjust the production of both immune effectors and immune regulators to the type of bacteria present in the gut. In particular, gut recolonization with *L. plantarum* induces a PGRP-LE-dependent burst of amidase production. Since these enzymes have the capacity to modify PGN immunostimulatory properties and to regulate bacterial growth, we tested the consequences of the lack of such enzymes on gut immune response and bacterial loads. No differences in *L. plantarum* bacterial loads were noticed between WT and *PGRP-LE* mutant guts suggesting that amidase levels do not directly impact *L. plantarum* growth in the gut (Figures S4A and S4B). We next analyzed the influence of removing *PGRP-SC1* and *PGRP-LB* on local immune response in the different gut domains (removing PGRP-LE would have been irrational, since this receptor is also required for IMD target gene activation by *L. plantarum*). While removing *PGRP-SC1* function had only moderate effects on IMD pathway activation (Figures 5A and 5B), eliminating *PGRP-LB* had profound consequences on gut IMD pathway activation by *L. plantarum* (Figures 5A and 5B). The Pv and Vtr which are in wild-type conditions unresponsive to *L. plantarum* became highly reactive when *PGRP-LB* was lacking. These results suggest that PGRP-LE-dependent production of amidases in the Vtr is required to tune down local gut immune response, thereby establishing immune tolerance to colonizing bacteria.

PGRP-LB Mutant Lethality Is Suppressed by Simultaneous Removal of PGRP-LE

PGRP-LB mutants present an infection-dependent IMD overactivation that provokes a reduced lifespan (Figure 6 and Paredes et al. [2011]). Although these phenotypes can be suppressed by the simultaneous inactivation of the intracellular IMD pathway member DREDD (Paredes et al., 2011), the tissue in which excessive IMD pathway overactivation is deleterious to the fly remains unknown. We anticipated that removing the main gut bacterial sensor PGRP-LE would suppress this *PGRP-LB* adult mutant susceptibility to infection. To test this hypothesis, *PGRP-LB*-only or *PGRP-LB;PGRP-LE* double mutants were infected with the innocuous bacteria *Ecc* and the survival rates measured. In such conditions, while *PGRP-LB* mutant flies died after 2 weeks, *PGRP-LB;PGRP-LE* double mutant flies could survive up to 30 days (Figure 6). These data suggest that by triggering a burst of amidases in the gut, PGRP-LE is providing an appropriate IMD pathway activation level which could, otherwise, be deleterious to the flies.

PGRP-LE Acts Locally in the Gut to Prevent Systemic Immune Activation

Upon ingestion, *Ecc* triggers a systemic immune response via the production of PGN that crosses the gut epithelium barrier and reaches the fat body (Gendrin et al., 2009). By cleaving PGN, PGRP-LB, and PGRP-SC1 contribute to reduce this systemic immune activation. It is however unclear whether such negative regulators are acting locally in the gut or in the fat body, where the systemic immune response is taking place. Since our data indicate that PGRP-LE is required to ensure proper transcription of *pirk, PGRP-SC1*, and *PGRP-LB* in the gut (Figures 3B, 3C, 3E, 3F, 4D, S5A, S5B, and S5C), we hypothesized that *PGRP-LE* loss-of-function would also present an excessive systemic immune response after *Ecc* ingestion. As expected, *Dpt* was produced at abnormally high levels in the fat body of *PGRP-LE* mutants fed with *Ecc* (Figures 7A and 7B). In wild-type controls, *Ecc* ingestion induced the expression of *PGRP-LB* and *pirk* in both gut and fat body (Figure 7A) and of *PGRP-SC1* only in the gut. In *PGRP-LE* mutants, while *Ecc* induced gut expression of all three mRNAs was abolished, fat body expression of *PGRP-LB* or *pirk* were not downregulated (Figure 7A). This suggests that the excessive systemic immune response in *PGRP-LE* mutant flies results from a reduced expression of these negative regulators specifically in the gut. In support to this conclusion, gut-specific overexpression of

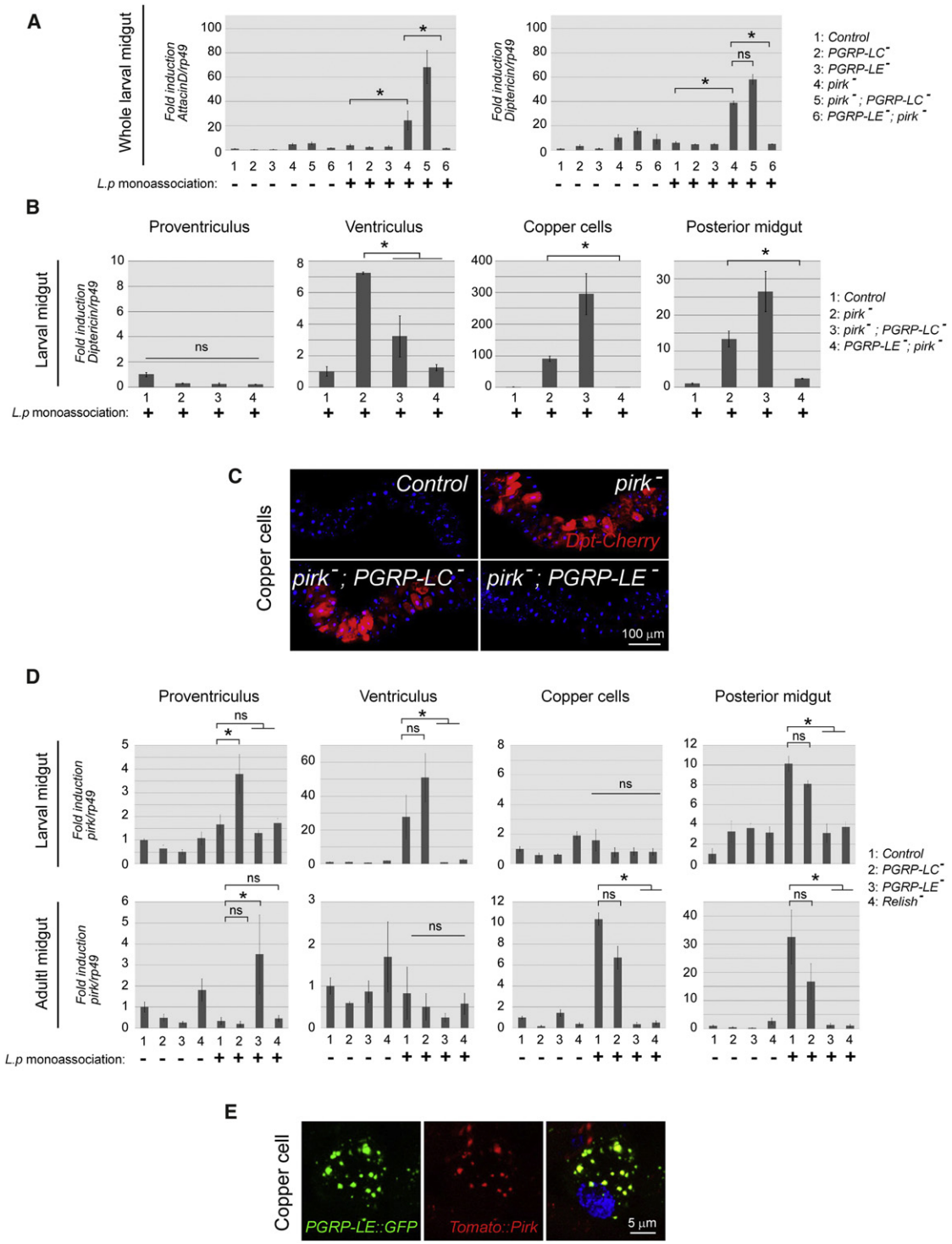


Figure 4. Loss of Immune Tolerance in *Pirk* Mutant Gut Is PGRP-LE-Dependent

(A and B) AMP overexpression in *pirk* mutant gut monoassociated with *L. plantarum* requires PGRP-LE but not PGRP-LC.

(C) Loss of immune tolerance, visualized by the expression of the *Dpt-Cherry* reporter gene is restricted to the Cc region of *pirk* mutant gut. Confocal images of *L. plantarum*-associated wild-type and mutant guts. Scale bar is 100 μm.

(D) *L. plantarum*-dependent induction of *pirk* requires PGRP-LE in both larval and adult guts.

(E) PGRP-LE: GFP colocalizes with pirk::Tomato. Confocal images of a Copper cell from an *Ecc*-infected *pgrp-PGRP-LE::GFP*; *ubi-pirk::Tomato* larval gut. Pictures were taken 24 hr post infection. Scale bar is 5 μm. For (A), (B), and (D), mRNA levels in axenic control flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histograms correspond to the mean value ± SD of three experiments. Values indicated by symbols (*) are statistically significant ($p < 0.05$). ns: not significantly different. See also Figure S3.

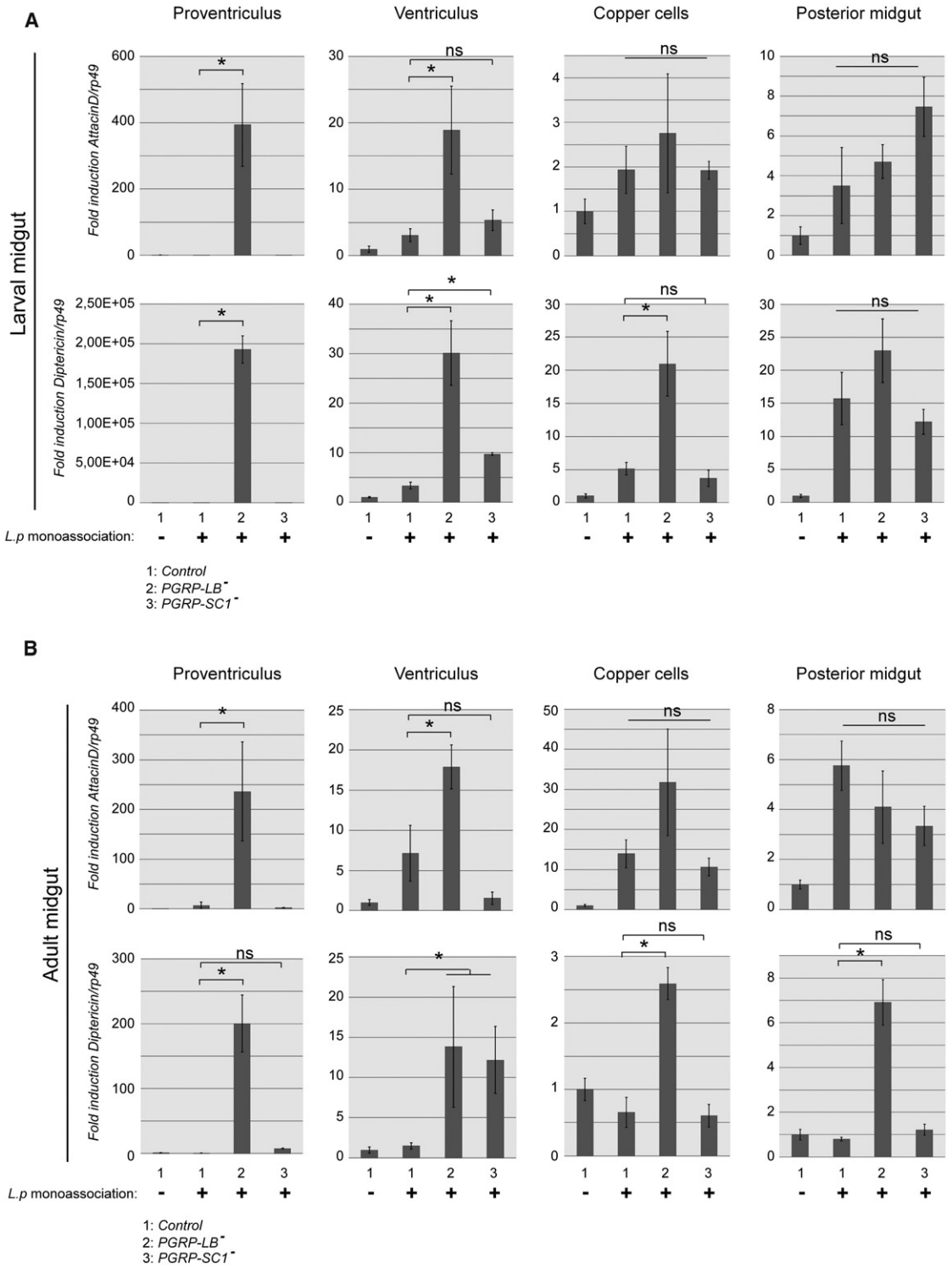


Figure 5. Amidase PGRPs Prevent Excessive IMD Activation in *L. plantarum*-Colonized Guts

(A and B) *L. plantarum*-triggered *Attacin-D* and *Diptericin* mRNA expressions are shown in larval (A) and adult (B) midgut domains. mRNA levels in axenic control flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histogram corresponds to the mean value ± SD of three experiments. Values indicated by symbols (*) are statistically significant ($p < 0.05$). ns: not significantly different. See also Figure S4.

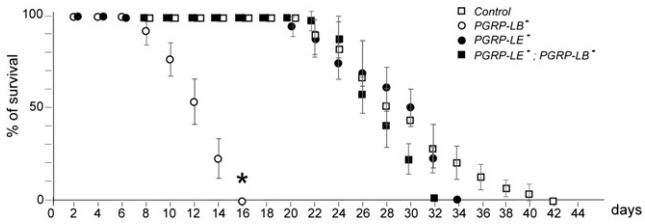


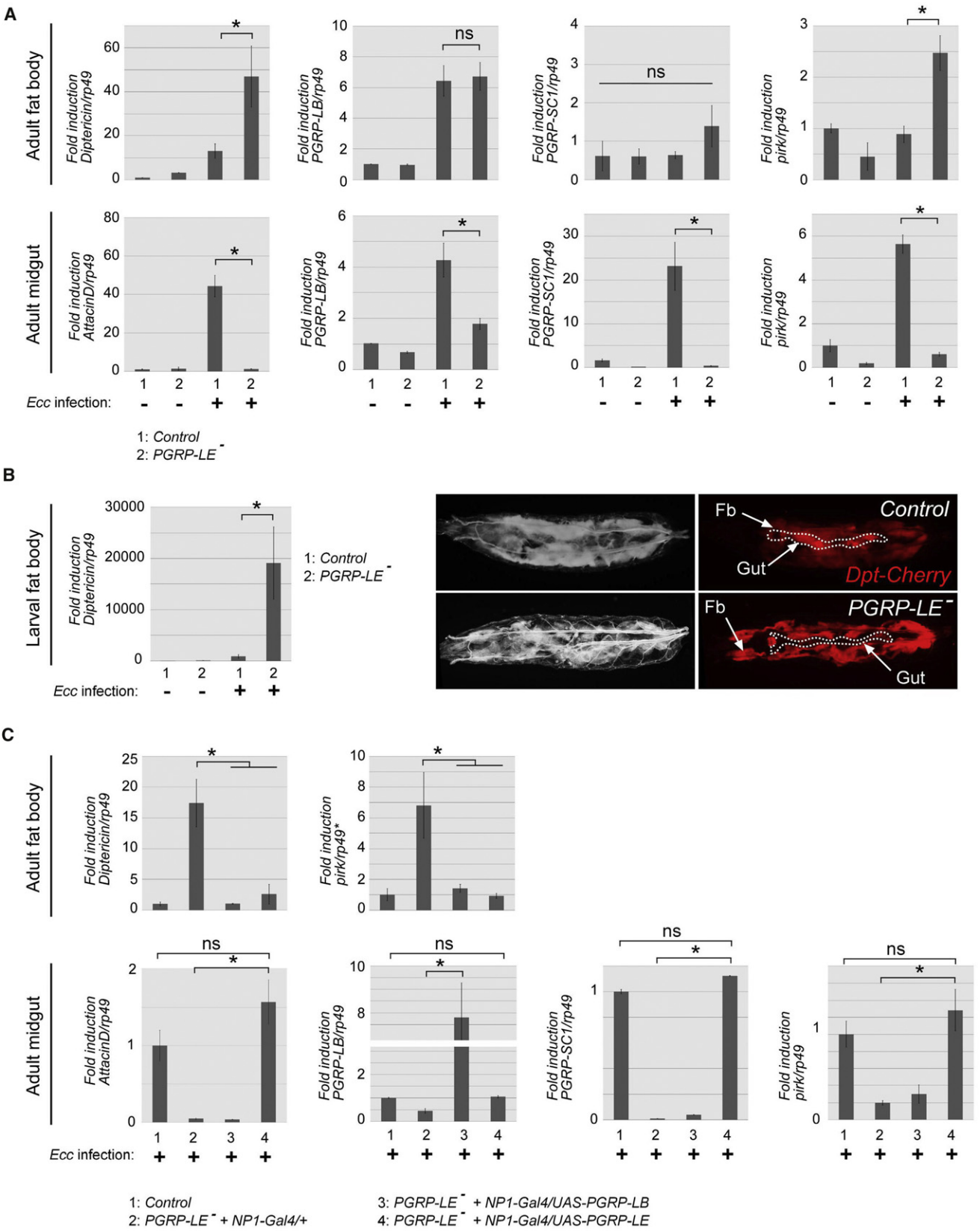
Figure 6. PGRP-LB Mutant Lethality Is Suppressed by Removing PGRP-LE Function

Survival curves of *Ecc* orally infected flies revealing that *PGRP-LB* mutant susceptibility can be partially suppressed by removing PGRP-LE function. Each survival curve corresponds to seven independent experiments with ten flies for each experiment. Survival curves of PGRP-LB only and PGRP-LE, PGRP-LB double mutant are statistically significantly different (*).

PGRP-LE was sufficient to restore wild-type level of *Dpt* expression in the fat body of *Ecc*-fed *PGRP-LE* mutants (Figures 7C and S5D). The fact that the simple gut overexpression of PGRP-LB was sufficient to rescue the *PGRP-LE* mutant phenotype indicates that PGRP-LB is probably the main PGRP-LE-dependent effector in this process. Altogether, these results suggest that by regulating gut expression of immune regulators and especially PGRP-LB, PGRP-LE ultimately prevents systemic immune activation after bacteria ingestion.

DISCUSSION

The present data are consistent with a model in which PGRP-LE functions as a gut master bacteria sensor, which by recognizing a unique bacterial cell wall component, PGN, is either providing a balanced immune response to infectious bacteria or mounting a protective immune tolerance to microbiota. Mammalian PGRP orthologs are also implicated in controlling gut-bacteria interactions (Royet et al., 2011; Saha et al., 2010). It should however be emphasized that while mammalian PGRPs, which are rather immune effectors than signaling regulators, directly impact on microbiota establishment and composition, PGRP-LE acts more downstream by limiting the damaging effects associated with the permanent presence of commensals, with no detectable effects on the gut flora itself (Dziarski and Gupta, 2006; Kashyap et al., 2011). Results from this work and others rather suggest that PGRP-LE exerts in *Drosophila* a function that more resembles that of Nucleotide-binding-oligomerisation domain 2 (NOD2) in mammals. Indeed, NOD2 recognizes bacterial muramyl dipeptide and is regarded as a pivotal sensor molecule of the intestinal barrier. For both PGRP-LE and NOD2, their intracellular detection of PGN regulates NF-κB activation via, respectively, the IMD adaptor protein or its mammalian ortholog RIP2. In addition, detection of intracellular bacteria such as *Listeria monocytogenesis by* NOD2 in mammalian cells and by PGRP-LE in *Drosophila* hemocytes triggers an autophagic program that is independent of both RIP and IMD (Travassos et al., 2010; Yano et al., 2008). It should however be mentioned that in some PGN-induced arthritis mouse models, both NOD2 and PGLYRP-2 play interdependent roles in promoting chemokine production (Saha et al., 2009). Our results show that PGRP-LE-mediated gut response after ingestion of infectious bacteria is shifted toward AMP response while gut recolonization by commensals preferentially induces production of negative regulators. We propose a model in which integration of two parameters, bacterial loads and duration of IMD activation, will translate into differential IMD pathway outputs. While transient contact between gut epithelium and high loads of infectious bacteria that are not able to persist in the gut (such as *Ecc*) will trigger AMP production, durable interaction between low amounts of recolonizing gut-associated bacteria will rather provoke IMD-dependent production of negative regulators such as amidases or pirk. This constant but moderate IMD pathway activation will not be strong enough to induce AMP production, which otherwise would alter gut microbiota, but sufficient to induce high levels of IMD negative regulators, establishing immune tolerance toward microbiota. Our results also highlighted the strong regionalization of the gut immune response, which is globally conserved between larval and adult stages, although subtle differences have been uncovered. This suggests that different domains along the gut perform different roles in setting up an ad hoc response to bacteria. It is for example interesting that pirk-mediated tolerance to the microbiota seems to specifically take place in the Cc region while amidase production upon *L. plantarum* recolonization is highest in Cc and Pmg. The different responsiveness of the gut regions to bacteria could reflect the existence of prepatterning of the different regions that would be acquired by the expression of "permissive" genes such as *dGATAe* (Senger et al., 2006). It could also be due to the nonuniform bacterial distribution in the gut, as observed upon ingestion or colonization, which would imply that the different gut domains are in contact with variable quantities of bacteria. However, although ingested bacteria have different distributions along the adult and larval midguts, immune responses in the different gut domains are very similar at both developmental stages. This suggests that it is not the bacteria themselves but rather the immune elicitor(s) they release that are the main trigger of the gut immune response. Consistently, posterior domains with no detectable live bacteria are highly responsive to bacteria ingestion. In addition, TCT feeding mimicks immune activation due to intact bacteria. The gut immune regionalization could also reflect qualitative and quantitative differences in the expression patterns of PGRP-LE and PGRP-LC. It is relevant that the differential PGRP receptor requirements are well correlated with the respective expression levels of *PGRP-LC* and *PGRP-LE*, since the *PGRP-LC/LE* mRNA ratios are highest in both the Pv and the fat body, the two tissues in which IMD activation is almost completely PGRP-LC dependent (Figure S1D). The respective roles for PGRP-LE and PGRP-LC in the different gut domains is intriguing and, in some respects, unexpected. For simplicity, we will reason that the IMD pathway in the gut is only triggered by TCT. It is clear that TCT recognition in the Pv is mediated by PGRP-LC, a result in accordance with data from the literature showing that PGRP-LCx and PGRP-LCa cooperate to detect TCT extracellularly (Chang et al., 2006). In this respect, the Pv behaves similarly to the fat body. This could be explained by the fact that both tissues are needed to react promptly to PGN. In the case of the fat body, PGN traces in the hemolymph could reflect the presence of infectious bacteria that need to be eliminated and therefore require immediate IMD pathway activation. Since the Pv is the first midgut domain to encounter bacteria, we propose that it


A
Adult fat body
Fold induction Diptericin/rp49
Fold induction PGRP-LB/rp49
Fold induction PGRP-SC1/rp49
Fold induction pirk/rp49
Adult midgut
Fold induction AttacinD/rp49
Fold induction PGRP-LB/rp49
Fold induction PGRP-SC1/rp49
Fold induction pirk/rp49
ns
Ecc infection: - - + +
1: Control
2: PGRP-LE-
B
Larval fat body
Fold induction Diptericin/rp49
Ecc infection: - - + +
1: Control
2: PGRP-LE-
Fb
Gut
Control
Dpt-Cherry
PGRP-LE-
C
Adult fat body
Fold induction Diptericin/rp49
Fold induction pirk/rp49*
Adult midgut
Fold induction AttacinD/rp49
Fold induction PGRP-LB/rp49
Fold induction PGRP-SC1/rp49
Fold induction pirk/rp49
Ecc infection: + + + +
1: Control
2: PGRP-LE- + NP1-Gal4/+
3: PGRP-LE- + NP1-Gal4/UAS-PGRP-LB
4: PGRP-LE- + NP1-Gal4/UAS-PGRP-LE

acts as a sentinel tissue, which by detecting PGN in the first place eventually conveys a signal to the rest of the body. In the Pv, PGRP-LE acts negatively on IMD pathway activation. While it is well documented that constant IMD pathway activation can be deleterious, and that the fly has engineered processes to dampen IMD signaling, PGRP-LE has so far never been implicated in such negative feedback (Bischoff et al., 2006; Maillet et al., 2008; Paredes et al., 2011). It remains to be understood how TCT detection by either PGRP-LC or PGRP-LE triggers opposite outputs on the IMD pathway in this domain. The mode of TCT recognition by PGRP-LE and PGRP-LC in the Vtr is quite different from that in the Pv and is also intriguing. If either PGRP-LE or PGRP-LC is removed, IMD pathway activation by bacteria or by TCT is abolished indicating that the absence of one receptor cannot be compensated by the other. In others words, PGRP-LE and PGRP-LC behave in the Vtr as if they were two subunits of a single TCT receptor. None of the available models for PGRP-LE and PGRP-LC modes of action could account for this result. Finally, IMD pathway activation in the Cc and Pmg is uniquely dependent on PGRP-LE. These posterior regions which, in larvae, are not in contact with bacteria, are probably activated through a PGRP-LE-mediated intracellular detection of TCT released by bacteria resident in anterior domains. Finally, our results demonstrate the importance of PGN sensing by PGRP-LE in the gut to restrict to this organ the activation of an energy-consuming immune response. We show that, by inducing the local production of amidase with PGN-cleaving properties, PGRP-LE prevents PGN diffusion into the hemolymph, a transfer that would in turn activate fat body IMD pathway activation in a PGRP-LC-dependent manner. This burst of PGRP-LE-dependent amidase also prevents lethality due to permanent IMD pathway activation in the gut.

Altogether our data show that PGRP-LE is the master bacteria sensor in the intestinal tract and that by sensing bacteria-derived PGN, it adjusts the level of NF-κB signaling to the nature of the bacteria that are present in the gut. Given the conserved role of PGN as an elicitor of immune responses (Chaput and Boneca, 2007) and its ability to translocate from the mammalian gut to neutrophils in the bone marrow (Clarke et al., 2010), we expect that our results could have implications in other metazoans, including vertebrates.

EXPERIMENTAL PROCEDURES

Bacterial Strains

The following microorganisms were used: *Erwinia carotovora carotovora 15* 2141, *Erwinia carotovora carotovora 15 pOM1-GFP*, *Lactobacillus plantarum*^{WJL}.

Drosophila melanogaster Strains and Maintenance

Drosophila yw are used as controls. *PGRP-LE*^{112} is a loss-of-function mutant partially removing the *PGRP-LE* locus (Takehana et al., 2004). *PGRP-LC*^{DE12} is a complete deletion of the *PGRP-LC* locus (Gottar et al., 2002). Other strains used in this work are: *Dpt-Cherry* (Charroux and Royet, 2009), *UAS-PGRP-LB::YFP* (Gendrin et al., 2009), *Relish*^{E20} (Hedengren et al., 1999), *pirk*^{EY00723} (Lhocine et al., 2008), *pgrp-le-PGRP-LE::GFP* (this work), *Ubi-Tomato::Pirk* (a gift from F.Leulier), *UAS-PGRP-LC::GFP* (Schmidt et al., 2008), *PGRP-LB*$^{\Delta}$ and *PGRP-SC*$^{\Delta}$ KO lines (Paredes et al., 2011), *UAS-PGRP-LE* (this work), and *NP1-Gal4*. Fly stocks were raised at 25°C on a yeast/cornmeal medium supplemented with propionic acid (CARLOERBA, cat. #409553). For antibiotic treatment, standard medium was supplemented with Ampicillin (50 μg/ml), Kanamycin (50 μg/ml), Tetracyclin (10 μg/ml), and Erythromycin (5 μg/ml).

Natural Infection of Larvae and Adults

Overnight bacterial cultures were centrifuged at 4000 g for 10 min at RT and resuspended in fresh Luria-Bertani media. Cells were serially diluted in PBS and the concentration of cells was determined by optical density (OD) measurement at 600 nm. An overnight bacterial culture (200 μl) of *Ecc-15* (OD = 200) or indicated bacteria were directly added on top of feeding third-instar larvae (96 hr after egg laying) into a standard cornmeal-agar medium at 25°C. For adult oral infection, flies were first incubated 2 hr at 29°C in empty vials and then placed in a fly vial with food. The food solution was obtained by mixing a pellet of an overnight culture of bacteria *Ecc-15* (OD = 200) or TCT (0.04 μM) with a solution of 5% sucrose (50/50) and added to a filter disk that completely covered the agar surface of the fly vial. Flies were incubated at 25°C for 4 hr or 24 hr. Bromophenol Blue (200 μl) (SIGMA # B8026) at 10 g/l was directly added on top of feeding third-instar larvae.

Monoassociation of Germ-Free Flies

Germ-free embryos laid on standard culture medium by germ-free females were covered with 150 μl of OD = 1 *L. plantarum* suspension. Emerging larvae were allowed to develop on the contaminated media. Feeding third-instar larvae (96 hr after egg laying) were then dissected. For adult studies, since adults emerging from *L.plantarum* monoassociated larvae have a highly variable load of *L.plantarum* (Storelli et al., 2011), we used the following procedure in order to standardize such monoassociation. Five-day-old germ-free females were first incubated 2 hr at 29°C in an empty vial and then placed in a fly vial with food. The food solution was obtained by mixing a culture of bacteria *L.plantarum* (OD = 2) with a solution of 5% sucrose (50/50) and was added to a filter disk that completely covered the agar surface of the fly vial. Flies were incubated at 25°C for 24 hr before dissection.

Bacterial Loads

Bacterial load of surface-sterilized individuals was quantified by plating serial dilutions of lysates obtained from eight individuals (larvae or adults) or eight dissected midguts (from larvae) on nutrient agar plates (MRS for *L.plantarum* and LB plates supplemented with Spectinomycin [100 μg/ml] for *Ecc-GFP*). Homogenization of individuals or tissues was performed using the Precellys 24-tissue homogenizer (Bertin Technologies, France) and 0.75/1 mm glass beads in 800 μl of the appropriate bacterial culture medium. Bacterial loads were analyzed 72 hr and 24 hr post monoassociation in larval and adults flies, respectively.

Figure 7. Gut PGRP-LE Prevents Systemic Immune Response Activation after Oral Infection with *Ecc*

(A) Transcriptional activation of various IMD targets in fat body and gut of adult flies 24 hr post oral infection with *Ecc*. mRNA levels in axenic flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histogram corresponds to the mean value ± SD of nine experiments.

(B) Expression of *Dpt* in fat body of *Ecc* infected larvae, 24 hr post infection. mRNA levels in axenic control flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histogram corresponds to the mean value ± SD of three experiments. Dorsal view of yw;;*Dpt-Cherry* and *yw PGRP-LE*^{112};;*Dpt-Cherry Ecc*-infected third instar larvae. Pictures were taken 24 hr post infection. Fb: fat body.

(C) Expression of UAS-PGRP-LE or PGRP-LB specifically in the gut is sufficient to rescue activation of the systemic response in PGRP-LE mutant flies. Expression of *Dpt* and *pirk* in the fat body and expression of *PGRP-LB*, *PGRP-SC1* and *pirk* in midgut are shown in *Ecc*-infected adult flies, 24 hr post infection. mRNA levels in *Ecc* infected control flies were set to 1, and values obtained with other genotypes were expressed as a fold of this value. Histograms correspond to the mean value ± SD of six experiments. For (A), (B), and (C), values indicated by symbols (*) are statistically significant ($p < 0.05$). ns: not significantly different. See also Figure S5.

Fly Survival Experiments

Adult flies were orally infected every 2 days with a solution of *Ecc-15* (OD = 200) 5% sucrose (50/50). Results are expressed as a percentage of living flies at different time points after the first infection.

Imaging

Larval or adult tissue was dissected in PBS and fixed for 20 min in 4% paraformaldehyde on ice. After several rinses in PBT (PBS + 0.1% Triton X-100), the tissues were mounted in Vectashield (Vector Laboratories) fluorescent mounting medium, with DAPI. Images were captured with a LSM 780 Zeiss confocal microscope.

Quantitative Real-Time PCR

Quantitative real-time PCR analysis was performed as previously described (Charroux and Royet, 2009). The amount of mRNA detected was normalized to control *rp49* mRNA values. Normalized data was used to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.06.002.

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REFERENCES

Aggarwal, K., Rus, F., Vriesema-Magnuson, C., Ertürk-Hasdemir, D., Paquette, N., and Silverman, N. (2008). Rudra interrupts receptor signaling complexes to negatively regulate the IMD pathway. PLoS Pathog. *4*, e1000120.

Basset, A., Khush, R.S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J.A., and Lemaitre, B. (2000). The phytopathogenic bacteria Erwinia carotovora infects Drosophila and activates an immune response. Proc. Natl. Acad. Sci. USA *97*, 3376–3381.

Bischoff, V., Vignal, C., Duvic, B., Boneca, I.G., Hoffmann, J.A., and Royet, J. (2006). Downregulation of the Drosophila immune response by peptidoglycan-recognition proteins SC1 and SC2. PLoS Pathog. *2*, e14.

Chandler, J.A., Lang, J.M., Bhatnagar, S., Eisen, J.A., and Kopp, A. (2011). Bacterial communities of diverse Drosophila species: ecological context of a host-microbe model system. PLoS Genet. *7*, e1002272.

Chang, C.I., Ihara, K., Chelliah, Y., Mengin-Lecreulx, D., Wakatsuki, S., and Deisenhofer, J. (2005). Structure of the ectodomain of Drosophila peptidoglycan-recognition protein LCa suggests a molecular mechanism for pattern recognition. Proc. Natl. Acad. Sci. USA *102*, 10279–10284.

Chang, C.I., Chelliah, Y., Borek, D., Mengin-Lecreulx, D., and Deisenhofer, J. (2006). Structure of tracheal cytotoxin in complex with a heterodimeric pattern-recognition receptor. Science *311*, 1761–1764.

Chaput, C., and Boneca, I.G. (2007). Peptidoglycan detection by mammals and flies. Microbes Infect. *9*, 637–647.

Charroux, B., and Royet, J. (2009). Elimination of plasmatocytes by targeted apoptosis reveals their role in multiple aspects of the Drosophila immune response. Proc. Natl. Acad. Sci. USA *106*, 9797–9802.

Charroux, B., and Royet, J. (2012). Gut-microbiota interactions in non-mammals: what can we learn from Drosophila? Semin. Immunol. *24*, 17–24.

Choe, K.M., Werner, T., Stöven, S., Hultmark, D., and Anderson, K.V. (2002). Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in Drosophila. Science *296*, 359–362.

Choe, K.M., Lee, H., and Anderson, K.V. (2005). Drosophila peptidoglycan recognition protein LC (PGRP-LC) acts as a signal-transducing innate immune receptor. Proc. Natl. Acad. Sci. USA *102*, 1122–1126.

Clarke, T.B., Davis, K.M., Lysenko, E.S., Zhou, A.Y., Yu, Y., and Weiser, J.N. (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat. Med. *16*, 228–231.

Dziarski, R., and Gupta, D. (2006). Mammalian PGRPs: novel antibacterial proteins. Cell. Microbiol. *8*, 1059–1069.

Ferrandon, D., Imler, J.L., Hetru, C., and Hoffmann, J.A. (2007). The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. Nat. Rev. Immunol. *7*, 862–874.

Garrett, W.S., Gordon, J.I., and Glimcher, L.H. (2010). Homeostasis and inflammation in the intestine. Cell *140*, 859–870.

Gendrin, M., Welchman, D.P., Poidevin, M., Hervé, M., and Lemaitre, B. (2009). Long-range activation of systemic immunity through peptidoglycan diffusion in Drosophila. PLoS Pathog. *5*, e1000694.

Gottar, M., Gobert, V., Michel, T., Belvin, M., Duyk, G., Hoffmann, J.A., Ferrandon, D., and Royet, J. (2002). The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature *416*, 640–644.

Ha, E.M., Oh, C.T., Bae, Y.S., and Lee, W.J. (2005). A direct role for dual oxidase in Drosophila gut immunity. Science *310*, 847–850.

Ha, E.M., Lee, K.A., Park, S.H., Kim, S.H., Nam, H.J., Lee, H.Y., Kang, D., and Lee, W.J. (2009a). Regulation of DUOX by the Galphaq-phospholipase Cbeta-Ca2+ pathway in Drosophila gut immunity. Dev. Cell *16*, 386–397.

Ha, E.M., Lee, K.A., Seo, Y.Y., Kim, S.H., Lim, J.H., Oh, B.H., Kim, J., and Lee, W.J. (2009b). Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in drosophila gut. Nat. Immunol. *10*, 949–957.

Hedengren, M., Asling, B., Dushay, M.S., Ando, I., Ekengren, S., Wihlborg, M., and Hultmark, D. (1999). Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol. Cell *4*, 827–837.

Kallio, J., Leinonen, A., Ulvila, J., Valanne, S., Ezekowitz, R.A., and Rämet, M. (2005). Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells. Microbes Infect. *7*, 811–819.

Kaneko, T., Goldman, W.E., Mellroth, P., Steiner, H., Fukase, K., Kusumoto, S., Harley, W., Fox, A., Golenbock, D., and Silverman, N. (2004). Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the Drosophila IMD pathway. Immunity *20*, 637–649.

Kaneko, T., Yano, T., Aggarwal, K., Lim, J.H., Ueda, K., Oshima, Y., Peach, C., Erturk-Hasdemir, D., Goldman, W.E., Oh, B.H., et al. (2006). PGRP-LC and PGRP-LE have essential yet distinct functions in the drosophila immune response to monomeric DAP-type peptidoglycan. Nat. Immunol. *7*, 715–723.

Kashyap, D.R., Wang, M., Liu, L.H., Boons, G.J., Gupta, D., and Dziarski, R. (2011). Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems. Nat. Med. *17*, 676–683.

Kleino, A., Myllymäki, H., Kallio, J., Vanha-aho, L.M., Oksanen, K., Ulvila, J., Hultmark, D., Valanne, S., and Rämet, M. (2008). Pirk is a negative regulator of the Drosophila Imd pathway. J. Immunol. *180*, 5413–5422.

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J.M., and Hoffmann, J.A. (1995). A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. Proc. Natl. Acad. Sci. USA *92*, 9465–9469.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., and Hoffmann, J.A. (1996). The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell *86*, 973–983.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., Lee, W.J., Mengin-Lecreulx, D., and Lemaitre, B. (2003). The Drosophila immune system detects

bacteria through specific peptidoglycan recognition. Nat. Immunol. *4*, 478–484.

Lhocine, N., Ribeiro, P.S., Buchon, N., Wepf, A., Wilson, R., Tenev, T., Lemaitre, B., Gstaiger, M., Meier, P., and Leulier, F. (2008). PIMS modulates immune tolerance by negatively regulating Drosophila innate immune signaling. Cell Host Microbe *4*, 147–158.

Liehl, P., Blight, M., Vodovar, N., Boccard, F., and Lemaitre, B. (2006). Prevalence of local immune response against oral infection in a Drosophila/Pseudomonas infection model. PLoS Pathog. *2*, e56.

Lim, J.H., Kim, M.S., Kim, H.E., Yano, T., Oshima, Y., Aggarwal, K., Goldman, W.E., Silverman, N., Kurata, S., and Oh, B.H. (2006). Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. J. Biol. Chem. *281*, 8286–8295.

Maillet, F., Bischoff, V., Vignal, C., Hoffmann, J., and Royet, J. (2008). The Drosophila peptidoglycan recognition protein PGRP-LF blocks PGRP-LC and IMD/JNK pathway activation. Cell Host Microbe *3*, 293–303.

Mellroth, P., Karlsson, J., and Steiner, H. (2003). A scavenger function for a Drosophila peptidoglycan recognition protein. J. Biol. Chem. *278*, 7059–7064.

Mellroth, P., Karlsson, J., Håkansson, J., Schultz, N., Goldman, W.E., and Steiner, H. (2005). Ligand-induced dimerization of Drosophila peptidoglycan recognition proteins in vitro. Proc. Natl. Acad. Sci. USA *102*, 6455–6460.

Mengin-Lecreulx, D., and Lemaitre, B. (2005). Structure and metabolism of peptidoglycan and molecular requirements allowing its detection by the Drosophila innate immune system. J. Endotoxin Res. *11*, 105–111.

Paredes, J.C., Welchman, D.P., Poidevin, M., and Lemaitre, B. (2011). Negative regulation by amidase PGRPs shapes the Drosophila antibacterial response and protects the fly from innocuous infection. Immunity *35*, 770–779.

Rämet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., and Ezekowitz, R.A. (2002). Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. coli. Nature *416*, 644–648.

Royet, J., Gupta, D., and Dziarski, R. (2011). Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. Nat. Rev. Immunol. *11*, 837–851.

Ryu, J.H., Kim, S.H., Lee, H.Y., Bai, J.Y., Nam, Y.D., Bae, J.W., Lee, D.G., Shin, S.C., Ha, E.M., and Lee, W.J. (2008). Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. Science *319*, 777–782.

Saha, S., Qi, J., Wang, S., Wang, M., Li, X., Kim, Y.G., Núñez, G., Gupta, D., and Dziarski, R. (2009). PGLYRP-2 and Nod2 are both required for peptidoglycan-induced arthritis and local inflammation. Cell Host Microbe *5*, 137–150.

Saha, S., Jing, X., Park, S.Y., Wang, S., Li, X., Gupta, D., and Dziarski, R. (2010). Peptidoglycan recognition proteins protect mice from experimental colitis by promoting normal gut flora and preventing induction of interferon-gamma. Cell Host Microbe *8*, 147–162.

Schmidt, R.L., Trejo, T.R., Plummer, T.B., Platt, J.L., and Tang, A.H. (2008). Infection-induced proteolysis of PGRP-LC controls the IMD activation and melanization cascades in Drosophila. FASEB J. *22*, 918–929.

Senger, K., Harris, K., and Levine, M. (2006). GATA factors participate in tissue-specific immune responses in Drosophila larvae. Proc. Natl. Acad. Sci. USA *103*, 15957–15962.

Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I., and Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA *107*, 20051–20056.

Shin, S.C., Kim, S.H., You, H., Kim, B., Kim, A.C., Lee, K.A., Yoon, J.H., Ryu, J.H., and Lee, W.J. (2011). Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science *334*, 670–674.

Storelli, G., Defaye, A., Erkosar, B., Hols, P., Royet, J., and Leulier, F. (2011). Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. Cell Metab. *14*, 403–414.

Takehana, A., Yano, T., Mita, S., Kotani, A., Oshima, Y., and Kurata, S. (2004). Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. EMBO J. *23*, 4690–4700.

Travassos, L.H., Carneiro, L.A., Ramjeet, M., Hussey, S., Kim, Y.G., Magalhães, J.G., Yuan, L., Soares, F., Chea, E., Le Bourhis, L., et al. (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat. Immunol. *11*, 55–62.

Werner, T., Borge-Renberg, K., Mellroth, P., Steiner, H., and Hultmark, D. (2003). Functional diversity of the Drosophila PGRP-LC gene cluster in the response to lipopolysaccharide and peptidoglycan. J. Biol. Chem. *278*, 26319–26322.

Wong, C.N., Ng, P., and Douglas, A.E. (2011). Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ. Microbiol. *13*, 1889–1900.

Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., Ueda, R., Takada, H., Goldman, W.E., Fukase, K., Silverman, N., et al. (2008). Autophagic control of listeria through intracellular innate immune recognition in drosophila. Nat. Immunol. *9*, 908–916.

Zaidman-Rémy, A., Hervé, M., Poidevin, M., Pili-Floury, S., Kim, M.S., Blanot, D., Oh, B.H., Ueda, R., Mengin-Lecreulx, D., and Lemaitre, B. (2006). The Drosophila amidase PGRP-LB modulates the immune response to bacterial infection. Immunity *24*, 463–473.