Immunity
Article

Negative Regulation by Amidase PGRPs Shapes the *Drosophila* Antibacterial Response and Protects the Fly from Innocuous Infection

Juan C. Paredes,[1,3] David P. Welchman,[1,3] Mickaël Poidevin,[2] and Bruno Lemaitre[1,*]
[1]Global Health Institute, Station 19, EPFL, 1015 Lausanne, Switzerland
[2]Centre de Génétique Moléculaire (CGM), CNRS, 91198 Gif-sur-Yvette, France
[3]These authors contributed equally to this work
*Correspondence: bruno.lemaitre@epfl.ch
DOI 10.1016/j.immuni.2011.09.018

SUMMARY

Peptidoglycan recognition proteins (PGRPs) are key regulators of insect immune responses. In addition to recognition PGRPs, which activate the Toll and Imd pathways, the *Drosophila* genome encodes six catalytic PGRPs with the capacity to scavenge peptidoglycan. We have performed a systematic analysis of catalytic PGRP function using deletions, separately and in combination. Our findings support the role of PGRP-LB as a negative regulator of the Imd pathway and brought to light a synergy of PGRP-SCs with PGRP-LB in the systemic response. Flies lacking all six catalytic PGRPs were still viable but exhibited deleterious immune responses to innocuous gut infections. Together with recent studies on mammalian PGRPs, our study uncovers a conserved role for PGRPs in gut homeostasis. Analysis of the immune phenotype of flies lacking all catalytic PGRPs and the Imd regulator Pirk reveals that the Imd-mediated immune response is highly constrained by the existence of multiple negative feedbacks.

INTRODUCTION

Microbial detection is emerging as a multistep process that ultimately requires direct contact between a host pattern-recognition receptor and a microbial molecule. A major issue in the field of innate immunity is to understand the microbial recognition process in tissues such as the gut where mechanisms to differentiate pathogenic infections from beneficial interactions with indigenous microbiota are essential. In this study, we analyzed the role of the six amidase peptidoglycan recognition proteins (PGRPs) of *Drosophila* that are predicted to influence bacterial sensing by their capacity to scavenge peptidoglycan.

Peptidoglycan is a highly complex and essential component of the cell wall of virtually all bacteria. It consists of long glycan chains made of alternating N-acetylglucosamine and N-acetylmuramic acid (MurNAc) residues that are crosslinked to each other by short peptide bridges (Chaput and Boneca, 2007). Peptidoglycan from Gram-negative bacteria differs from most Gram-positive peptidoglycan by the replacement of lysine with meso-diaminopimelic acid (DAP) at the third position in the peptide chain. The polymeric nature of peptidoglycan, as well as its diversity, makes this molecule a unique signature for the host to detect and even differentiate different types of bacteria. Pattern-recognition receptors involved in the recognition of peptidoglycan include PGRPs in insects and NODs in mammals (Royet and Dziarski, 2007). Interestingly, the peptidoglycan polymer can also be processed and degraded by several host enzymes, namely lysozymes and amidase PGRPs, thereby indirectly influencing bacterial sensing by pattern-recognition receptors. The most diverse functional family of peptidoglycan-interacting proteins are the PGRPs that have recently been implicated in the dialogue between microbes and their host in several symbiotic and pathogenic interactions (Anselme et al., 2006; Dziarski and Gupta, 2010; Li et al., 2007; Royet and Dziarski, 2007; Troll et al., 2010; Wang et al., 2009; Yu et al., 2010).

PGRPs are highly conserved from insects to mammals and share a conserved 160 amino acid domain with similarities to the bacteriophage T7 lysozyme, a zinc-dependant amidase that hydrolyzes peptidoglycan (Royet and Dziarski, 2007). Like T7 lysozyme, some PGRPs, referred to as catalytic PGRPs, hydrolyze peptidoglycan by cleaving the amide bond between MurNAc and the peptidic bridge. In contrast, noncatalytic PGRPs bind to peptidoglycan but lack amidase activity because of the absence of key cysteine residues for zinc binding. Noncatalytic PGRPs are crucial for the sensing of bacteria in insects such as *Drosophila*. The *Drosophila* genome encodes seven noncatalytic PGRPs, four of which (PGRP-SA, -SD, -LC, and -LE) mediate bacterial sensing upstream of the Toll and Imd pathways that regulate the production of antimicrobial peptides (AMPs) (Ferrandon et al., 2007). PGRP-SA and PGRP-SD are secreted proteins circulating in the hemolymph that have been shown to activate the Toll pathway in response to the lysine-type peptidoglycan found in most Gram-positive bacteria (Royet and Dziarski, 2007). PGRP-LC acts as a transmembrane receptor upstream of the Imd pathway and is activated by the DAP-type peptidoglycan of Gram-negative bacteria or *Bacillus* (Royet and Dziarski, 2007). Recent studies indicate that both polymeric and monomeric Gram-negative peptidoglycan mediate Imd pathway activation via various PGRP-LC isoforms (Kaneko et al., 2004; Stenbak et al., 2004). Finally, PGRP-LE, a secreted PGRP that binds preferentially to

DAP-type peptidoglycan, functions synergistically with PGRP-LC in both autophagy and Imd pathway activation (Ferrandon et al., 2007; Yano et al., 2008). The *Drosophila* genome also encodes six catalytic PGRPs (PGRP-SC1A, -SC1B, -SC2, -LB, -SB1, and -SB2) that have been less studied. The predicted catalytic activity of amidase PGRPs led to the proposal that they might either modulate the immune response by scavenging peptidoglycan or act as directly antibacterial agents (Mellroth et al., 2003). This catalytic activity has been demonstrated for PGRP-LB, PGRP-SC1B, and PGRP-SB1 (Mellroth et al., 2003; Mellroth and Steiner, 2006; Zaidman-Rémy et al., 2006; Zaidman-Rémy et al., 2011). In the case of PGRP-SC1B and PGRP-LB, this enzymatic activity was shown to be required for their capacity to downregulate the immune response (Mellroth et al., 2003; Mellroth and Steiner, 2006; Zaidman-Rémy et al., 2006; Zaidman-Rémy et al., 2011). Various studies have addressed the in vivo roles of these proteins through RNAi or single mutations. In spite of these studies, no clear picture of the overall role of the amidase PGRPs has emerged, with a role for PGRP-LB in regulation of the Imd pathway (Zaidman-Rémy et al., 2006), conflicting evidence for roles of PGRP-SCs (PGRP-SC1A, -1B, and -SC2) in regulation of the Imd and Toll pathways and of phagocytosis of Gram-positive bacteria (Bischoff et al., 2006; Garver et al., 2006), and thus far no overt phenotype in flies deleted for PGRP-SB1 and SB2 (Zaidman-Rémy et al., 2011). In this study, we have generated *Drosophila* lines deleted for PGRP-LB and the PGRP-SC1A, -SC1B, and -SC2 gene cluster by homologous recombination. By analyzing these mutations singly and in combination, we clarify the functions of this class of PGRPs in the fine-tuning of the *Drosophila* immune response.

RESULTS

A Gene-Deletion Strategy to Address Amidase PGRP Function

Through homologous recombination, we previously obtained a deletion of *PGRP-SB1* and *PGRP-SB2* (referred to as *PGRP-SB$^{\Delta}$*), which showed no immune phenotype and gave no clues as to the function of these two genes (Zaidman-Rémy et al., 2011). This raised the possibility of functional redundancy among the amidase PGRPs. In this study, we have generated further mutant lines deleted for either *PGRP-LB* (referred to as *PGRP-LB$^{\Delta}$*) or the *PGRP-SC* gene cluster (referred to as *PGRP-SC$^{\Delta}$*), with the latter encompassing *PGRP-SC1A*, *PGRP-SC1B*, *PGRP-SC2*, and an uncharacterized gene *CG14743* (Figure S1 available online). To address possible redundancy between amidase PGRPs, we recombined these three deletions to generate double (*PGRP-SC$^{\Delta}$;LB$^{\Delta}$; PGRP-SC$^{\Delta}$;SB$^{\Delta}$; PGRP-LB$^{\Delta}$,SB$^{\Delta}$*) or triple (*PGRP-SC$^{\Delta}$;LB$^{\Delta}$,SB$^{\Delta}$*) deficiency stocks. The triple mutant stock lacks all members of the amidase PGRP family in *Drosophila*. In this study, we present the most important results focusing on the role of amidase PGRPs in systemic immunity (i.e., production of antimicrobial peptide by the fat body) and the gut immune response.

PGRP-LB Is a Negative Regulator of the Imd Pathway

PGRP-LB functions as a negative regulator of the Imd pathway in both local and systemic immune responses (Zaidman-Rémy et al., 2006). *PGRP-LB$^{\Delta}$* flies failed to express PGRP-LB mRNA (data not shown), as anticipated, and were viable and fertile, with no obvious developmental defects. After septic injury with the Gram-negative bacterium *Erwinia carotovora carotovora 15* (*Ecc15*), *PGRP-LB$^{\Delta}$* flies had stronger and more sustained immune response than wild-type flies, as measured by the expression of the antibacterial peptide gene *Diptericin* (*Dpt*), a readout of the Imd pathway (Figure 1A). In contrast to the *PGRP-LB* RNAi phenotype, this *Dpt* expression was maintained in *PGRP-LB$^{\Delta}$* until 2 days post-infection and then declined by 4 days post-infection. An enhanced immune response was also observed when flies were infected with another Gram-negative bacterium, *Enterobacter cloacae* (Figure S2A, left graph). The same phenotype, albeit with more rapid kinetics, was observed after injection of inert DAP-type peptidoglycan, confirming that the increase in immune response was a result of increased stimulation of Imd signaling and not of increased bacterial proliferation (Figures 1B and S2A, middle graph). This conclusion was further supported by the absence of any short-term susceptibility of *PGRP-LB$^{\Delta}$* flies to *Ecc15* septic injury (Figure S2B, top graph).

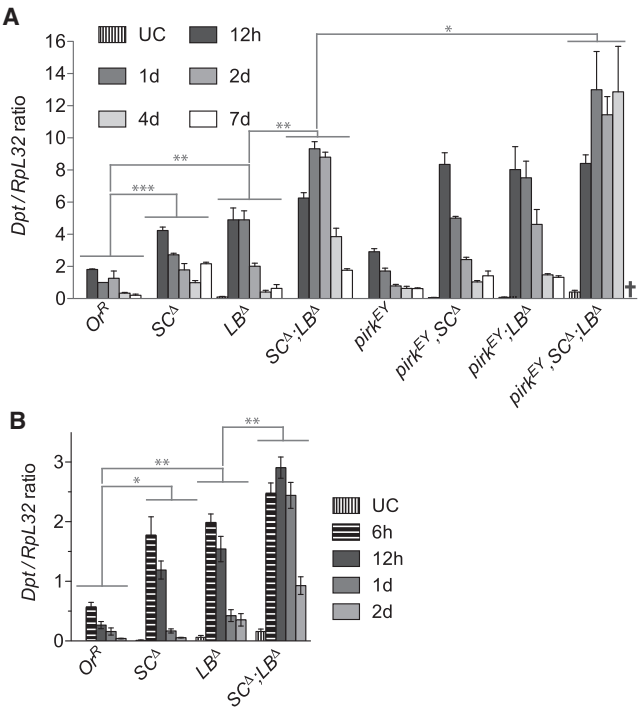

Figure 1. PGRP-LB, PGRP-SCs, and Pirk Contribute to the Downregulation of the Imd Pathway during the Systemic Immune Response

Diptericin (*Dpt*) expression was monitored at different time points in whole flies by RT-qPCR, representing the systemic activation of the Imd pathway. Flies carrying various combinations of amidase PGRP and *pirk* mutations present a higher activation of the Imd pathway compared to wild-type (Oregon^{R}, *Or^{R}*) flies after infection by septic injury with the Gram-negative bacteria *Ecc15* (A), or injection with the Gram-negative peptidoglycan of *E. coli* (B). A cross indicates that data could not be analyzed because many of the flies were dead at this time point. Data are representative of at least three independent experiments (mean + SEM). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ with a Student's t test. *SC$^{\Delta}$: PGRP-SC$^{\Delta}$, LB$^{\Delta}$: PGRP-LB$^{\Delta}$, SC$^{\Delta}$;LB$^{\Delta}$: PGRP-SC$^{\Delta}$;LB$^{\Delta}$, pirk^{EY}: pirk^{EY00723}*.

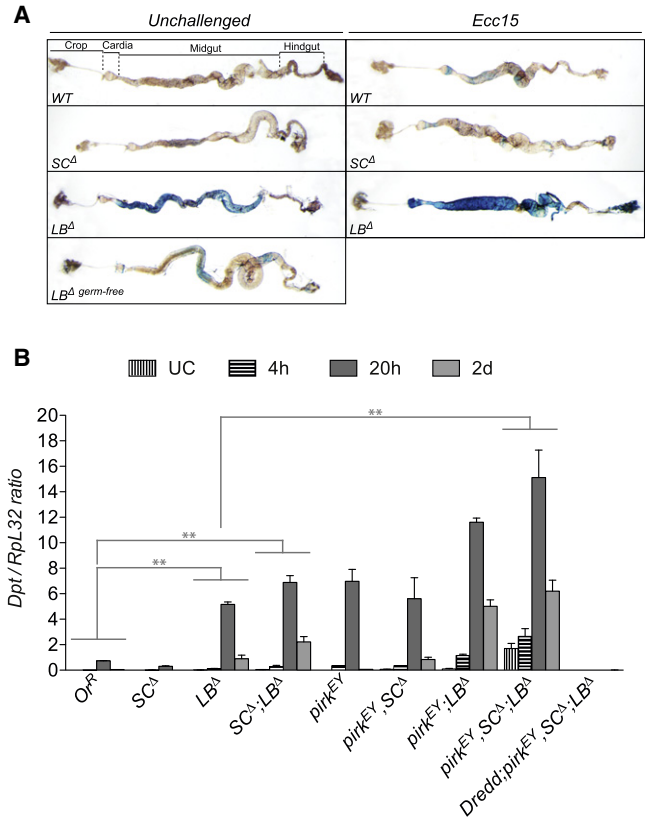

Figure 2. PGRP-LB, PGRP-SCs, and Pirk Contribute to the Downregulation of the Imd Pathway during the Local Immune Response

(A) *Dpt* expression was measured with the β-galactosidase activity (X-gal staining in blue) of *Dpt::LacZ* reporter lines in unchallenged guts (A, left panel) or guts orally challenged (20 hr) with *Ecc15* (A, right panel). *Dpt* is highly induced by gut microbiota (A, left panel) and ingested bacteria (A, right panel) in *PGRP-LB*$^{\Delta}$ compared to the wild-type. *Dpt::LacZ* expression is reduced when *PGRP-LB*$^{\Delta}$ mutant flies are raised in a germ-free environment (*LB*$^{\Delta\ germ\text{-}free}$). Representative images are shown of at least ten dissected guts.

(B) Endogenous *Dpt* expression was monitored by RT-qPCR at different time points after oral infection with *Ecc15*. Data are representative of at least three independent experiments (mean + SEM). *$p < 0.05$ and **$p < 0.01$ with a Student's t test.

The local immune response of the *Drosophila* gut is also mediated by the Imd pathway upon detection of DAP-type peptidoglycan (Zaidman-Rémy et al., 2006). We observed that the gut of *PGRP-LB*$^{\Delta}$ flies showed enhanced expression of *Dpt* in response to an oral infection with *Ecc15*. Using the reporter gene *Dpt-lacZ*, we showed that orally infected *PGRP-LB*$^{\Delta}$ flies expressed *Dpt-lacZ* to a much higher level in the cardia and midgut than the wild-type control (Figure 2A, right panel). This observation was also borne out by quantification of the endogenous *Dpt* transcript (Figure 2B).

A principal role for amidase PGRPs in the gut could be to prevent unnecessary immune responses to commensal microbiota. Indeed Ryu et al. (2008) showed that the basal expression of *PGRP-LB* in the adult midgut is lost in germ-free conditions, suggesting that it is induced in the presence of microbiota to prevent an Imd pathway response. This role was confirmed by the observation that *PGRP-LB*$^{\Delta}$ guts showed substantially higher *Dpt-lacZ* expression than the wild-type control in the absence of any infection (Figure 2A, left panel). Furthermore, in germ-free conditions, this *Dpt-lacZ* expression was reduced, demonstrating that it reflects an unsuppressed immune response to microbiota (Figure 2A, left panel).

PGRP-LB Prevents Systemic Immune Activation after Ingestion of Bacteria

Oral infection with certain Gram-negative bacteria, including *Pseudomonas entomophila*, leads not only to a local but also a systemic fat body immune response (Vodovar et al., 2005). It has been proposed that this systemic reaction to a local infection is mediated by translocation of peptidoglycan fragments across the gut epithelium (Gendrin et al., 2009; Zaidman-Rémy et al., 2006). This was supported by the observation that *PGRP-LB* RNAi flies with reduced amidase activity showed a systemic immune response to oral infection with *Ecc15*, which induced no systemic response in wild-type flies. *PGRP-LB*$^{\Delta}$ mutant flies likewise showed a strong response to oral *Ecc15* infection to a level similar to that observed after infection by septic injury with the same bacteria (Figure 3A). The use of a lacZ reporter gene and RT-qPCR experiments confirmed that the *Dpt* gene was expressed in the fat body of *PGRP-LB*$^{\Delta}$ flies orally infected by *Ecc15* (Figures 3B and S2C). This contrasts sharply with wild-type flies that showed very little systemic *Dpt* expression after oral infection. The same experiment was performed with trans-heterozygous flies carrying one allele of *PGRP-LB*$^{\Delta}$ over a larger deletion removing the *PGRP-LB* gene region (*Df(3R)Exel8153*), with the same result (data not shown). Similarly, the systemic response to oral infection of *PGRP-LB*$^{\Delta}$ flies could be completely suppressed by overexpression of PGRP-LB in the gut (*NP1-Gal4*), in the fat body and hemocytes (*C564-Gal4*), or ubiquitously (*da-Gal4*) (Figure 3C).

Thus, our study confirmed that PGRP-LB is a negative regulator of the Imd pathway response in both epithelia and the fat body of adults and larvae (Supplemental Results and Figure S3).

The PGRP-SC Family Negatively Regulates the Imd Pathway during Systemic Infection

A strain in which *PGRP-SC1A*, *-1B* and *2*, and *CG14743* have been deleted, named *PGRP-SC*$^{\Delta}$, failed to express mRNA for any of the *PGRP-SC* family members (data not shown), as anticipated, and was viable and fertile with no obvious developmental defects. After septic injury with *Ecc15* or injection of DAP-type peptidoglycan, *PGRP-SC*$^{\Delta}$ flies showed a stronger immune response than wild-type controls from 12 hr after infection, to a level similar to that of *PGRP-LB*$^{\Delta}$ flies (Figures 1A, 1B, and S2A, left and middle graphs). This alteration in the immune response did not correlate with any increased short-term susceptibility to this infection (Figure S2B, top graph). To demonstrate that the immune phenotype observed with *PGRP-SC*$^{\Delta}$ was indeed caused by the lack of amidase PGRP-SC, we carried out a rescue experiment. Figure 4 shows that *PGRP-SC*$^{\Delta}$ flies carrying a transgene containing a modified *PGRP-SC* locus lacking *CG14743* (Figure S1) exhibited a wild-type expression of *Dpt*. This rescue experiment demonstrates that the PGRP-SC family plays a similar role to PGRP-LB in negatively regulating the systemic Imd pathway response. These observations are in agreement with those of Bischoff et al. (2006). In contrast to

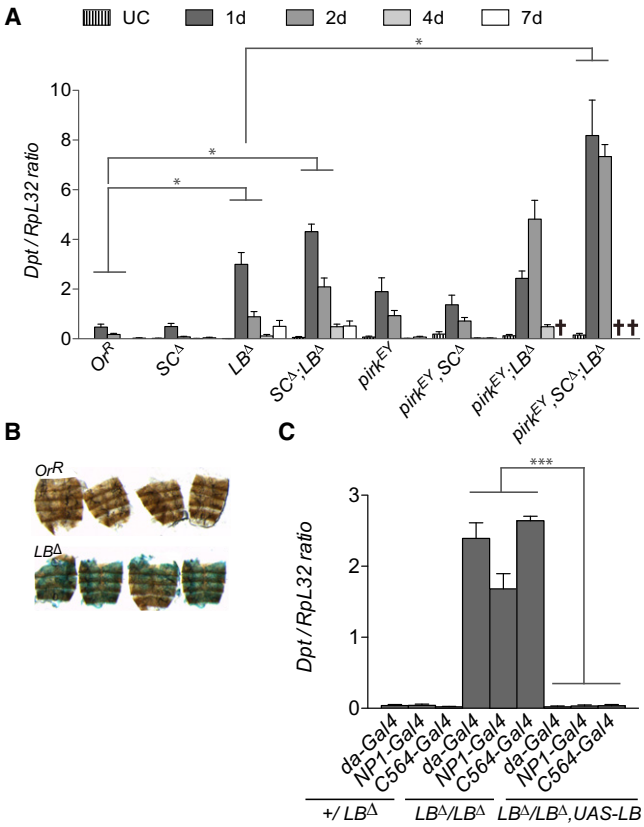

Figure 3. PGRP-LB, PGRP-SCs, and Pirk Prevent the Activation of the Systemic Immune Response after Oral Infection with *Ecc15*

(A) *Dpt* expression was monitored at different time points in whole flies by RT-qPCR, representing the systemic activation of the Imd pathway. Oral infection with *Ecc15* induced strong systemic *Dpt* expression in amidase *PGRP*-deficient flies but not in wild-type flies. The levels measured in this experiment correspond to systemic expression of *Dpt* by the fat body since the contribution of gut *Dpt* expression is negligible (see Figure S2C).

(B) The same enhancement of *Dpt* expression is revealed with the *Dpt::LacZ* reporter line in fly carcasses in the presence (Or^R) or absence (LB^{Δ}) of PGRP-LB. Carcasses of flies were fixed and stained 1 day after the oral infection with *Ecc15*.

(C) The use of ubiquitous (*da-Gal4*), fat body (*C564-Gal4*) and gut (*NP1-Gal4*) Gal4 drivers show that expression of *PGRP-LB* in the whole body, gut, or fat body and hemocytes is sufficient to block the systemic immune response 1 day after oral infection with *Ecc15*. A cross indicates that data could not be analyzed because many of the flies were dead at this time point. Data are representative of at least three independent experiments (mean + SEM). *p < 0.05 and **p < 0.01 with a Student's t test.

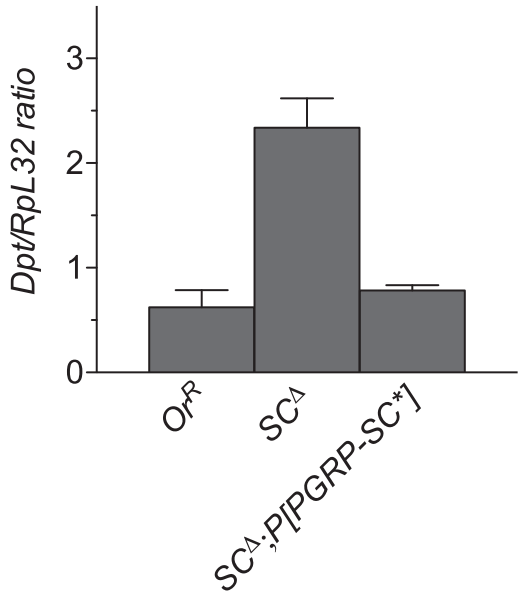

Figure 4. A Transgene, *P[PGRP-SC*]*, Containing the *PGRP-SC* Gene Cluster Devoid of *CG14743* Gene Rescued the PGRP-SC^{Δ} Phenotype

Dpt expression was monitored in whole flies after 1 day of infection by septic injury with *Ecc15* by RT-QPCR. Data are representative of at least three independent experiments (mean + SEM).

a previous report (Garver et al., 2006), we did not detect any effect of the *PGRP-SC*$^{\Delta}$ deletion on Toll pathway activation (See Supplemental Results, Figure S2A, right graph, and Figure S2B, bottom graphs).

Members of the PGRP-SC family are strongly expressed in the gut of adult flies and induced there upon oral infection with *Ecc15* (Buchon et al., 2009b; Werner et al., 2000). We therefore assayed the immune response of *PGRP-SC*$^{\Delta}$ guts to *Ecc15* oral infection. In contrast to *PGRP-LB*$^{\Delta}$, *Dpt* expression in *PGRP-SC*$^{\Delta}$ was similar to or even lower than that in wild-type guts either by RT-qPCR or with the *Dpt-lacZ* reporter gene (Figures 2A and 2B). This was the case in both unchallenged and *Ecc15*-infected conditions. In addition, *PGRP-SC*$^{\Delta}$ flies showed no systemic response to oral infection with *Ecc15* (Figure 3A). Thus, the PGRP-SC family does not appear to have a major role in the regulation of the gut immune response of adult flies or in the systemic response to gut infections. In contrast to the Bischoff et al. (2006) study, which used an RNAi approach, we did not uncover any major role for the PGRP-SC family in the regulation of the gut immune response in adults nor in the systemic response to gut infections at the larval stage (Supplemental Results and Figure S3).

Phenotypic Analysis of Flies Lacking Multiple Amidase PGRPs

We next analyzed the immune phenotype of *PGRP-SC*$^{\Delta}$*;PGRP-LB*$^{\Delta}$ flies (referred to as *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$) to investigate the effect of the absence of multiple amidase PGRP members. After septic injury with *Ecc15*, *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies showed greatly increased *Dpt* expression at 12 and 24 hr postinfection, reflecting the importance of both PGRPs in the regulation of this response (Figure 1A). Strikingly, the *Dpt* expression remained higher in *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies at 2 and 4 days postinfection than the peak *Dpt* expression in wild-type flies. As with the single-mutant strains, this increased response did not reflect an increased bacterial load given that no early susceptibility to infection was observed (Figure S2B, top graph) and a similar increase and extension of the immune response was seen after injection of DAP-type peptidoglycan (Figures 1B and S2A, middle graph).

In contrast to the response to septic injury, no striking effect of *PGRP-SC*$^{\Delta}$ was observed on the response to oral infections. In agreement, *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ guts showed only a modest

increase in *Dpt* expression over *PGRP-LB*$^{\Delta}$ guts in both *Ecc15* infection and unchallenged conditions (Figure 2B). Nevertheless, a slightly stronger and more sustained systemic immune response was observed in *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies orally infected with *Ecc15* (Figure 3A). Overall, our data indicate that PGRP-LB and the PGRP-SC family play overlapping roles in the systemic response but that PGRP-LB makes a greater contribution to gut immunity.

A recent analysis revealed no clear immune phenotype of *PGRP-SB*$^{\Delta}$ deficiency flies, in spite of the strong induction of PGRP-SB1 by infection and its demonstrated amidase activity (Zaidman-Rémy et al., 2011). We successfully generated viable fly lines lacking all amidase PGRPs by recombining *PGRP-SB*$^{\Delta}$ to the two other deficiency stocks. It was our hope that, in combination with the *PGRP-SC*$^{\Delta}$ and/or *LB*$^{\Delta}$, some cryptic phenotype would be detected for *PGRP-SB1* and *-SB2*. However, no consistent difference was observed between the immune responses of *PGRP-LB*$^{\Delta}$ and *PGRP-LB*$^{\Delta}$*,SB*$^{\Delta}$ or *PGRP-SC*$^{\Delta}$ and *PGRP-SC*$^{\Delta}$*;SB*$^{\Delta}$ or *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ and *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$*,SB*$^{\Delta}$ (Figure S4). Thus, the amidases PGRP-SB1 and -SB2 do not play any additional role in the regulation of the Imd pathway and our results leave open the nature of its function.

Loss of Pirk Further Enhances the Immune Responses of PGRP-LB and SC Mutants

Recent studies in *Drosophila* have revealed that multiple levels of regulation are employed to suppress Imd pathway activity. Pirk, a protein interacting with PGRP-LC and regulated by the Imd pathway, has been shown to regulate the Imd pathway receptor and thus participate in the precise control of Imd pathway induction (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). A resolution of the immune response was still observed at late time points in flies deleted for either Pirk or amidase PGRPs (Figures 1A, 2B, and 3A), demonstrating that they still possess the capacity to downregulate the response. We wanted to find out whether the removal of Pirk and amidase PGRPs together would have an effect on the immune response or viability. *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ and *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$*,SB*$^{\Delta}$ flies were indeed viable, although they were not fully fertile and could not be maintained as homozygous stocks, partly because of a low number of viable males. In the response to *Ecc15* septic injury, the addition of *pirk*^{EY} to either *PGRP-SC*$^{\Delta}$ or *PGRP-LB*$^{\Delta}$ alone resulted in a significant enhancement of the *Dpt* expression (Figure 1A). This is to be expected because Pirk modulates the level of the Imd response to a given pool of ligand and not, as do the amidases, the amount of available ligand. However the addition of *pirk*^{EY} to the *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ double mutant resulted not only in a further increase in the early *Dpt* expression but also the maintenance of this level up to 4 days postinfection when flies start to die (see below). Of note, the level of *Dpt* expression was more than 8-fold higher in *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies than wild-type flies at 24 hr (Figure 1A).

After oral infection with *Ecc15*, the addition of *pirk*^{EY} to *PGRP-LB*$^{\Delta}$ results in an enhancement of the immune response locally (Figure 2B) and systemically (Figure 3A). The addition of *pirk*^{EY} to the *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ genotype resulted in a much higher level of *Dpt* expression locally (Figure 2B) and much higher and more persistent expression systemically (Figure 3A) than are ever observed in wild-type flies with standard modes of infection.

As with septic injury, *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies began to die within 4 days of oral infection with *Ecc15*. Although *pirk*^{EY}*,PGRP-SC*$^{\Delta}$ showed the same level of response as *pirk*^{EY} alone, it is important to note that the response of *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ is significantly higher than that of *pirk*^{EY}*;PGRP-LB*$^{\Delta}$, indicating that the PGRP-SC family plays a redundant role in the gut that is hidden, in the case of *Ecc15* infection, by the activity of PGRP-LB or Pirk.

Collectively, our results indicate that Pirk and the amidase PGRPs, PGRP-LB, and the PGRP-SC family strongly limit the immune response to bacteria. Removal of these three "brakes" led to an excessive and indefinite immune response.

Negative Regulators Prevent Lethal Host Immune Responses to Innocuous Infections

A key question concerning the role of amidase PGRPs and Pirk is their significance for the viability of infected flies. To address this question, we assayed the lifespan of the single, double, and triple mutants upon transient oral infection with *Ecc15* at 25°C. *Ecc15*-infected *PGRP-LB*$^{\Delta}$ and *pirk*^{EY} and to a lesser extent *PGRP-SC*$^{\Delta}$ single mutant flies showed reductions in mean lifespan (as much as 20 days for *PGRP-LB*$^{\Delta}$) compared to wild-type Oregon^{R} (Figure 5A). This indeed suggested that suppression of an excessive immune response to transient *Ecc15* infection has a fitness benefit. *PGRP-SB*$^{\Delta}$ flies showed no or only a small decrease in mean lifespan after oral *Ecc15* infection (data not shown). The stronger immune responses to *Ecc15* oral infection of *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies were correlated with a further decrease in the lifespan compared to single-mutant flies (Figure 5A). Strikingly *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies do not simply show an incremental reduction in their lifespan, but rather die rapidly to oral *Ecc15* infection, deceasing by 50% after only 5 days (Figure 5A). In order to verify that these flies were not dying as a result of bacterial accumulation, we dissected guts at several time points after infection and assessed the persistence of *Ecc15* by plating extracts on Luria Broth agar. No significant difference in bacterial persistence was observed between *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$, *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ and wild-type flies (Figure S5A). Of note, we did not see any translocation of *Ecc15* from the gut lumen to the hemolymph in the triple mutant flies (data not shown). Furthermore, these flies were also susceptible to oral infection with dead sonicated *Ecc15* (Figure 5B), demonstrating that it is not bacteria that are killing the fly but rather its own excessive immune response. Surprisingly, *PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ and *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies succumbed even faster upon ingestion of sonicated versus live *Ecc15* (compare Figure 5A with Figure 5B). A plausible explanation of this counterintuitive observation is that sonicated *Ecc15* is more immunostimulatory than live *Ecc15* because of the release and solubilization of peptidoglycan. Importantly, *Dredd*^{1}*;pirk*^{EY}*,SC*$^{\Delta}$*;LB*$^{\Delta}$ and *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$*,Relish*^{E20} flies, with impaired Imd pathway activity due to the presence of the *Dredd* or *Relish* mutations, exhibited an increased lifespan upon oral infection with *Ecc15* compared to *pirk*^{EY}*,PGRP-SC*$^{\Delta}$*;LB*$^{\Delta}$ flies (Figures 5A and S5B). This demonstrates that the lethality is due to the excessive activation of the Imd pathway upon *Ecc15* infection. Additional experiments demonstrate that the lethality and higher immune response in the absence of amidase PGRPs and Pirk are still observed when flies

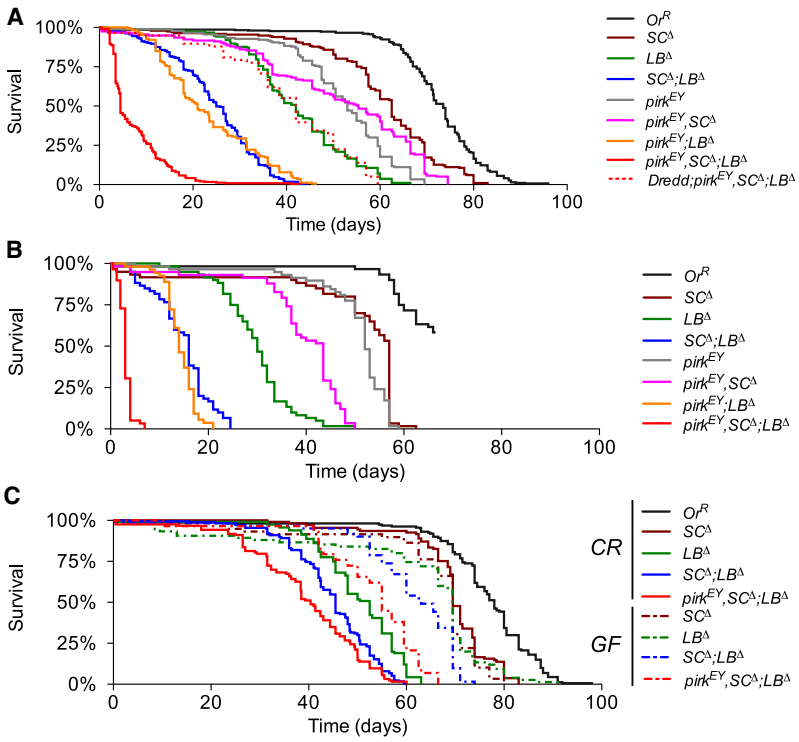

Figure 5. Amidase PGRPs and Pirk Enhance Fly Fitness in Response to Innocuous Infections

(A) Survival analysis of flies orally infected with *Ecc15* reveals a marked decrease in the survival rate of *PGRP-SC$^\Delta$;LB$^\Delta$* and *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies ($p < 0.001$).

(B) Mortality rates of flies orally infected with sonicated *Ecc15* indicate that the cause of the rapid death of *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* is the strong immune activation rather than bacterial proliferation in the gut (see also Figure S6). In agreement, the use of the *Dredd* mutation indicates that this susceptibility is mostly due to excessive activation of the Imd pathway ($p < 0.001$) (A).

(C) Lifespan analysis of unchallenged flies reveals an increase in mortality rate of *PGRP-SC$^\Delta$;LB$^\Delta$* and *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies that can be partially rescued in germ-free conditions ($p < 0.001$). Each survival curve corresponds to at least three independent experiments of 3 tubes of 20 flies each. p values were calculated with the Log-rank and Wilcoxon test. A detailed statistical analysis is shown in Table S1.

are raised in germ-free conditions or on a different medium (Supplemental Results and Figure S6). Thus, the lethality of *PGRP-SC$^\Delta$;LB$^\Delta$* and *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* mutants upon *Ecc15* infection is not an indirect consequence of a change in the microbiota composition and is not influenced by the medium composition.

We next monitored the lifespan of *PGRP-SC$^\Delta$;LB$^\Delta$* and *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* mutant flies in unchallenged conditions at 25°C. Figure 5C shows that double- or triple-mutant flies lacking several negative regulators showed a marked reduction of lifespan of more than 30 days for *PGRP-SC$^\Delta$;LB$^\Delta$* and *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$*. These results indicate that amidase PGRPs and Pirk contribute to the fitness of flies in the absence of infection. We observed that the survival rate of *PGRP-SC$^\Delta$;LB$^\Delta$* and *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies in unchallenged conditions was variable and correlated with the frequency at which flies were flipped on to freshly autoclaved medium. This suggested that in the absence of negative regulators, chronic activation of the Imd pathway by the indigenous flora or bacteria ingested with their food is deleterious to the fly. Supporting this hypothesis, we found that germ-free *PGRP-SC$^\Delta$;LB$^\Delta$* or *pirk^{EY},PGRP-SC$^\Delta$;LB* flies had substantially longer life spans than their conventionally raised counterparts (Figure 5C).

Lack of Negative Imd Pathway Regulation in Amidase *PGRP* and *pirk* Mutants Causes a Rupture of Gut Homeostasis

The survival analyses described above underline the importance of negative regulation of the Imd pathway in fly fitness. They also raise the question of what causes the reduced lifespan observed in *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies. Several reports have recently underlined a link between abnormal proliferative activities of intestinal stem cells and fly health (Biteau et al., 2010; Buchon et al., 2009a). Given the shorter lifespan of *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies, it was plausible to consider that a lack of negative Imd regulation could lead to cell death and increased epithelium renewal. To test this hypothesis, we stained guts with an anti-phosphohistone H3 (anti-PH3) antibody that marks dividing stem cells. As previously reported, a low number of PH3-positive cells were detected in the gut of unchallenged wild-type flies while the number of mitotic cells increased upon *Ecc15* infection, indicative of higher epithelium renewal (Figure 6A). Strikingly, the level of epithelium renewal, as evidenced by the number of mitotic cells along the midgut, was already very high in *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies in the absence of infection, approaching the level seen in infected wild-type guts. The mitotic index of *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies then only doubles from unchallenged to *Ecc15* oral infection conditions, suggesting that the level of epithelium renewal in the triple mutant was approaching the limit of cells available to undergo mitosis. Recent studies have demonstrated that epithelium renewal is stimulated by the release of a secreted ligand, Upd3, from stressed enterocytes which activates the JAK-STAT pathway in intestinal stem cells to promote both their division and differentiation, establishing a homeostatic regulatory loop (Buchon et al., 2009a; Jiang et al., 2009). Consistent with this, we observed a higher level of JAK-STAT activity in the guts of unchallenged *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies as monitored by the expression of *upd3* and the JAK-STAT target gene *Socs36E* (Figures 6B and 6C). The presence of the *Dredd* mutation fully suppressed both the high mitotic count (Figure 6A) and the elevated JAK-STAT activity (Figures 6B and 6C) observed in *pirk^{EY},PGRP-SC$^\Delta$;LB$^\Delta$* flies in the absence of infection, demonstrating that excessive Imd pathway activation is required for the gut damage which leads to epithelium renewal in these flies. We concluded that tight control of Imd pathway activity by amidase PGRPs and Pirk prevents the chronic and deleterious stimulation of intestinal stem cell activity by microbiota and ingested bacteria.

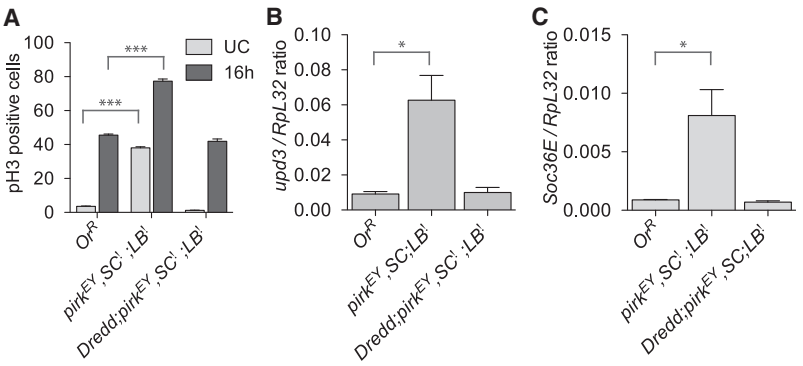

Figure 6. Amidase PGRPs and Pirk Protect the Gut from a Damaging Immune Response

(A) Phospho-Histone-3-positive cells were counted in the dissected guts of wild-type, $pirk^{EY}$,$PGRP\text{-}SC^{\Delta}$;LB^{Δ}, and *Dredd*;$pirk^{EY}$,$PGRP\text{-}SC^{\Delta}$;LB^{Δ} flies in unchallenged conditions or after 16 hr of *Ecc15* oral infection.

(B) JAK-STAT pathway activation was measured by the expression of *Socs*36E and *upd3* in unchallenged dissected guts. Data are representative of at least three independent experiments (mean + SEM). *p < 0.05 and ***p < 0.001 with a Student's t-test (A) or Mann Withney test (B and C).

DISCUSSION

In this study, we performed a systematic analysis of amidase PGRP function in *Drosophila*. Using three independent deletions, we were able to remove the three amidase PGRP families. Previous studies using an RNAi approach have suggested that PGRP-LB and the PGRP-SC family are required for fly viability (Bischoff et al., 2006; Zaidman-Rémy et al., 2006). In contrast, the use of null mutation lines reveals that $PGRP\text{-}LB^{\Delta}$ and SC^{Δ} flies are viable under laboratory conditions. Furthermore, we were surprised to find that viable flies lacking the whole set of amidase PGRPs could be obtained, albeit at lower frequency than expected. This indicates that amidase PGRPs do not play any essential role in *Drosophila* development.

The first aim of our project was to clarify the respective roles of PGRP-LB and the PGRP-SC family in the immune response. Our study confirms that PGRP-LB negatively regulates the Imd pathway both in barrier epithelia and in the fat body in agreement Zaidman-Rémy et al. (2006). Our present study uncovers a new role of PGRP-LB in downregulating the Imd pathway in the adult gut by commensals under unchallenged conditions. PGRP-SC1 and -SC2 has been reported to have conflicting roles in regulation of the Imd and Toll pathways and in the phagocytosis of Gram-positive bacteria (Bischoff et al., 2006; Garver et al., 2006). The use of this deletion reveals a narrower role for this family of PGRP. Indeed, we observed no major impact of the PGRP-SC deletion on the activity of either the Toll pathway or local Imd pathway activity in response to oral infection, in contrast with previous studies. Our study reveals instead that the PGRP-SC family negatively regulates the Imd pathway during systemic infection and synergizes with PGRP-LB and Pirk in the systemic immune response to ingested bacteria. We have not addressed the individual contribution of each of the three PGRP-SC isoforms, PGRP-SC1A, PGRP-SC1B, and PGRP-SC2, to these phenotypes. PGRP-SC1A and PGRP-SC1B have probably arisen from a recent duplication given that the two genes differ only by a synonymous mutation, and because their expression is confined to the gut it seems likely that PGRP-SC2 might be responsible for the higher immune activation during systemic infection. Our studies leave open the possibility that PGRP-SC1A and -SC1B have additional functions in the gut such as the digestion of peptidoglycan or regulation of commensals.

The observation that the contribution of the PGRP-SC family to the local immune response is largely masked by PGRP-LB is intriguing. The phenotype observed could be explained if PGRP-LB were capable of fully processing ingested peptidoglycan while the PGRP-SC family members had a lower activity because of a more restricted expression pattern and/or different enzymatic properties. Biochemical studies on PGRP-LB, PGRP-SB1, and to a lesser extent the PGRP-SC family indicate that amidase PGRPs differ in their enzymatic efficiencies and substrate specificities (Mellroth et al., 2003; Zaidman-Rémy et al., 2006; Zaidman-Rémy et al., 2011). Further studies should explore the enzymatic characteristics of PGRP-SC1A, SC1B, and PGRP-SC2. Nevertheless, it is possible that PGRP-SC has additional independent functions that may be revealed by the use of specific bacterial strains.

The involvement of several amidase PGRPs in the downregulation of the Imd pathway is interesting. Experimental and modeling analyses have suggested that one advantage of multiple layers of negative regulation is to reduce the noise inherent in the system, by limiting oscillation of signaling activity (Mengel et al., 2010). Thus, the involvement of multiple amidase PGRPs in the control of Imd signaling would reinforce the tight control of this pathway and make it less sensitive to variation. Moreover, differences in the expression pattern of amidase PGRPs in different gut regions, along with the superimposition of inducible and constitutive levels of expression, will add to the precise patterning of the spatial and temporal activity of the Imd pathway in this tissue.

Finally, our study did not reveal any cryptic phenotype for PGRP-SB1 and SB2 in combination with the PGRP-SC and/or LB gene deletion. We can conclude that PGRP-SB1 and SB2 are, at most, only marginally involved in the regulation of the Imd pathway. The observation that PGRP-SB1 is induced to high levels after infection, with an expression level similar to that of antimicrobial peptide genes, and that PGRP-SB2 is also strongly induced during metamorphosis point to a putative role as immune effectors as described for zebrafish amidase PGRPs (Li et al., 2007). This function might be masked by the plethora of other immune effectors present in the genome of *Drosophila* (see discussion in Zaidman-Rémy et al., 2011).

Our study reveals that both Pirk, which reduces the level of Imd signaling downstream of PGRP-LC, and amidase PGRPs (LB and SC), which limit the availability of PGRP-LC ligand, synergize to dampen the immune response. Although flies lacking

one, or even two, of these negative regulators exhibit higher immune responses, the level of immune activity declines at late time points, indicating that they still possess some regulatory capacities. In sharp contrast, removing both amidases (PGRP-SCs and PGRP-LB) as well as Pirk leads to uncontrolled immune responses. The level of immune response in infected flies does not peak and then decline, but remains extremely high at 4 days after infection, after which the flies die rapidly as a result of their excessive immune response.

In *Drosophila*, bacterial infection triggers a massive expression of antimicrobial peptide genes, which are among the most highly expressed genes in the genome. Thus, we were surprised to find that removing Pirk, PGRP-LB, and the PGRP-SCs can still lead to AMP expression levels eight to ten times higher than those observed during infections of wild-type flies. This indicates that the immune response is highly constrained by the existence of negative regulators. The observation that the extent of the immune response to severe infections is far below the maximum possible response is intriguing and highlights the importance of negative regulation in shaping the antibacterial response.

The tight constraints on the level of Imd signaling suggest a strong selection to limit the antibacterial response, but previous studies have not addressed the relevance of amidase PGRPs and/or Pirk to the fitness of flies. Indeed, taking into account possible background effects, the fitness outcome of deleting a single negative regulator is modest. Here, we have observed that $pirk^{EY}$,$PGRP\text{-}SC^{\Delta}$;LB^{Δ} flies, and to a lesser extent flies lacking only the amidase PGRP loci ($PGRP\text{-}SC^{\Delta}$;LB^{Δ}), have a reduced lifespan compared to their wild-type counterparts. This lifespan reduction was in part rescued in germ-free conditions, indicating that it results from stimulation of the Imd pathway by commensals or ingested bacteria. Interestingly, guts from old flies contain higher counts of indigenous bacteria than in their younger counterparts (Buchon et al., 2009a). This would explain why unchallenged $PGRP\text{-}SC^{\Delta}$;LB^{Δ} *and* $pirk^{EY}$,$PGRP\text{-}SC^{\Delta}$;LB^{Δ} flies succumb late in life (60 days) at a stage when the microbiota are abundant. Ingestion of either live or dead *Ecc15* resulted in an even more severe reduction of the lifespan of $pirk^{EY}$,$PGRP\text{-}SC^{\Delta}$;LB^{Δ} flies. Use of germ-free flies shows that this higher immune response and lethality result from an excessive immune response rather than a change in microbiota composition. Strikingly, this effect was largely suppressed by blocking the activity of the Imd pathway. In conclusion, our study highlights the importance of tight regulation of the Imd pathway by the amidase PGRPs and Pirk to prevent excessive immune responses to innocuous bacteria and basal activation by commensals, which reduce lifespans.

Several studies have shown that low intestinal stem cell activity is a good indicator of gut homeostasis (Biteau et al., 2010; Buchon et al., 2009a). For instance, old flies show abnormal gut morphology due to higher proliferation of stem cells and their aberrant differentiation (Choi et al., 2008). Biteau et al. (2010) have recently shown that proliferative activity in aging intestinal epithelia correlates negatively with longevity, with maximal lifespan when intestinal proliferation is reduced but not completely inhibited. Interestingly, we observed a very high level of stem cell activity in the midgut of unchallenged $pirk^{EY}$,$PGRP\text{-}SC^{\Delta}$;LB^{Δ} flies, which also show a markedly reduced life span. This suggests a model in which a chronic immune response, due to the lack of negative regulators, leads to gut cell damage and compensatory production of enterocytes via stem cell activity. This will lead to a dysfunction of the gut, as observed in old flies, which is expected to cause defects in nutrient absorption and metabolic homeostasis. Thus, our study underlines the key role played by negative regulators of the Imd pathway in the maintenance of gut homeostasis. The rupture of gut homeostasis is not the only factor that reduces fly fitness, given that $pirk^{EY}$,$PGRP\text{-}SC^{\Delta}$;LB^{Δ} flies also succumb to a septic injury.

PGRPs are highly conserved from insects to mammals. Mammals have four PGRPs: three of them, PGLYRP1, PGLYRP3, and PGLYRP4, are directly bactericidal, whereas PGLYRP2 is an amidase that hydrolyzes peptidoglycan (Gelius et al., 2003; Lu et al., 2006; Wang et al., 2007). Although both mammalian and insect PGRPs are involved in the host response to infection, they have distinct roles. In insects, PGRPs are mostly involved in activating or downregulating defense pathways after microbial sensing (Royet and Dziarski, 2007). By contrast, mammalian PGRPs have primarily antimicrobial activities. Interestingly, all four mammalian PGRPs have recently been implicated in protecting the host from colitis induced by dextran sulfate sodium (DSS) (Saha et al., 2010). Mice deleted for each of the PGLYRP genes were all shown to be more sensitive than wild-type mice to DSS-induced colitis because of the presence of a more inflammatory gut microbiota, higher production of interferon-γ, and an increased number of NK cells in the colon. Together with our paper, this recent finding uncovers a conserved role of PGRPs in the maintenance of proper gut homeostasis by inhibiting the immune response induced by commensals or innocuous ingested bacteria. This goal is accomplished, however, by different strategies. *Drosophila* PGRPs (LB and the SC family) reduce Imd pathway activation by reducing the biological activity of peptidoglycan, whereas mammalian PGRPs seem to have a direct effect on the microflora composition.

Collectively, our study and others underline the multiple roles of PGRPs in the *Drosophila* immune response as pattern-recognition receptors, negative regulators, and potentially bactericidal molecules. The *Drosophila* genome encodes 26 genes (13 PGRPs and 13 lysozymes) with the potential to detect and/or lyse peptidoglycan and consequently modulate the relationship between *Drosophila* and bacteria. To date, *Drosophila* lysozymes have only been proposed to be involved in the digestion process, on the basis of their strong expression in the gut (Daffre et al., 1994), although a role in modulation of the immune response is not excluded. The fact that PGRPs are key players in the *Drosophila* immune response raises some questions regarding their emergence as pattern-recognition receptors during evolution. A possible scenario would be that catalytic PGRPs emerged first as digestive and/or antibacterial enzymes participating in the elimination and utilization of ingested bacteria, in synergy with lysozymes. Noncatalytic PGRPs may then have been selected for bacterial sensing, whereas some catalytic PGRPs (such as PGRP-LB and the PGRP-SCs) might have differentiated into modulators of the immune response. Diversification of the PGRP domain to allow it to distinguish between DAP- versus Lys-type peptidoglycan and monomeric

versus polymeric peptidoglycan, because of its capacity to sense the peptidic-glycan bridge of peptidoglycan, has probably allowed PGRPs to adopt a broad range of functions in the insect immune system. Future studies should investigate the possibilities that amidase PGRPs also play a role in the digestive process and lysozymes in the modulation of the immune response.

EXPERIMENTAL PROCEDURES

Fly Stocks and Mutant Generation

Oregon^R (Or^R) flies were used as wild-type controls. The $Dredd^1$, $Relish^{E20}$, *UAS-PGRP-LB-YFP*, $pirk^{EY00723}$, and $PGRP\text{-}SB^{\Delta}$ lines are described in Gendrin et al. (2009), Lhocine et al. (2008), and Zaidman-Rémy et al. (2011). The $PGRP\text{-}LB^{\Delta}$ and $PGRP\text{-}SC^{\Delta}$ KO lines were generated by homologous recombination (Figure S1). *PGRP-SC* rescue transgene: *P[PGPR-SC*]* is a third chromosomal *P* insertion containing the DNA sequence of the *PGRP-SC* cluster (corresponding to the sequence deleted in $PGRP\text{-}SC^{\Delta}$) with a deletion of *CG14743*. $PGRP\text{-}LB^{\Delta}$ flies carrying *da-Gal4*, *NP1-Gal4*, or *C564-Gal4* were crossed with control, $PGRP\text{-}LB^{\Delta}$, or $PGRP\text{-}LB^{\Delta}$, *UAS-PGRP-LB-YFP* flies for rescue experiments. The F1 progeny carrying *Gal4* and $PGRP\text{-}LB^{\Delta}$, with or without *UAS-PGRP-LB-YFP*, was transferred to 29°C 3 days prior to the infection for optimal GAL4 efficiency. *Drosophila* stocks were maintained at 25°C with standard fly medium.

Bacterial Strains and Infection Experiments

The bacterial strains used and their respective optical density (O.D.) at 600 nm were as follows: Gram-negative bacteria *Ecc15* (O.D. 200) and *E. cloacae* (O.D. 200), the Gram-positive bacteria *L. innocua* (O.D. 200), *M. luteus* (O.D. 200), *S. aureus* (O.D. 200), and *E. faecalis* (O.D. 30). We performed systemic bacterial infections by pricking adult females in the thorax with a thin needle previously dipped into a concentrated bacterial pellet. Oral bacterial infection was performed on female flies after a 2 hr starvation at 29°C by application of a concentrated bacterial solution (O.D 180) supplemented with sucrose (final concentration, 5%) to a filter disk in a fly medium tube. Flies were infected for 24 hr, then flipped to a fresh fly medium tube and maintained at 29°C for *diptericin* quantification or at 25°C for survival analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, one table, and six figures and can be found with this article online at doi:10.1016/j.immuni.2011.09.018.

ACKNOWLEDGEMENTS

We thank our colleagues M. Frochaux, L. Mury, D. Brandalise, and J.P. Boquete for technical support; M. Gendrin for stimulating discussions; C. Neyen and Nichole Broderick for comments on the manuscript; D. Mengin-Lecreulx for peptidoglycan samples; and the Bloomington stock centre and National Institute of Genetics (NIG) for fly stocks. This work was funded by the Bettencourt-Scheller Foundation, the ERC Advanced Grant and the Swiss National Fund (3100A0-12079/1).

Received: March 4, 2011
Revised: July 12, 2011
Accepted: September 6, 2011
Published online: November 23, 2011

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