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Functional Analysis of PGRP-LA in Drosophila Immunity

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Abstract

PeptidoGlycan Recognition Proteins (PGRPs) are key regulators of the insect innate antibacterial response. Even if they have been intensively studied, some of them have yet unknown functions. Here, we present a functional analysis of PGRP-LA, an as yet uncharacterized Drosophila PGRP. The PGRP-LA gene is located in cluster with PGRP-LC and PGRP-LF, which encode a receptor and a negative regulator of the Imd pathway, respectively. Structure predictions indicate that PGRP-LA would not bind to peptidoglycan, pointing to a regulatory role of this PGRP. PGRP-LA expression was enriched in barrier epithelia, but low in the fat body. Use of a newly generated PGRP-LA deficient mutant indicates that PGRP-LA is not required for the production of antimicrobial peptides by the fat body in response to a systemic infection. Focusing on the respiratory tract, where PGRP-LA is strongly expressed, we conducted a genome-wide microarray analysis of the tracheal immune response of wild-type, Diptericin, and PGRP-LA mutant larvae. Comparing our data to previous microarray studies, we report that a majority of genes regulated in the trachea upon infection differ from those induced in the gut or the fat body. Importantly, antimicrobial peptide gene expression was reduced in the tracheae of larvae and in the adult gut of PGRP-LA-deficient Drosophila upon oral bacterial infection. Together, our results suggest that PGRP-LA positively regulates the Imd pathway in barrier epithelia.

Introduction

Drosophila, in contrast to mammals, lacks adaptive immunity and therefore relies entirely on innate immunity for defense against invading pathogens [1,2]. Microorganisms are recognized through the interaction between microbial compounds and host pattern-recognition receptors. In insects, the peptidoGlycan recognition proteins (PGRPs) are a major class of pattern-recognition receptors that sense bacteria by interacting with peptidoglycan and regulate host antibacterial defenses. In Drosophila, the Toll and Imd pathways are the two major signaling cascades regulating the massive expression of antimicrobial peptide genes and other immune genes by the fat body following a systemic infection [3–5]. The Toll pathway is strongly induced by Gram-positive bacteria and fungi, and controls the expression of several genes, notably the antifungal peptide gene Drosomycin; the Imd pathway is strongly induced by Gram-negative and bacillus-shaped Gram-positive bacteria and regulates the expression of genes such as Diptericin, encoding an antibacterial peptide [6]. Activation of both pathways by bacteria is achieved through the sensing of specific forms of peptidoglycan by PGRPs. Peptidoglycan is an essential cell wall component of bacteria, composed of long glycan chains with alternating N-acetylmuramoyl and N-acetylmuramic acid residues that are cross-linked to each other by short peptide bridges. The third residue of these stem peptides differs between bacteria: it is a lysine in Gram-positive cocci and a meso-diaminopimelic acid (DAP) in both Gram-negative bacteria and Gram-positive bacilli, such as Bacillus and Listeria species [7]. Studies using highly purified bacterial compounds have shown that the highest Toll pathway activity is observed upon injection of Lysine-type peptidoglycan, while the Imd pathway is activated by DAP-type peptidoglycan [8]. Further studies have shown that both polymeric and monomeric DAP-type peptidoglycan can activate the Imd pathway. A specific monomer, the GlcNAc-MurNAc(anhydro)-L-Ala-γ-D-Glu-meso-DAP-D-Ala, also known as tracheal cytotoxin (TCT), has been identified as the minimal peptidoglycan motif capable of efficient induction of the Imd pathway [9,10].

PGRPs form a conserved family of proteins sharing a 160 amino acid domain (the PGRP domain) with similarities to bacteriophage T7 lysozyme, a zinc-dependent N-acetylmuramoyl-L-alanine amidase that removes peptides from the glycan chains of peptidoglycan [11,12]. The Drosophila genome encodes 13 PGRPs, some of which retain amidase properties. The PGRPs of this subgroup, referred to as catalytic PGRPs, have demonstrated (PGRP-SC1A/B, LB, SB1) or predicted (PGRP-SB2, SC2) zinc-dependent amidase activity, which reduces or eliminates the ability of peptidoglycan to elicit an immune response [13–15]. PGRP-LB
and to a lesser extent PGRP-SC1A/SC1B/SC2 have been shown to down-regulate the Imd pathway activity by scavenging peptidoglycan [16–18]. The exact function of PGRP-SB1/SB2 is not yet clear: it was proposed that this secreted PGRP could function as an antibacterial protein [15], but a recent genetic analysis did not identify any immune phenotype [19]. The non-catalytic PGRPs (PGRP-SA, SD, LA, LC, LD, LE, LF) lack the zinc-binding resiheres required for amidase activity but some of them retain the ability to bind peptidoglycan and function as bacteria sensors. 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 [20,21]. The receptor PGRP-LC, located at the plasma membrane, induces the Imd pathway when activated by DAP-type peptidoglycan [22–24]. PGRP-LE is produced in both extracellular and intracellular forms and has been shown to participate in the sensing of bacteria containing DAP-type peptidoglycan in two different manners. A secreted fragment of PGRP-LE corresponding to the PGRP domain alone enhances PGRP-LC-mediated peptidoglycan recognition on the cell surface [25]. In contrast, the full-length form of PGRP-LE is cytoplasmic and acts as an intracellular receptor for monomeric peptidoglycan, effectively bypassing the requirement for PGRP-LC [26]. While PGRP-LE is the main receptor upstream of the Imd pathway in the fat body, both PGRP-LC and PGRP-LE account for the sensing of Gram-negative bacteria upstream of the Imd pathway in the gut [27,28]. A gene in cluster with PGRP-LC, PGRP-LF encodes a transmembrane protein with two PGRP domains. Studies have indicated that PGRP-LF does not bind peptidoglycan but inhibits the activation of PGRP-LC by competing with PGRP-LC dimerization [29,30]. The functions of PGRP-LD and PGRP-LA are not yet known.

In this study, we report a functional analysis of PGRP-LA, a non-catalytic PGRP encoded by a gene of the PGRP-LC genomic cluster. PGRP-LA expression is enriched in several barrier epithelia such as the hindgut and tracheae whereas its expression in the fat body is low [31]. Based on over-expression, deletion and rescue experiments, this work suggests that PGRP-LA has a regulatory role and is involved in the fine-tuning of the Imd pathway in barrier epithelia. Our study also includes a genome-wide analysis of gene expression in tracheae in the presence or absence of epithelia. Our study also includes a genome-wide analysis of gene expression in tracheae in the presence or absence of epithelia.

Results

Structure predictions indicate that PGRP-LA would not bind to peptidoglycan

PGRP-LA is located at the 5’ boundary of a cluster of three genes that includes PGRP-LC and PGRP-LF. It encodes three isoforms, which are referred to here as PGRP-LA1, 2 and 3 (Figure 1A), following Flybase nomenclature, but which were previously referred to as PGRP-LAa, LAB and LAc respectively [32]. Sequence analysis predicted that the isoforms encoded by PGRP-LA differ considerably in their protein domain organization. PGRP-LA1 encodes a putative transmembrane protein with an intracellular domain containing a RIP Homotypic Interaction Motif (RHIM) [26,33], but lacking the PGRP domain. The RHIM domain is also found in PGRP-LC and PGRP-LF and has been shown to be necessary in these receptors for induction of the Imd pathway [26]. PGRP-LA2 contains both a putative transmembrane domain and a PGRP domain, a structure similar to that of the PGRP-LC receptor, except its lack of a RHIM domain. PGRP-LA3 encodes a short protein of 138 amino acids composed exclusively of a N-terminus-truncated PGRP domain: although the typical PGRP domain structure comprises a central β-sheet composed of six β-strands surrounded by three α-helices, PGRP-LA3 lacks the β1 and β2 sheets and a part of the α1 helix (see Figure 1B).

The PGRP domain of most PGRPs has been shown to interact with peptidoglycan. Nevertheless, biochemical studies have shown that some PGRPs, namely PGRP-LF and PGRP-LCa, have lost the capacity to bind peptidoglycan and function as a negative regulator and co-receptor of PGRP-LCx, respectively [30,34]. To get an insight on PGRP-LA function, we analyzed the sequence of its PGRP domain and its conservation among species. PGRP-LA is found in several insect species and its sequence is well conserved across species; the Drosophila PGRP-LA domain shares 60% identity with Aedes and Culex and 52% with A anonymus (Figure 1B).

In addition, the PGRP-LA domain sequence shares only 35% identity with PGRP-LF (60/168), 32% with PGRP-LCx (46/142), and 31% with PGRP-LF (52/164). These percentages are lower than the identity rate among other PGRPs (e.g. PGRP-LCx shares 40 to 52% with PGRP-LF, SD, SC1 and SA), but are above the 30% threshold necessary to predict that the folding of PGRP-LA is similar to the folding of other PGRPs [35]. Study of the putative peptidoglycan binding site of PGRP-LA using both the 3D model obtained with the Phyre software [36] and the sequence alignment with PGRP-LCx leads to three main observations. First, among the 10 residues of PGRP-LCx implicated in the binding to TCT [37], only two are conserved in PGRP-LA (Figure 1B), whereas these residues are highly conserved in PGRPs [14,38]. In particular, His388, which binds to GlcNAc, is replaced by an alanine, Tyr399, which is located in the central part of the binding crevice, is replaced by a serine, and Trp394, which stacks against the elongated side chain of DAP, is replaced by a leucine. In addition several residues, which are not directly in contact with TCT, but are engaged in shaping the binding crevice, are also not conserved in PGRP-LA. This is the case for Thr366, which is replaced by an isoleucine. Second, the PGRP domain of PGRP-LA displays a deletion of four amino acids in the β4–β5 loop (Figure 1B), which is known to be crucial for the binding to peptidoglycan, as an insertion of two residues in this loop prevents the binding to peptidoglycan in PGRP-LCa [34]. Third, an insertion of two residues occurs in the β2–α1 loop (Figure 1B), which has been shown to stabilize the pyranose ring of the MurNAc sugar of TCT. Considering these three points, it seems very unlikely that PGRP-LA binds peptidoglycan, suggesting that this PGRP is not a receptor but could have a regulatory role.

PGRP-LA is expressed in barrier epithelia and is up-regulated in response to infection

PGRP-LA was shown to be expressed at a moderate level during most developmental stages and its level of expression is higher in late larvae and prepupa [32,39]. We confirmed these results by RT-qPCR analysis (Figure S1A). Data from FlyAtlas reveal a strong expression of PGRP-LA in barrier epithelia, especially in salivary glands and tracheae of larvae and in the hindgut and eyes of adults, while it was weakly expressed in the fat body (9% and 21% of the average expression respectively for larvae and adults, Figure 2A) [31]. RT-qPCR with PGRP-LAD or PGRP-LAD/F specific primers shows a similar distribution of these isoforms in all the tissues tested except in Malpighian tubules where PGRP-LAD and 1 were absent (Figure S1A). Previous studies have shown that PGRP-LA expression is induced about two-fold in adults upon septic injury [3,30]. Upon oral infection with E. coli, PGRP-LA was also shown to be induced 1.6-fold and 10-fold in adult gut and
larval tracheae, respectively [40,41]. Using RT-qPCR, we confirmed that PGRP-LA expression is induced in whole flies after septic injury and in the midgut after oral infection with the Gram-negative bacterium *Erwinia carotovora carotovora* 15 (Ecc15) (Figures 2B, S1B). Together, these data indicate that PGRP-LA is induced after epithelial and septic infection.

Over-expression of PGRP-LAD induces the Imd pathway

Over-expression of PGRP-LE and PGRP-LC is sufficient to activate the Imd pathway, in agreement with their function upstream of this signaling cascade [22,42]. This prompted us to investigate the effect of the over-expression of PGRP-LA isoforms on the Imd pathway activation. Figure 3A shows that, using the da-Gal4 driver, over-expression in unchallenged flies of PGRP-LAD but not that of PGRP-LAF or PGRP-LAC was sufficient to induce a very high expression level of *Diptericin*, an antibacterial peptide gene used as a read-out of the Imd pathway. *Diptericin* induction by PGRP-LAD required Dredd and Tak1, but not PGRP-LC (Figure 3B). We also observed that ubiquitous over-expression of PGRP-LAD with the da-Gal4 driver induces some lethality, as observed upon PGRP-LC ubiquitous over-expression (data not shown). The observation that PGRP-LAD can activate the Imd pathway, the presence of a RHIM domain, and the location of PGRP-LA in the same cluster as PGRP-LC and LF are

![Figure 1. Description of PGRP-LA genomic locus and isoforms. A. Scheme of the locus containing PGRP-LA, PGRP-LC and PGRP-LF. Each gene contains at least one PGRP domain (orange) and PGRP-LA and LC contain a transmembrane domain (TM, green) and a RHIM motif (blue). No signal peptide has been predicted in the PGRP-LA sequence, and the C-terminal sequence (purple) contains 2 Cys residues. PGRP-LA encodes three isoforms, depicted under the gene: boxes represent the exons, of which the coding sequence is colored in red. PGRP-LAD (LA2A) deletion was performed by imprecise excision of the P-element G14937 (KAIST library) and PGRP-LAD (LALC) by FRT mediated deletion of the region between the P-elements 1930 and 4396. PGRP-LC12 (LC12) deletion has already been published [22]. In PGRP-LC, the hatched box represents a segment between a start and a stop codon, but which is not predicted to be the coding sequence (Flybase). Fp, Rg: localization of the primers used for RT-qPCR. B. Alignment of the proteic sequences of the PGRP domains of PGRP-LA in *Drosophila* (Dm), *Anopheles gambiae* (Ag) and *Culex quinquefasciatus* (Cq) and of *Drosophila* PGRP-LCx, of which the crystal structure has already been solved. Blue boxes contain conserved amino acids (identities and similarities are highlighted and written in red respectively). The residues that are directly in contact with TCT in the structure of the complex with PGRP-LCx [37] are marked with yellow triangles. The numbering corresponds to PGRP-LCx. The two residues insertion in the b2-a1 loop and the four residues deletion in the b4-b5 loop are denoted with green and red stars, respectively.

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larval tracheae, respectively [40,41]. Using RT-qPCR, we confirmed that *PGRP-LA* expression is induced in whole flies after septic injury and in the midgut after oral infection with the Gram-negative bacterium *Erwinia carotovora carotovora* 15 (Ecc15) (Figures 2B, S1B). Together, these data indicate that *PGRP-LA* is induced after epithelial and septic infection.
suggestive of a role of PGRP-LA in the regulation of the Imd pathway.

PGRP-LA is dispensable for the induction of a systemic immune response

In order to investigate the role of PGRP-LA in vivo, we generated a Drosophila strain deficient for PGRP-LA by imprecise excision of the P-element G14937 (from the Korea Advanced Institute of Science and Technology library). PGRP-LA2A mutant bears a deletion of 1401 bp upstream of the P-element insertion site, uncovering the PGRP domain sequence. This deletion includes the exons encoding the whole of the PGRP-LAC and LAF isoforms and the last four exons of the PGRP-LAD isoform (Figure 1A). In agreement with the molecular characterization, we found that PGRP-LA2A adults did not express PGRP-LA mRNA (see below). In addition, PGRP-LA2A mutants were viable and fertile and did not show any apparent developmental defects as observed for all the other PGRP deficient lines described so far. We introgressed the PGRP-LA2A mutation into the wild-type CantonS background by backcrossing PGRP-LA2A males with CantonS females for three generations in order to reduce possible effects of the genetic background.

As PGRP-LA expression is induced upon infection and as its over-expression up-regulates antibacterial gene transcription, we hypothesized that this gene was involved in the immune response. In order to clarify its role, we analyzed the effect of PGRP-LA deletion on the systemic immune response to different classes of microorganisms injected into the body cavity. Inactivation of PGRP-LA did not impact fly survival to injection with Gram-negative bacteria (Ecc15, Salmonella typhimurium), Gram-positive bacteria (L. monocytogenes, Enterococcus faecalis), or fungi (Aspergillus glaucus), whereas inhibition of the Imd pathway in a Relish mutant or the Toll pathway in a Spaetzle mutant had a dramatic effect upon survival (Figure 4A, B and Figure S2 A–C). Consistent with these survival analyses, we did not detect an effect of PGRP-LA2A mutation on the expression levels of Diptericin after systemic
infection with Ecc15 in larvae and adults, infection with L. monocytogenes or injection of DAP-type peptidoglycan or TCT in adults, nor on levels of Drosomycin (a read-out of the Toll pathway) after systemic infection with the Gram-positive bacterium Micrococcus luteus in adults (Figure 1C–E and Figure S2 D, E). These data indicate that PGRP-LA does not function as an essential recognition receptor in either the Toll or the Imd pathway during the systemic immune response of adults. Given that PGRP-LA expression is enriched in epithelia, we hypothesized that this PGRP might be involved in peptidoglycan translocation and long-range activation of the systemic response observed upon oral bacterial infection in PGRP-LB deficient flies or upon genital infection [43,44]. However, we did not find any role of PGRP-LA in the activation of the systemic response upon gut infections with Ecc15 or P. entomophila, or genital infections with Ecc15 (Figure 5F–H).

Finally, we generated a PGRP-LA, PGRP-LC double mutant in order to test if any involvement of PGRP-LA in the systemic response was masked due to a redundancy between PGRP-LA and PGRP-LC, as reported for PGRP-LE [25]. This mutant, referred to as PGRP-[LALC]4, was produced by flip-flop excision of a 15 kb region encompassing both genes, as depicted in Figure 2A. No difference in the susceptibility to infection or in the immune response activation was observed between PGRP-[LALC]4 and PGRP-LC[22], a deletion containing only the PGRP-LC gene [22] (Figures 4A, C, F and S2A, C). We conclude that PGRP-LA does not play a major role in the systemic immune response.

A microarray analysis reveals a role of PGRP-LA in antimicrobial genes expression in tracheae

In the absence of any overt immune function for PGRP-LA in the fat body, we next explored its role in the tracheae of larvae, a tissue in which PGRP-LA expression is enriched (Figure 2A) and up-regulated in response to infection [41]. Since the tracheal immune response is poorly characterized, we first used an unbiased approach and performed a genome-wide microarray analysis to compare the list of genes induced in the tracheae upon infection and monitor the effect of the PGRP-LA[22] mutation.

To determine the genes specifically induced in tracheae, we investigated transcriptome variations in dissected tracheae of larvae infected with the Gram-negative bacterium Ecc15. We chose Ecc15 as this bacterial strain strongly induces the Imd pathway in the tracheae upon bacterial infection, as revealed by the induction of the Drosomycin gene (which can be used as a read-out of Imd pathway in the trachea, see [41,45]). The transcriptomes of wild-type, Relish[29] and PGRP-LA[22] third-instar larvae were analyzed in unchallenged conditions and 24 h after placing larvae in Ecc15-contaminated fly medium at 18°C (see Materials and Methods and [46]), using Affymetrix GeneChip Drosophila Genome 2.0 Array. Our analysis identified 898 genes whose expression significantly varied in response to Ecc15 infection in the wild-type strain. We focused our attention on the genes that differ by at least a 2-fold change over unchallenged condition, corresponding to 119 induced and 105 repressed transcripts, 30% of which vary by more than 4-fold (Figure 5A; see Figure 5B for a selection of up-regulated genes and Table S1 for complete data set of regulated genes). Using a global classification, more than half of the tracheae-regulated genes were assigned to six functional categories: immunity, stress response, signaling, proteases and inhibitors, metabolism and transport, and chitin/cuticle metabolism (Figure 5C). Moreover, our analysis revealed a large set of previously unidentified bacteria-responsive genes, which are specific to the tracheae (71/119 and 96/105 in up and down-regulated genes respectively, Figure 5B and Table S1). To determine the contribution of the Imd pathway to antimicrobial defense in the tracheae, we examined the effect of the Relish mutation on gene expression. The expression of 54 up-regulated genes and 20 down-regulated genes was altered at least 2-fold in a Relish background compared to wild-type, with clear enrichment of Relish target genes among the most strongly induced genes (Figure 5A and Table S1). We found that 79% (19/24) of the genes annotated as immune genes were affected in the tracheae of Relish mutant flies (Table S1). Of these immune genes 71% (17/24) have been previously reported to be induced in the systemic or gut immune responses in adults [40,47]. These genes may represent the “core” of Imd pathway-regulated genes and include PGRP-SD, SBI and LE, most antibacterial peptide genes, genes coding for Imd pathway components (Ple, Relish, PGRP-LB), as well as TopI and Transferin 1.

The tracheal response to bacteria appears quantitatively less complex than the response occurring in the gut: 224 genes were modulated in the trachea using a two-fold criteria compared to 900 genes in the gut [40]. Although we cannot completely rule out an effect of the differences in stages or experimental protocols, we tend to attribute this difference to the fact that the gut response to bacteria also comprises an epithelium renewal response through stem cell proliferation and differentiation (Figure 5B) [40]. In the tracheae, infection induced a new set of genes notably involved in the stress response and oxidoreduction. Prominent among the repressed genes is a large set of chitin binding proteins, especially the Tidol family, of which 7 members are down-regulated in the tracheae, suggesting a remodeling of the highly structured intima, thin chitinous cuticle covering the tracheae [48], in response to infection. Thus, infection with Ecc15 alters the physiology of larval tracheae, with a repression of chitin metabolism and the stimulation of immune and stress responses, as well as changes in signaling and metabolism.

We then investigated the impact of PGRP-LA[22] deletion on the transcriptome of tracheae. We confirmed that the expression of PGRP-LA was lost in the mutant (Figure 6A) and that the expression of PGRP-LC, which is located just upstream of the 3’ end of PGRP-LA, was not impaired (fold change LA[22]/Cs: 1.4 both in unchallenged and infected conditions). We observed that 143 genes were more than 2-fold up- or down-regulated in PGRP-LA[22] as compared to wild-type (45 of them, whose expression varies more than 3-fold threshold in the mutant, are shown in Figure 6A). The most significant difference between wild-type and PGRP-LA[22] was the lower expression of many targets of the Imd pathway, notably antibacterial peptide genes, in both unchallenged and challenged conditions. For instance, expression of Defensin, Drosomycin and Drosacin were respectively 34, 14 and 13-fold lower in unchallenged PGRP-LA[22] compared to wild-type larvae (Figure 6A). Antimicrobial peptide genes were induced in PGRP-LA[22] tracheae in response to Ecc15, but reached a lower level than in wild-type tracheae. RT-qPCR using independent unchallenged tracheal samples confirmed that Defensin and Drosomycin transcripts were significantly lower in PGRP-LA[22] compared to wild-type (Figure 6B).

PGRP-LA participates in the activation of the Imd pathway in several barrier epithelia

The result above suggests a role of PGRP-LA in antimicrobial peptide gene expression in the tracheae, but not in the fat body. Nevertheless, the antimicrobial genes remain largely inducible in PGRP-LA[22] mutant tracheae indicating that PGRP-LA is not a core member of the Imd pathway, but rather might participate in the fine-tuning of the epithelial immune response. It could not be fully excluded that our microarray results were caused by the
Figure 4. PGRP-LA is not required for the systemic immune response. A,B. Survival analysis upon septic injury with Ecc15 in females (A) and E. faecalis in males (B). Full results of log-rank tests corrected with Bonferroni’s method: in A, wt vs RelE20: **, wt vs LA2A: ns, wt vs LALC: **, LA2A vs LALC: *; in B, wt vs RelE20: **, wt vs LA2A: ns, wt vs LALC: ns. C–E. RT-qPCR analysis of Dpt (C, D) and Drs (E) expression in whole females after septic injury with Ecc15 (C), L. monocytogenes (D), and M. luteus (E). F–H. RT-qPCR analysis of Dpt expression in whole females after oral infection with P. entomophila (F) or Ecc15 (G), and in males 6 h after genital infection by Ecc15 (H). In G, H, data are shown as a ratio of LB16 h (G) and wt (H). In C–G, data were analyzed by 2-way ANOVA with Bonferroni’s multiple comparison post-tests (in C, F, G, a and b groups are statistically different in infected flies: *, ** and *** respectively. In D, wt vs RelE20 (24 h): **, wt vs LA2A: ns (Spzrm7 is not included in the tests). In F and G, no significant differences were observed in unchallenged flies). In B, C, G, H, data are the mean of two repeats and error bars indicate data variation. In A, D, E, data are the mean of three independent repeats and error bars indicate standard errors. In F, data are the mean of 8 repeats from two independent experiments and error bars indicate standard errors. wt – wild type; LA2A, LB2A – PGRP-LA2A; LA2A – PGRP-LA2A; LA2A, LB2A, LA2A, LB2A – PGRP-LA2A; LA2A; PGRP-LB2A, RelishE20 – RelishE20; Spzrm7 – Speztlem7; LB2A – PGRP-LB2A; LA2A, LB2A (1) and (2) are two strains derived from independent recombination events between LA2A and LB2A; nd – no data; ns: non significantly different; *, **, *** show statistical differences with p<0.05, p<0.01 and p<0.001 respectively.

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Figure 5. Microarray characterization of the tracheal immune response in wild-type larvae. A. Distribution of regulated genes, based on their up or down-regulation and their fold change in the microarray. Black and white bar portions represent the genes whose expression is affected or not affected in RelE20 respectively. B. Comparison of the distribution of genes up-regulated in the tracheae upon Ecc15 bacterial infection to that of genes induced in the gut upon Ecc15 ingestion and in whole flies upon septic injury with Ecc15 [40,47]. * indicates that the gene expression is affected in RelE20. The number of genes induced in each tissue is indicated in brackets. C. Repartition of induced (left) and repressed (right) genes in defined categories of gene ontology. doi:10.1371/journal.pone.0069742.g005
Figure 6. PGRP-LA promotes epithelial antibacterial responses. A. List of the genes that are down-regulated (left) or up-regulated (right) in the PGRP-LA<sup>LA2A</sup> mutant, with a fold change versus wild-type Canton S (Cs) > 3 in either unchallenged or infected conditions, in the microarray analysis. For each gene the fold change in Ecc15-challenged versus unchallenged Cs larvae, the fold-change in PGRP-LA<sup>LA2A</sup> mutant versus Cs in unchallenged condition (UC) and after Ecc15 infection, and the fold change in RelE20 versus Cs Ecc15-challenged larvae are provided. B. RT-qPCR analysis Def and Drs expression in tracheae of unchallenged wild-type and LA2A larvae. C. Observation of Drs-GFP larvae 4 days after bacterial infection with Ecc15 at 18°C. All the larvae observed here (including of PGRP-LA<sup>LA2A</sup>) were showing Drs-GFP signal in the tracheae in more than half of the larvae, classified as ++++. D. Drs-GFP signal coverage observed in unchallenged tracheae of wild-type and LA2A larvae. Classification is the same as in B, +/− indicates a high background level of fluorescence compared to −. Data were analyzed by grouping − and +/− on one side, +, ++ and +++ on the other side for statistical analysis. *** show statistical difference between the proportion of larvae with Drs-GFP signal in PGRP-LA<sup>LA2A</sup> vs PGRP-LA<sup>LA2A</sup>, LB<sup>D</sup> strains. E–G. RT-qPCR quantification after Ecc15 infection of Def (E, F) and Dpt (G) expression in the larval gut regions (oral inf) and whole gut (oral inf).
Expression was less marked in the midgut. To confirm this, following oral infection with Ecc15, we observed that the proportion of Drosomycin-GFP expressing larvae was smaller in PGRP-LA2A mutants (Figure 3S) and that even when selecting larvae expressing the reporter, the fluorescence intensity was lower in PGRP-LA2A mutant than in wild-type tracheae (Figures 3S, 6C). The difference was less clear in unchallenged conditions, as the expression of Drosomycin-GFP was very low, even in the wild-type (Figure 6D). Thus, we decided to use a fly line deficient for PGRP-LB, which encodes a negative regulator of the Imd pathway [18]. As reported before, tracheae of larvae where PGRP-LB is down-regulated express a much higher level of Drosomycin-GFP reporter compared to wild-type (Figure 6). We observed that the GFP signal in double mutant PGRP-LA2A, LB1 larvae was significantly more restricted than in PGRP-LB1 larvae (Figure 6D).

To confirm that the effect seen on the activation of the Imd pathway was not due to the genetic background, we also performed a genomic rescue of the PGRP-LA deficiency line with a transgene containing the PGRP-LA locus including 4 kb upstream of the start codon (referred to as [PGRP-LA]/2M). In both the tracheae and the midgut, the expression of PGRP-LA in the rescue line (genotype: [PGRP-LA]/2M; PGRP-LA2A) was similar to wild-type levels (Figure S1B, C). In order to elucidate any effect of the microbiota, we generated axenic (germ-free) PGRP-LA2A and [PGRP-LA]/2M; PGRP-LA2A lines, reconstituted a gnotobiotic microbiota composed of Lactobacillus plantarum and Lactobacillus brevis only, two bacteria commonly found in Drosophila microbiota (reviewed in [50]) and maintained these germ-free and gnotobiotic lines in autoclaved fly medium. In these conditions, the levels of Drosomycin and Defensin in unchallenge tracheae were very low and too variable, preventing us to analyze the effect of the PGRP-LA2A deletion on basal Imd pathway activation by RT-qPCR. We therefore focused our analysis on tracheae of larvae collected 24 h after bacterial infection with Ecc15 at 29°C. Defensin and Drosomycin expression was 3 to 10-fold lower in the tracheae of PGRP-LA2A compared to [PGRP-LA]/2M; PGRP-LA2A infected larvae (Figures 6E, S4). The effect of PGRP-LA on tracheal antimicrobial genes upon Ecc15 infection was still observed when larvae were raised in germ-free conditions. The results were however variable and statistical significance could only be observed when monitoring Defensin after infecting germ-free larvae (Figures 6E).

To extend our analysis, we next investigated whether PGRP-LA was involved in the Imd pathway activation in the gut of adults since PGRP-LA is also enriched in this tissue (Figure 2A). Figure 6F shows that the level of Defensin was significantly lower in the hindgut of PGRP-LA2A flies compared to wild-type following oral infection with Ecc15. The effect of PGRP-LA on Defensin expression was less marked in the midgut. To confirm this result, we also monitored Dipterin expression in the gut of PGRP-LA2A and [PGRP-LA]/2M; PGRP-LA2A adult flies raised in either germ-free or gnotobiotic conditions, and then infected with Ecc15. Figure 6G shows that Dipterin expression was also lower in the gut of PGRP-LA2A mutant compared to [PGRP-LA]/2M; PGRP-LA2A adults 20 h after oral infection with Ecc15 although this effect was only significant when infecting previously germ-free flies (Figure 6G).

Together with the microarray analysis, these data suggest that PGRP-LA positively regulates the Imd pathway in barrier epithelia such as the tracheae and the gut.

Discussion

In this manuscript, we present a first detailed analysis of PGRP-LA function. Our structural study predicts that the PGRP domain of PGRP-LA is unlikely to bind peptidoglycan by itself. We next show that over-expression of PGRP-LA isoform, but not of PGRP-LA1 and PGRP-LA2, leads to the activation of Dipterin expression in absence of infection. Our experiments placed PGRP-LA upstream of the Drdell caspase and of the Taka1 MAP3K. The intracellular domain of PGRP-LA contains a RHIM motif similar to that observed in PGRP-LC and PGRP-LE for which it is essential for Imd pathway activation [26]. This suggests that the RHIM motif confers to PGRP-LA the capacity to induce the Imd pathway. Studies involving short mutations in PGRP-LC and PGRP-LE reported that their RHIM motifs are not involved in any physical interaction with Imd, the downstream adaptor of the Imd pathway, but bind with Pirk, a negative regulator of the Imd pathway [26,51]. Further analysis will be required to test whether the different PGRP-LA isoforms physically interacts with Pirk and/or with PGRP-LC. Collectively, this initial molecular characterization of PGRP-LA suggests a modulatory role of this PGRP in the Imd pathway.

Using a PGRP-LA-deficient line, we showed that PGRP-LA is not required for the systemic production of antimicrobial peptides in the adult. Consistent with this observation, mutations in PGRP-LA did not increase the susceptibility to systemic bacterial infection. This matches with the very low expression of PGRP-LA in the fat body. Of note, phagocytosis was also not affected in the PGRP-LA2A mutant (Figure S5). Consistently, previous studies on S2-cells did not reveal any role of PGRP-LA in the induction of antimicrobial peptides by peptidoglycan or Gram-negative bacteria [23,24] or in the phagocytosis of Gram-negative or Gram-positive bacteria [24]. All these data clearly indicate that PGRP-LA is not compulsory for the systemic activation of the Imd or Toll pathways, although a more specific role under a very specific condition or in response to a specific form of peptidoglycan could formally not be excluded.

Several studies have shown that the antimicrobial response of Drosophila exhibits major differences depending on the tissue [1,4,5,15,52,53]. Notably, regulatory mechanisms controlling the antimicrobial response in barrier epithelia significantly differ from that involved in fat body-mediated systemic immune response. For instance, the expression of antimicrobial peptide genes (including Drosomycin) in the midgut or the tracheae relies only on the Imd pathway.
pathway [45]. In addition, it has recently been shown that PGRP-LA has a significant role in Imd pathway activation in the midgut while PGRP-LC is the main sensor of Gram-negative bacteria during systemic infection [27,28]. These differences are probably a consequence of the necessity to maintain tight control on immune activation according to the level of exposure to bacteria or microbial products; while the hemocoel surrounding the fat body remains sterile, organs such as the digestive tract and tracheae are constantly in direct contact with the external environment. This raises the possibility that PGRP-LA has a subder role in barrier epithelia where its expression is enriched. In support of this notion, our microarray analysis revealed a lower expression of antimicrobial peptides in PGRP-LA^−/− tracheae of both Ecc15-infected and unchallenged larvae. The idea that PGRP-LA could establish the basal level of Imd pathway in unchallenged conditions is intriguing. These results were confirmed in RT-qPCR (Figure 6B), but limitations due to the low and variable levels of antimicrobial gene expression in the tracheae and the gut in unchallenged conditions, when maintaining fly lines in autoclaved fly medium, did not allow us to confirm this hypothesis (data not shown). Nevertheless, we observed that the expression of several antimicrobial peptide genes was reduced in larval tracheae and adult guts of PGRP-LA^ΔΔ mutants upon Ecc15 infection. A rescue experiment confirms that the phenotype is specifically linked to the PGRP-LA deletion and not to the genetic background. However, in normal laboratory conditions the PGRP-LA phenotype is not very strong and we were unable to detect any infectious condition for which a contribution of PGRP-LA to adult survival was discernable.

Our results support the notion that PGRP-LA positively regulates the antibacterial response in infected epithelia. However, we cannot exclude subtle additional roles for PGRP-LA, such as its participation in inter-organ communication by spreading immune signaling from epithelia to another tissue (e.g. between the gut and the tracheae). Such immune communication between tissues occurs between several epithelia and the fat body in Drosophila [17,44,46,54]. However, no role of PGRP-LA could be discerned in the activation of the systemic response upon gut or genital infections (Figure 4F–H).

The implication of several pattern-recognition receptors in the gut highlights the complexity of mechanisms underlying bacterial sensing in barrier epithelia. The conservation of PGRP-LA in mosquito (contrary to PGRP-LE or PGRP-LF) where it is also located in cluster with PGRP-LC suggests the conservation of its function in other insect species. The genomic organization of the PGRP-LA, LC, LF cluster is intriguing since the Imd-receptor gene PGRP-LC is flanked by both a positive (PGRP-LA) and a negative (PGRP-LF) regulator of the pathway. Future studies should elucidate the mechanisms by which PGRP-LA modulates the Imd pathway, notably to determine which PGRP-LA isoforms are involved. Another question to address will be the respective contributions of PGRP-LA, LC, and LE in the sensing of bacteria in the intestine. Thus, our data add a layer of complexity to the mechanism regulating the Imd pathway and further investigation is needed to fully characterize the role of PGRP-LA.

The Drosophila tracheal immune response remained poorly characterized [41,53,56]. In this study, we also present a general analysis of tracheal transcriptome variations after bacterial infection in larvae. Our data reveal a major role of the Imd pathway, which controls the expression of half of the genes regulated upon infection and of most of the immunity-related genes, such as antimicrobial genes. This is in accordance with previous reports showing that this pathway controls the local production of antimicrobial peptide genes, in tracheae and the gut [40,45,57]. We note that it also regulates genes involved in other cellular functions such as metabolism. Interestingly, we observed that many genes encoding putative or characterized cuticle proteins are down-regulated upon infection. The shape of the tracheae is maintained by helicoidal thickenings of the intima called taenidiae [49]. Therefore, the down-regulation of structural genes highlighted in our microarray suggests a remodeling of this structure upon infection. Consistent with this down-regulation, an apical-basal enlargement of the cells of the airway epithelium has been previously reported in regions of the tracheae exhibiting a strong immune response [41]. This enlargement might be explained by a thinning of the cuticle and consequent loss of rigidity. Thus, infection with Ecc15 not only induces an immune and stress response, but also alters the metabolism and physiology of tracheae. Interestingly, microarray comparison of the immune response during systemic (fat body), gut, and tracheal immune response reveals that only a small group of common genes are induced, all regulated by the Imd pathway and encoding mainly antimicrobial peptides and other pathway components. These genes may therefore represent the “core” of Imd pathway that are complemented by tissue-specific genes to achieve an optimal immune response.

Materials and Methods

Fly Stocks

Oregon^R^ flies were used as wild-type controls for the PGRP-LA^ΔΔ original strain (Figure 4A, H, 6H, S2B), and Canton^S^ flies were used as wild-type controls of PGRP-LA^ΔΔ introgressed into the Canton^S^ background (all other figures). Over-expression experiments were controlled by crossing the da-Gal4 UAS-driver to w^1118^, the strain in which the UAS construct insertions were generated. Relish^E20^ (R^ef26^), Dredd^B118^, Tak1^, PGRP-LC^E12^ and Spaetzle^m7^ are described elsewhere [22,58–61]. The da-Gal4 line expresses Gal4 ubiquitously and constitutively. The UAS-PGRP-LA^C^ (insertion R1), UAS-PGRP-LA^P^ (insertion R4) and UAS-PGRP-LA^F^ (insertion R2) lines were obtained as follows. A full-length cDNA of each isoform of PGRP-LA (using the CG32042 CDNA gold GH4960, GH18280 and GH10945, respectively, from DGRC) was placed downstream of the UAS sequence using the pUAST vector. F1 progeny young adults carrying both the UAS construct and the Gal4 driver were transferred to 29°C for optimal efficiency of the UAS/Gal4 system.

Stocks were reared at 25°C on media prepared as follows: per liter of water, 58.8 g inactivated yeast (Biospringer Springaline® BA95/0), 58.8 grams maize flour (Westhove Farigel Maize H1), 7.5 g agar, 58 mL of 1:1 mix of grape and multi-fruit juice were combined with water and boiled at 80°C for 30 min. When the mixture had cooled to 65°C, 4.85 ml of 99% propionic acid and 30 ml of a 10% solution of methyl paraban in 85% ethanol were added. After cooling to room temperature, live yeast was added on the surface of the media, except for germ-free and gnotobiotic flies, which were reared on autoclaved media in glass tubes without the addition of live yeast.

PGRP-LA^ΔΔ^ mutant was obtained by imprecise excision of the G14937 P-element (KAIST library) and PGRP-[LALC]^Δ^ by FRT mediated deletion of the region between the P-elements 1930 and 4396, following previously published methods [62]. PGRP-LA^ΔM^ rescue line was generated using gap-repair and recombineering, and final rescue construct carried by F[acman] vectors was inserted into the Phic31 landing site 51C on chromosome 2 (BDSC strain 24482) [63],[64]. Vector with PGRP-LA contain the PGRP-LA gene including the following sequence (based on Flybase release r5.47): 3'L: 9323736–9331619.

Characterization of PGRP-LA in Drosophila Immunity
Drosophila stocks and crosses were maintained at 25°C in yeasted tubes containing corn-meal fly medium. Germ-free lines were generated by egg bleaching and kept in autoclaved fly medium. Gnotobiotic lines were generated by introducing cultured L. plantarum and L. brevis previously isolated from our fly lines into the medium of germ-free lines and were also kept in autoclaved fly medium.

Bacterial and fungal stocks

All bacteria were stored as frozen stocks (15% DMSO). E. coli, L. monocytogenes, Micrococcus luteus, Salmonella typhimurium, and L. brevis were described previously [61]. They were grown on LB-Agar plates and grown overnight in LB-medium at 29°C and generally used as pellets of OD <sub>600</sub> = 200, i.e. the OD <sub>600</sub> of a 1/1000<sup>th</sup> dilution of the pellet in PBS was 0.2 corresponding to 4.10<sup>11</sup> CFU.mL<sup>-1</sup> (exceptions are mentioned below). Salomonella typhimurium, Listeria monocytogenes and Candida albicans were described previously [61]. They were grown overnight at 37°C, respectively in LB-medium, BHI, and YPG. Aspergillus glaucus was kept as a spore-suspension at 4°C and injected as such into the flies. While testing the susceptibility to septic injury E. coli was tested at OD <sub>600</sub> 200 and 50, Salmonella typhimurium at OD <sub>600</sub> 0.65 and 10<sup>-5</sup>, L. monocytogenes at OD <sub>600</sub> 0.65 and 10<sup>-5</sup>, Enterococcus faecalis at OD <sub>600</sub> 5, 10, 15.

Infection and survival experiments

Septic injuries were made by prickling adults in the thorax with a thin needle dipped into a concentrated bacterial pellet. Genital infections were performed by touching the tip of the abdomen with a 200 µL pipette-tip containing 10 µL of bacterial pellet [44]. For gut infection, flies were starved for 2 h, then allowed to feed on a 1:1 mixture of mashed banana and peptidoglycan (5 mg/ml), or TCT (tracheal cytotoxin; 0.046 mM) applied to a filter disk completely covering the surface of standard fly medium. Flies were maintained at 29°C and guts were dissected 16–24 h after contact with infected food.

In Figures 6F, S1, midgut was defined as the section of the gut between the proventriculus (included) and the pylorus (midgut/hindgut junction), while hindgut corresponds to the section between the pylorus and the anus. Malpighian tubules were excluded from both midgut and hindgut samples. In Figure 6G, whole guts include the section between the crop (included) and the pylorus, excluded from both midgut and hindgut samples. In Figure 6A, C, D, S3: adults were allowed to lay eggs for 3 days then removed, and 500 µl E. coli 15 pellet (OD <sub>600</sub> = 200) were added on the 4<sup>th</sup> day into 28.5 mm-wide vials where larvae were developing.

Microarray Analysis

Larvae were infected with method 1 and dissected by gently pulling the posterior spiracles backwards until the whole tracheae went out. If needed, the anterior part of the tracheae was pulled out in a second similar step. RNA pools from the tracheae (including anterior spiracles) of 50 3<sup>rd</sup> instar larvae were isolated, purified with RNA clean-up purification kits (Macherey Nagel), and DNase treated. The samples were controlled for fat body contamination by RT-qPCR on Fat body protein P6 (Flp2). RNA quality was controlled on Agilent 2100 Bioanalyzer chips. As the quality of some samples was not good enough after this first purification, RNA of all samples was ethanol precipitated to pass Bioanalyzer quality control. For each sample, 100 ng of total RNA was amplified and labeled using the GeneChip IVT Labeling Kit according to the protocol provided by the supplier. Affymetrix Drosophila Genome 2.0 arrays were hybridized with 30 mg of labeled cRNA, washed, stained, and scanned according to the protocol described in the Affymetrix Manual. Three independent repeats were performed for each condition and gene expression profiles from challenged larvae were normalized to their controls. Statistical analyses were performed using the R and Bioconductor statistical packages. Full dataset can be found at http://mairetrelab.epfl.ch/ and has been deposited at EMBL-EBI database (Accession number: E-MEXP-3925).

Phagocytosis assay

The phagocytosis assay was performed as previously published [65]. Briefly, 41.1 nL of S. aureus or E. coli bioparticles (20 mg.mL<sup>-1</sup>) were injected in the fly abdomen. Flies were left for 30–40 min at 25°C and injected with 6×69 nL of 0.4% trypan blue.

Accession Numbers

The Flybase (www.flybase.org) accession numbers for genes mentioned in the microarray are indicated in the data (Figure 6A, Table S1). The accession numbers for genes mentioned in the rest of this study are: Defensin (CG1385), Dipterocin (CG12763), Drosocin (CG10816), Drosomycin (CG10816), PGRP-LA (CG32042), PGRPLB (CG14704), PGRP-LC (CG4432), PGRP-LF (CG4437), da (daughterless, CG5102), pik (CG15678) and Relish (CG11992). The vectorbase (www.vectorbase.org) accession numbers for Culex and Anopheles homologs of PGRP-LA are CPI006558 and AGAP005205 respectively.

Supporting Information

Table S1 Expression profile of the genes regulated in the tracheae upon bacterial infection in larvae. List of the genes showing a fold change >2, upon Ecc15 infection, in the tracheae of Canton<sup>s</sup> larvae. Fold changes in Canton<sup>s</sup> and Rel<sup>−/−</sup> are
indicated. In the “Rel” column, “R” indicates the genes whose regulation is affected in RelE<sup>GFP</sup> mutant. The columns “sys”, “gut” and “svys+gut” show respectively the genes regulated in whole flies upon septic injury with Ecc15 [47], in the gut upon Ecc15 ingestion [40], and in both conditions; for each tissue, “+” means that the gene is up-regulated “−” that it is repressed. AvgExp: mean signal over all chips.

(PDF)

**Figure S1 PGRP-LA expression in tissues.** RT-qPCR analysis of PGRP-LA expression in wild-type adult female tissues (A) and in adult female midguts (B) and larval tracheae (C) of wild-type and [PGRP-LA<sup>2M</sup>; PGRP-LA<sup>2A</sup>] strains. Data are normalized to Rpl32 and shown as a ratio of the expression in the wild-type. In A, a single experiment was performed. In B, data are the mean of three independent experiments, error bars indicate standard errors and data were analyzed by 2-way ANOVA with Bonferroni post-tests. In C, data are the mean of two independent experiments and error bars indicate data variation. (TIF)

**Figure S2 PGRP-LA is not required for the systemic immune response.** A–C. Survival analysis of flies after infection with S. typhimurium (A, OD 10<sup>−6</sup>, 69 nL injected), L. monocytogenes (B, OD 6.5, 9.2 nL injected), A. glaucescens (C, spore suspension, 69 nL injected). D, E. Dpt expression after injection of 9.2 nL of monomeric (tracheal cytotoxin, TCT, 0.46 mM) or polymeric peptidoglycan (PGN, 5 mg mL<sup>−1</sup>) (A) and after septic injury with Ecc15 in larvae (B).

(TIF)

**Figure S3 Tracheal Drosophila response in wt and PGRP-LA<sup>2A</sup> larvae (GFP).** Fluorescence observed in the trachea of wild-type and PGRP-LA<sup>2A</sup> larvae expressing the Drs-GFP reporter gene 4 days after bacterial infection with Ecc15 at 18°C. (−) no fluorescence (+) fluorescence in the spiracles only, (+++) in the tracheal trunks, (+++) in the tracheae in less than half of the larva or (++++) in the tracheae in more than half of the larva. •, ••, •••, ••••• increasing intensity of fluorescence. Data of one experiment representative of 3 independent experiments are shown. (TIF)

**Figure S4 Tracheal Drosophila response in wt and PGRP-LA<sup>2A</sup> deficient larvae (RT-qPCR).** RT-qPCR quantification of Drosophila expression in the larval trachea 24 h after Ecc15 infection in L<sup>2A</sup> and [PGRP-LA<sup>2M</sup>; LA2A]<sup>2A</sup> lines raised in germ-free conditions or gnotobiotic conditions (Lp<sub>2B</sub>) where the flora is composed of L. plantarum and L. brevis. Data show the mean of 4 repeats and error bars indicate standard errors. Data were analyzed by Mann-Whitney tests, differences are non significant. (TIF)

**Figure S5 PGRP-LA is not required for the phagocytosis.** Fluorescent images of fly abdomens after injection of S. marcescens or E. coli nanoparticles. Data show representative results of one experiment. (TIF)

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**Author Contributions**

Conceived and designed the experiments: MG AZR NAB JP BL. Performed the experiments: MG AZR NAB JP. Analyzed the data: MG AZR NAB JP AR BL. Contributed reagents/materials/analysis tools: AZR MP AR. Wrote the paper: MG AZR NAB BL.

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