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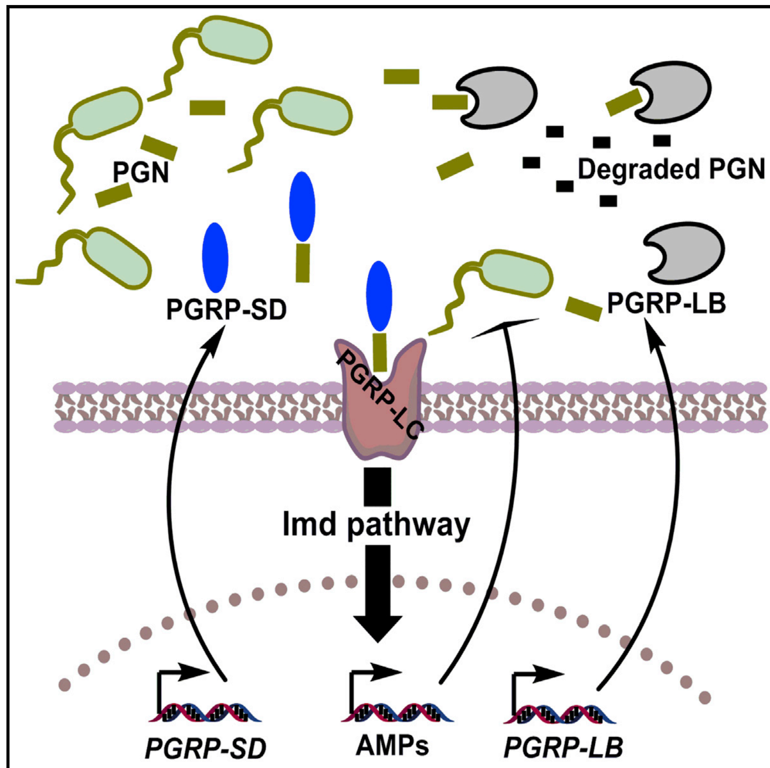
Immunity

# PGRP-SD, an Extracellular Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation of the Drosophila Imd Pathway

## Graphical Abstract




## Highlights

- PGRP-SD is a secreted pattern-recognition receptor required for Imd pathway activation
- PGRP-SD is critical for survival from Gram-negative bacterial infections
- PGRP-SD enhances Imd signaling upstream of the transmembrane receptor PGRP-LC
- PGRP-SD promotes peptidoglycan re-localization to the membrane receptor PGRP-LC

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## In Brief

Peptidoglycan recognition proteins execute diverse defense functions, which are not fully understood. Iatsenko et al. show that *Drosophila* PGRP-SD is a secreted pattern-recognition receptor required upstream of transmembrane receptor PGRP-LC to enhance activation of the Imd pathway by promoting peptidoglycan re-localization to the cell surface.

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# PGRP-SD, an Extracellular Pattern-Recognition Receptor, Enhances Peptidoglycan-Mediated Activation of the Drosophila Imd Pathway

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## SUMMARY

**Activation of the innate immune response in Metazoans is initiated through the recognition of microbes by host pattern-recognition receptors. In *Drosophila*, diaminopimelic acid (DAP)-containing peptidoglycan from Gram-negative bacteria is detected by the transmembrane receptor PGRP-LC and by the intracellular receptor PGRP-LE. Here, we show that PGRP-SD acted upstream of PGRP-LC as an extracellular receptor to enhance peptidoglycan-mediated activation of Imd signaling. Consistent with this, *PGRP-SD* mutants exhibited impaired activation of the Imd pathway and increased susceptibility to DAP-type bacteria. PGRP-SD enhanced the localization of peptidoglycans to the cell surface and hence promoted signaling. Moreover, PGRP-SD antagonized the action of PGRP-LB, an extracellular negative regulator, to fine-tune the intensity of the immune response. These data reveal that *Drosophila* PGRP-SD functions as an extracellular receptor similar to mammalian CD14 and demonstrate that, comparable to lipopolysaccharide sensing in mammals, *Drosophila* relies on both intra- and extracellular receptors for the detection of bacteria.**

## INTRODUCTION

The innate immune response is an important line of defense against invading pathogens in Metazoans (Janeway and Medzhitov, 2002; Lemaitre and Hoffmann, 2007). Like all invertebrates, the fruit fly, *Drosophila*, relies exclusively on innate immunity to fight off infections (Lemaitre and Hoffmann, 2007). Recognition of bacterial infection in this insect is mediated through the sensing of peptidoglycans by a class of pattern-recognition receptors (PRRs) called peptidoglycan recognition proteins (PGRPs) (Leulier et al., 2003; Royet et al., 2011). PGRPs are conserved from insects to mammals and are defined by the presence of a 160 amino acid PGRP domain, which is similar to that of bacterial *N*-acetylmuramyl-L-alanine amidases (Kurata, 2014; Yoshida et al., 1996). *Drosophila* has 13 PGRP genes encoding 19 known proteins, which execute multiple defense functions (Royet et al., 2011). Some *Drosophila* PGRPs, known as enzymatic PGRPs (e.g., PGRP-LB, PGRP-SC, and PGRP-SB), cleave peptidoglycan into non-immunostimulatory fragments by removing peptides from the glycan chains (Mellroth et al., 2003; Zaidman-Rémy et al., 2011, 2006). In this way, catalytic PGRPs either modulate the immune response by scavenging peptidoglycan or act as bactericidal molecules (Costechareyre et al., 2016; Mellroth and Steiner, 2006; Paredes et al., 2011; Zaidman-Rémy et al., 2011, 2006). In contrast, non-catalytic PGRPs (e.g., PGRP-LC, PGRP-LE, and PGRP-SA) have lost their enzymatic activity but have conserved their ability to bind peptidoglycan. Non-catalytic PGRPs often function as PRRs, which mediate microbial ligand-dependent activation of downstream signaling (Royet et al., 2011).

Peptidoglycan is an essential component of the bacterial cell wall, which consists of long glycan chains made of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues that are cross-linked by short peptide bridges (Schleifer and Kandler, 1972). All Gram-negative and a subset of Gram-positive bacteria (e.g., *Bacillus*) possess a diaminopimelic acid (DAP) residue at the third position of the peptide bridge, whereas all other Gram-positive bacteria possess an L-lysine at this position (Schleifer and Kandler, 1972). Application of highly purified peptidoglycan preparations has shown that *Drosophila* can discriminate between Lys-type and DAP-type peptidoglycan-containing bacteria to elicit distinct antimicrobial responses via the selective activation of the Toll and Imd pathways, respectively (Leulier et al., 2003; Stenbak et al., 2004; Kaneko et al., 2004). Lys-type Gram-positive bacteria are recognized by a secreted PGRP, PGRP-SA, which activates the Toll pathway to stimulate the expression of antimicrobial peptide genes (e.g., *Drosomycin*) via the NF-κB members Dif and Dorsal (Gobert et al., 2003; Valanne et al., 2011). The Toll pathway can also be activated by fungi upon sensing of glucan by beta-1,3-glucan binding protein GNBP3 or directly by microbial proteases (Gottar et al., 2006). In contrast, Gram-negative and DAP-type Gram-positive bacteria trigger the Imd pathway through the NF-κB transcription factor Relish (Ferrandon et al., 2007; Kleino and Silverman, 2014; Lemaitre and Hoffmann, 2007; Neyen and Lemaitre, 2016). The transmembrane protein PGRP-LC is the major receptor upstream of the Imd pathway (Gottar et al., 2002; Choe et al., 2002). It is expressed as three splice isoforms

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(LCa, LCx, and LCy), each consisting of the same intracellular domain linked to a different extracellular domain (Kaneko et al., 2004; Neyen et al., 2016). PGRP-LCx is sufficient to recognize polymeric peptidoglycan, whereas detection of monomeric peptidoglycan requires both PGRP-LCx and PGRP-LCa (Kaneko et al., 2004; Neyen et al., 2012). PGRP-LE is another DAP-type peptidoglycan-binding protein regulating the Imd pathway (Kaneko et al., 2006; Takehana et al., 2004). Whereas activation of the Imd pathway in the fat body during systemic infection relies mostly on PGRP-LC (Neyen et al., 2012; Takehana et al., 2004), PGRP-LE plays a major role in the response to Gram-negative bacteria in the midgut (Bosco-Drayon et al., 2012; Neyen et al., 2012). Negative regulation of the Imd pathway is also critical for survival and occurs at multiple levels. Notably in the hemolymph, secreted enzymatic PGRPs, such as PGRP-LB, scavenge peptidoglycan, the bacterial elicitor of the pathway (Zaidman-Rémy et al., 2006; Paredes et al., 2011). Thus, immune response involving the Imd pathway is thought to require a balance between peptidoglycan sensing by recognition PGRPs and peptidoglycan degradation by enzymatic PGRPs.

PGRP-SD is a short secreted protein that is strongly induced upon infection by the Imd pathway (De Gregorio et al., 2002). Crystal-structure analysis has revealed that PGRP-SD holds a peptidoglycan-binding groove characteristic of DAP-type recognition PGRPs (Leone et al., 2008). In vitro binding studies subsequently confirmed that PGRP-SD binds DAP-type but not Lys-type peptidoglycan (Leone et al., 2008), suggesting a role for PGRP-SD in the Imd pathway. However, a genetic analysis performed with a presumptive null *PGRP-SD* mutant did not reveal any role for PGRP-SD in the detection of Gram-negative bacteria (Bischoff et al., 2004). Instead, Bischoff et al. (2004) showed that *PGRP-SD* mutants are susceptible to Gram-positive bacteria and that some Lys-type bacteria cause defects in activation of the Toll pathway. Wang et al., (2008) confirmed that PGRP-SD interacts with PGRP-SA and GNBP1 upstream of the Toll pathway (Wang et al., 2008), and another study (Leone et al., 2008) has suggested a role for PGRP-SD in Toll pathway activation by DAP-type peptidoglycan-containing bacteria. These discrepancies between structural and genetic studies prompted us to re-investigate the role of PGRP-SD in the *Drosophila* immune response.

Using newly generated *PGRP-SD* mutants, we were able to show that PGRP-SD was not required for Toll pathway activation by Lys- or DAP-type bacteria. Instead, *PGRP-SD* mutants had a reduced systemic Imd pathway activation and increased susceptibility to Gram-negative bacteria. In addition, PGRP-SD strongly enhanced peptidoglycan-mediated activation of the Imd pathway by promoting peptidoglycan re-localization to the cell-surface receptor PGRP-LC. All together, our results provide strong genetic evidence that PGRP-SD is an important DAP-type pattern-recognition protein required upstream of the Imd pathway for defense against DAP-type peptidoglycan-containing bacteria.

## RESULTS

### PGRP-SD Is a Secreted Non-catalytic PGRP Induced by Infection

PGRP-SD, a secreted protein of 186 amino acid residues, contains a signal peptide and a PGRP domain with no additional regulatory domains (Figure 1A). In the absence of infection, *PGRP-SD* is moderately expressed in the fat body, midgut, and head of adult flies, whereas in larvae it is strongly expressed in the fat body (Figures S1A and S1B). In a previously published microarray dataset (De Gregorio et al., 2002), we showed that *PGRP-SD* is strongly induced by bacterial challenge in wild-type flies (Figure S1C). PGRP-SD induction is mediated by the Imd pathway, but not the Toll pathway (Figure 1B). To analyze PGRP-SD function, we first tested *PGRP-SD*$^{\Delta}$ flies (Bischoff et al., 2004) but realized that none of the available *PGRP-SD*$^{\Delta 3}$ labeled fly stocks originating from different laboratories carried the expected *PGRP-SD*$^{\Delta 3}$ mutation (Figure S1D). This prompted us to generate two independent *PGRP-SD* mutant alleles (*PGRP-SD*$^{sk1}$ and *PGRP-SD*$^{sk2}$) by using the CRISPR-Cas9 method. Both alleles have a small deletion (Figure 1A), inducing a frameshift causing a premature stop codon and leading to a peptide of 50 residues lacking the PGRP domain (Figure S1E). Given that both alleles behaved similarly, results are shown only for one of them (*PGRP-SD*$^{sk1}$). To eliminate the influence of any second site mutations and to limit background effect, we backcrossed the *PGRP-SD*$^{sk1}$ mutant seven times to *white* isogenic Drosdel flies, and these isogenized flies with their wild-type counterpart were used in all experiments unless stated otherwise.

*PGRP-SD*$^{sk1}$ flies exhibited a wild-type lifespan at 29°C (Figure 1C), were viable and fertile, and had no obvious developmental defects, indicating that under normal conditions, the *PGRP-SD*$^{sk1}$ mutation has no detrimental effects on fly physiology. Considering that *PGRP-SD* is strongly induced by bacterial infection (Figure S1C) and that some PGRPs in mammals and insects have bactericidal activity, we explored whether PGRP-SD could function in a similar manner as an effector molecule. To test whether it participates in bacterial elimination, we monitored the persistence of the Gram-negative bacterium *Erwinia carotovora* and the DAP-type Gram-positive bacterium *Listeria monocytogenes* in wild-type and *PGRP-SD*$^{sk1}$ flies. As shown by colony-forming unit (CFU) counts (Figures 1D and 1E), the *PGRP-SD* mutation did not affect persistence of either bacterial species. In line with this result, incubation of several bacterial species with a recombinant PGRP-SD protein did not affect bacteria viability in comparison to that of untreated controls (Figure 1F), indicating that PGRP-SD lacks bactericidal activity. Moreover, we did not observe any role for PGRP-SD in the melanization response upon septic injury (Figures S1F and S1G).

### PGRP-SD Is Not Required for Activation of the Toll Pathway

Previous studies have suggested a role for PGRP-SD in the activation of the Toll pathway in response to Lys-type bacteria (Bischoff et al., 2004; Wang et al., 2008) or DAP-type Gram-positive bacteria (Leone et al., 2008). To test the involvement of PGRP-SD in activation of the Toll pathway, we measured by qRT-PCR the expression of *Drs* (the gene that encodes Drosomycin), a typical readout of the pathway, in *PGRP-SD*$^{sk1}$ flies. As shown in Figures 2A–2C, the level of *Drosomycin* expression in *PGRP-SD*$^{sk1}$ flies was similar to that in wild-type flies both after infection with *Micrococcus luteus* and *Enterococcus faecalis* and after injection of purified Lys-type peptidoglycan. Consistently, *PGRP-SD*$^{sk1}$ flies did not show an increased susceptibility to these infections (Figures 2D and 2E). Similarly, knocking down *PGRP-SD* by

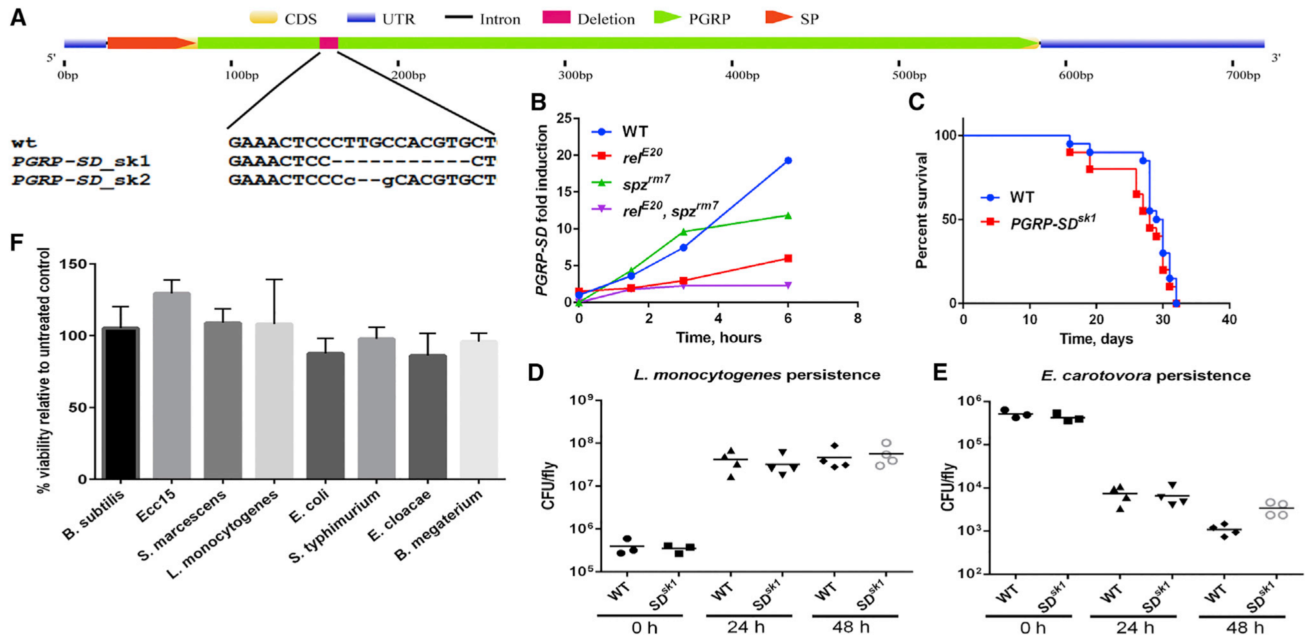


**Figure 1. PGRP-SD Is an Inducible Protein Lacking Bactericidal Activity**

(A) Schematic representation of the *PGRP-SD* locus (generated with GSDS 2.0; Hu et al., 2015) and *PGRP-SD* mutant alleles generated by the CRISPR-Cas9 method. Deleted sequences are represented by a dashed line. The $PGRP\text{-}SD^{sk1}$ allele is caused by an 11 bp deletion (nucleotides 129–139), the $PGRP\text{-}SD^{sk2}$ allele is caused by a 2 nt deletion (130–131) and two nucleotide substitutions. SP, signal peptide; UTR, untranslated region; CDS, coding sequence.

(B) The *PGRP-SD* mRNA expression level in different mutants after septic injury with a mixture of *E. coli* and *M. luteus*. The data for this figure (n = 3) were extracted from De Gregorio et al. (2002).

(C) Lifespan of wild-type (WT) and *PGRP-SD* mutant flies at 29°C (p = 0.2774, WT versus $PGRP\text{-}SD^{sk1}$). The experiment was repeated three times with two to three cohorts of 20 flies within each independent experiment. The results of one representative experiment are shown.

(D and E) Persistence of *L. monocytogenes* (D) and *E. carotovora* (E) in $PGRP\text{-}SD^{sk1}$ mutants and WT flies (p > 0.8, WT versus $PGRP\text{-}SD^{sk1}$, one-way ANOVA and Sidak's test). The horizontal axis indicates the median number of colony-forming units (CFUs) per fly of two experiments, each done in duplicate.

(F) Effect of recombinant PGRP-SD (300 μg/L) on bacterial viability after 4 hr incubation in comparison viability of untreated controls (set to 100%).

Results are shown as means ± SD of three independent experiments. See also Figure S1.

RNAi did not lead to reduced *Drosomycin* expression after *M. luteus* infection (Figure S2A).

To test a possible redundancy between PGRP-SD and PGRP-SA or GNBP1, two known PRRs acting upstream of the Toll pathway (Gobert et al., 2003; Pili-Floury et al., 2004), we generated double-mutant $PGRP\text{-}SA^{seml}$;;$PGRP\text{-}SD^{sk1}$ and $GNBP1^{osi}$,$PGRP\text{-}SD^{sk1}$ flies. We then infected flies with *M. luteus* and *Streptococcus pyogenes*. Whereas the immune response to *M. luteus* is strictly PGRP-SA- and GNBP1-dependent, *S. pyogenes* has been shown to activate the Toll pathway in a PGRP-SA- and GNBP1-independent manner (Bischoff et al., 2004). Nevertheless, it was reported (Bischoff et al., 2004) that the activation of the Toll pathway by this bacterium was reduced in a *PGRP-SA;;PGRP-SD* double mutant, leading to the initial conclusion that PGRP-SD is involved in the sensing of certain Gram-positive bacteria upstream of the Toll pathway. Figures S2B–S2E show, however, that *Drosomycin* expression in the double mutant was not significantly different from its expression in single *PGRP-SA* or *GNBP1* mutants after challenge with *M. luteus* and *S. pyogenes*. In line with this, double-mutant flies did not exhibit increased susceptibility to *S. aureus* and *S. pyogenes* infection in comparison to *PGRP-SA* and *GNBP1* single mutants (Figures 2F–2G; Figures S2F and S2G).

We then investigated whether PGRP-SD might be involved in Toll pathway activation by DAP-type bacteria, as was suggested by Leone et al. (2008). Again, we did not find evidence for this, given that *Drosomycin* expression after challenge with the DAP-type bacteria *Bacillus subtilis* and *Escherichia coli* was not affected in $PGRP\text{-}SD^{sk1}$ flies (Figure 2H). Thus, in contrast to previous reports, we did not find that PGRP-SD alone or in combination with other Toll pathway PRRs was required for Toll pathway activation in response to Lys- or DAP-type bacteria. Similarly, results shown in Figures S2H–S2J did not reveal any role for PGRP-SD in Toll pathway activation by fungi or in resistance to fungi.

## Systemic Imd Pathway Activation Is Reduced in $PGRP\text{-}SD^{sk1}$ Mutant

The facts that PGRP-SD is a DAP-type peptidoglycan-binding protein (Leone et al., 2008) and is regulated by *Relish* (Figure 1B) suggest that it might be involved in the activation of the Imd pathway. To test this, we monitored by qRT-PCR the expression of the Imd target gene *Diptericin* (*Dpt*) in $PGRP\text{-}SD^{sk1}$ flies infected with the Gram-negative bacteria *E. carotovora*. We observed that *Dpt* expression in $PGRP\text{-}SD^{sk1}$ flies was similar to that in wild-type flies 1.5 and 3 hr after infection. However, *Dpt* expression was significantly lower in the mutants 6 and

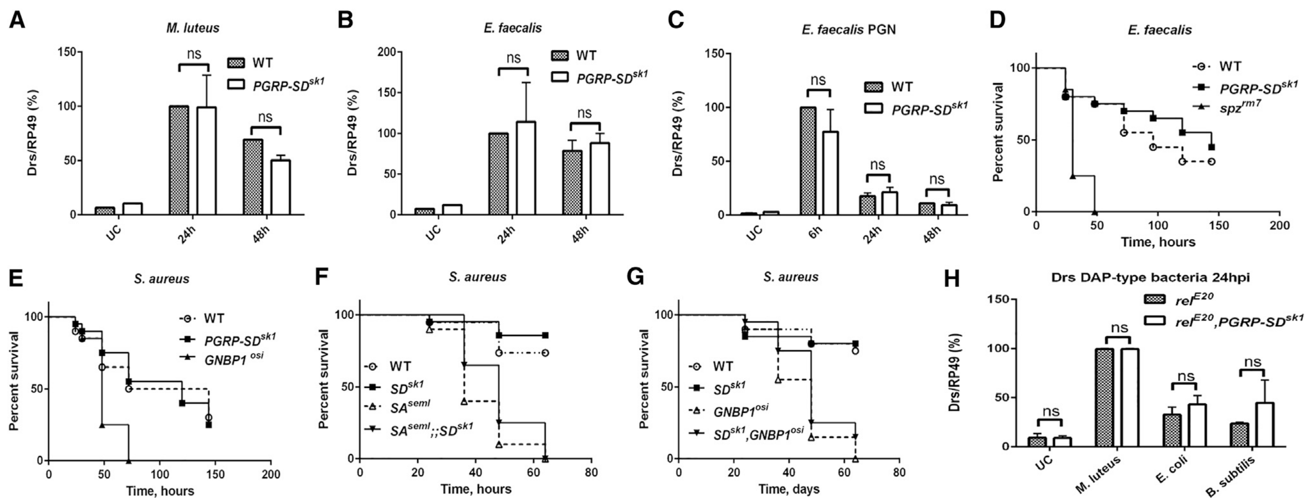


**Figure 2. Effect of *PGRP-SD* Mutation on Toll Pathway Activation**

(A–C) Induction of *Drosomycin* (*Drs*) expression in *PGRP-SD*$^{sk1}$ mutants and WT flies in response to systemic challenge with *M. luteus* (A) and *E. faecalis* (B) and injection of *E. faecalis* peptidoglycan (C).

(D and E) Survival rates of WT and *PGRP-SD*$^{sk1}$ mutants systemically infected with *E. faecalis* (D) and *S. aureus* (E) ($p > 0.4$, WT versus *PGRP-SD*$^{sk1}$). Flies with a defective Toll pathway were used as positive controls ($p < 0.002$, *spz*$^{rm7}$ versus *PGRP-SD*$^{sk1}$; $p < 0.0001$, *GNBP1* versus *PGRP-SD*$^{sk1}$).

(F and G) Survival rates of *PGRP-SA* (F) and *GNBP1* (G) single mutants and of *PGRP-SA*$^{seml}$*;;PGRP-SD*$^{sk1}$ and *GNBP1*$^{osi}$*,PGRP-SD*$^{sk1}$ double mutants after systemic *S. aureus* infection ($p > 0.05$, single mutants versus double mutants).

(H) Systemic Toll pathway activation 24 hr after challenge with DAP-type bacteria *E. coli* and *B. subtilis*. The experiment was performed in a *relish*$^{E20}$ mutant background lacking a functional Imd pathway to monitor only the contribution of the Toll pathway to *Drs* expression. The *Drs* mRNA level in *M. luteus*-challenged *relish* flies was set to 100%, and all other values were expressed as a percentage of this value.

For qRT-PCR results, *Drs* mRNA levels in challenged WT flies (24 hr) were set to 100%, and all other values were expressed as a percentage of this value. qRT-PCR results are shown as mean ± SD from ten female flies per genotype from three independent experiments. All survival graphs show one representative experiment out of three independent repeats with two to three cohorts of 20 male flies per genotype. Two-tailed Student's t tests were used for data analysis in (A)–(C) and (H). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$; ns (non-significant), $p > 0.05$. See also Figure S2.

24 hr after infection (Figure 3A). This late effect is consistent with the inducible nature of PGRP-SD in adults, which is hypothesized to accumulate in the hemolymph before it exerts its function. Similarly, a reduction of *Dpt* expression was also observed in larvae systemically infected with *E. carotovora* (Figure S3A). Interestingly, we noticed that larvae responded faster and stronger than adult flies to *E. carotovora* infection (Figure S3B). We hypothesized that the higher immune reactivity of larvae than of adults is due to a high level of *PGRP-SD* basal expression at this stage in the absence of infection. Returning to the study of the adult immune system, we then confirmed the role of PGRP-SD in Imd pathway activation by two other DAP-type bacteria, *E. coli* and *Pseudomonas entomophila*. For these two bacterial species, the difference in *Dpt* expression between wild-type and *PGRP-SD* mutant flies was even more striking than with *E. carotovora* (Figures 3B and 3C). A similar effect was also observed when the DAP-type Gram-positive bacterium *B. subtilis* was used to infect the flies (Figure 3D). Furthermore, injection of *E. coli* polymeric peptidoglycan led to a lower *Dpt* induction in *PGRP-SD*$^{sk1}$ flies than in wild-type flies (Figure 3E). A similar effect of PGRP-SD was also observed for two other antimicrobial peptide genes regulated by the Imd pathway: *Attacin A1* and *Drosocin* (Figures S3C–S3H).

A peptidoglycan monomer, GlcNAc-1,6-anhydro-MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala, also called the tracheal cytotoxin (TCT), was identified as the minimal peptidoglycan motif able to elicit an Imd pathway response in *Drosophila* (Kaneko et al., 2004; Stenbak et al., 2004). Figure 3F shows that injection of TCT resulted in lower *Dpt* expression in *PGRP-SD*$^{sk1}$ mutants 6 hr after injection. This result indicates that PGRP-SD is required for proper Imd pathway activation in response to both polymeric and monomeric DAP-type peptidoglycan. Moreover, injection of serially diluted, heat-killed *E. carotovora* or purified *E. coli* peptidoglycan revealed a significantly lower expression of *Dpt* in *PGRP-SD*$^{sk1}$ mutants at all tested concentrations (Figures 3G; Figure S3I). Interestingly, the Imd pathway of wild-type flies strongly responded to *E. carotovora* even when the optical density (OD) of the bacterial pellet used to challenge the flies was 0.2, whereas the Imd response of *PGRP-SD*$^{sk1}$ flies at this concentration was not significantly different from that of flies injected with PBS alone (Figure 3G). Thus, our results indicate that the role of PGRP-SD in promoting the immune response is particularly important at low elicitor amounts.

We next used an in vivo RNAi approach and rescue experiments to confirm the *PGRP-SD*$^{sk1}$ mutant phenotypes. Silencing of *PGRP-SD* in the fat body (*c564-GAL4* and *lpp-GAL4* drivers) or ubiquitously (*act5C-GAL4*) led to reduced *Dpt* induction after *E. carotovora* challenge. In contrast, RNAi knockdown of *PGRP-SD* in hemocytes (*hml-GAL4*), the midgut (*Myo1A-GAL4*), or the nervous system (*elav-GAL4*) had no effect (Figure 3H). Therefore, the RNAi experiments confirm the results obtained with the *PGRP-SD* mutation and show that its function in the systemic activation of the Imd pathway requires *PGRP-SD* expression in the fat body. Consistent with this, overexpression of *PGRP-SD*

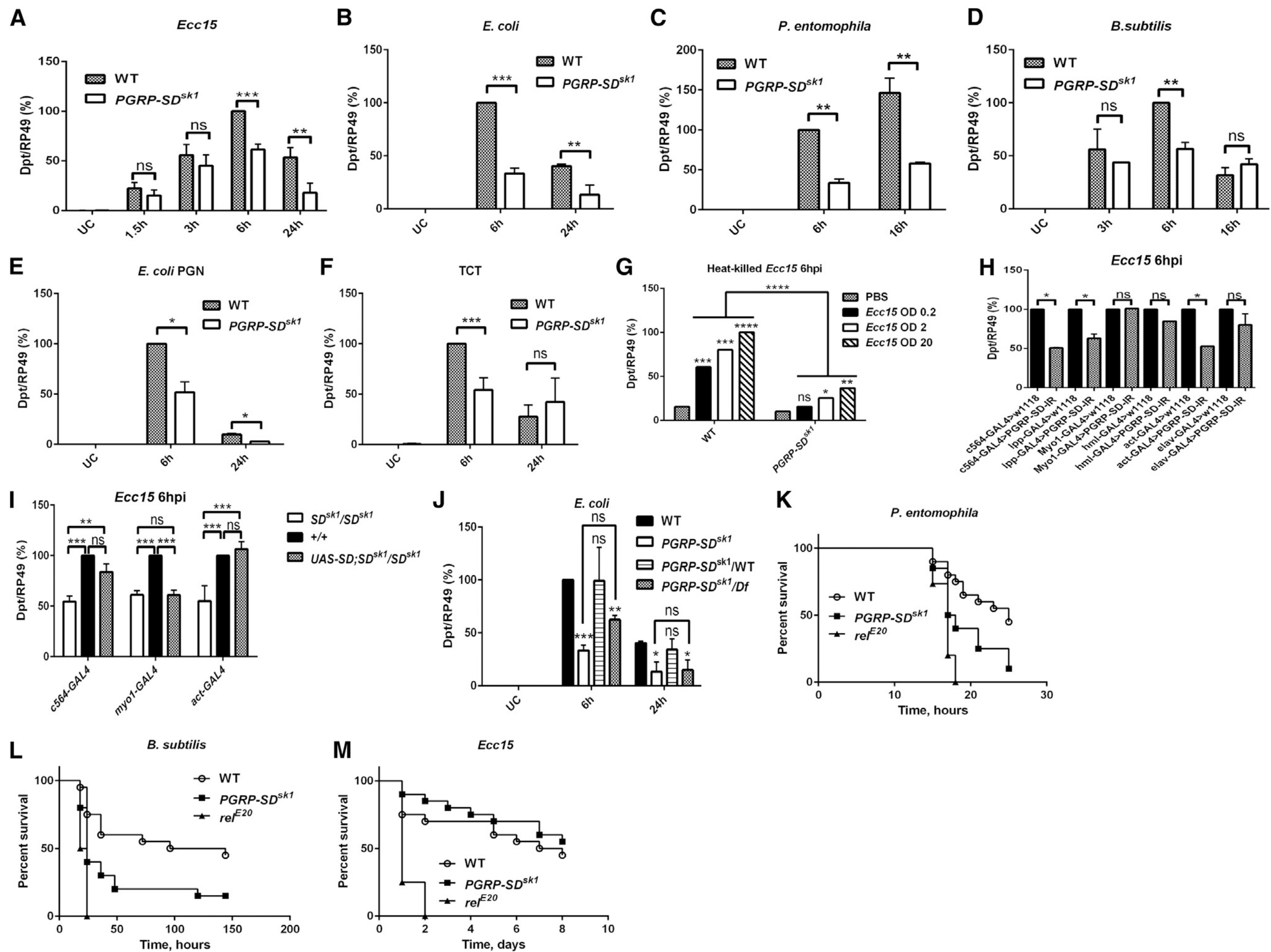


**Figure 3. PGRP-SD Is Required for Systemic Imd Pathway Activation**

(A–D) Induction of *Diptericin* (*Dpt*) expression in *PGRP-SD*$^{sk1}$ mutants and WT flies after systemic infection with *E. carotovora* OD200 (A), *E. coli* OD200 (B), *P. entomophila* OD1 (C), and *B. subtilis* OD30 (D).

(E and F) *Dpt* expression in response to injection of polymeric PGN (E) and monomeric PGN (F) in *PGRP-SD*$^{sk1}$ mutants and WT flies.

(G) Dose-dependent response of *PGRP-SD*$^{sk1}$ mutants and WT flies to heat-killed *E. carotovora*. Asterisks indicate significance levels in comparison to those of PBS-injected flies. One representative experiment out of three is shown.

(H) Effect of tissue-specific *PGRP-SD* knockdown by RNAi on *Dpt* expression 6 hr after systemic infection with *E. carotovora*. *Dpt* expression in *GAL4*-driver stocks crossed with $w^{1118}$ was set to 100%, and expression in *GAL4* stocks crossed with *UAS-PGRP-SD-IR* was expressed as a percentage of this value.

(I) Tissue-specific rescue of *Dpt* expression in *PGRP-SD*$^{sk1}$ mutants 6 hr after systemic infection with *E. carotovora*.

(J) *Dpt* expression in response to systemic *E. coli* infection in *PGRP-SD*$^{sk1}$ mutants and *PGRP-SD*$^{sk1}$ mutants transheterozygous with deficiency. Asterisks indicate significance levels in comparison to those of wild-type flies 6 and 24 hr after infection.

(K–M) Survival rates of *PGRP-SD*$^{sk1}$ mutants and WT flies systemically infected with *P. entomophila* (K, p = 0.0137, WT versus *PGRP-SD*), *B. subtilis* (L, p = 0.0149, WT versus *PGRP-SD*), and *E. carotovora* (M, p > 0.05, WT versus *PGRP-SD*). Flies with a defective Imd pathway (*rel*$^{E20}$) were used as positive controls. For qRT-PCR results, *Dpt* mRNA levels in challenged WT flies (6 hr) were set to 100%, and all other values were expressed as a percentage of this value. qRT-PCR results are shown as means ± SD from ten female flies per genotype from three independent experiments. Student's t tests were used for data analysis in (A)–(F); one-way ANOVA with Bonferroni post-test was used for data analysis in (H); and two-way ANOVA and Tukey's post-test were used for data analysis in (G), (I), and (J). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001; ns (non-significant), p > 0.05. See also Figure S3.

in the fat body of *PGRP-SD*$^{sk1}$ flies (genotype: *C564-Gal4/uas-PGRP-SD; PGRP-SD*$^{sk1}$*/PGRP-SD*$^{sk1}$) was sufficient to restore the strong *Dpt* expression upon infection with *E. carotovora* or *E. coli* (Figure 3I; Figure S3J). Rescue of the *PGRP-SD* phenotype was also obtained when *PGRP-SD* was ubiquitously expressed, but not when it was expressed only in the midgut. Finally, *Dpt* activation upon *E. coli* infection was lower in flies carrying the *PGRP-SD*$^{sk1}$ mutation than in those with a deficiency that removes the *PGRP-SD* locus and some flanking genes (Figure 3J). Thus, the *PGRP-SD* phenotype was consistent in two loss-of-function contexts with distinct genetic backgrounds.

We next investigated whether the loss of *PGRP-SD* had detrimental consequences on the ability of flies to fight off infections. We observed that *PGRP-SD*$^{sk1}$ flies displayed an enhanced susceptibility to a systemic infection with *P. entomophila* (Figure 3K):

the survival rate of *PGRP-SD*$^{sk1}$ flies was intermediate between those of Imd-deficient (*Relish*) and wild-type flies. Moreover, *PGRP-SD*$^{sk1}$ flies showed a higher susceptibility to the Gram-positive DAP-type *B. subtilis* than did wild-type flies (Figure 3L). Nevertheless, *PGRP-SD*$^{sk1}$ did not show higher susceptibility to *E. carotovora* (OD = 200) infection than did wild-type flies (Figure 3M). Because *E. carotovora* is weakly pathogenic, we speculate that the residual immune response observed in the *PGRP-SD* mutant upon *E. carotovora* infection is sufficient to allow survival in these conditions.

Collectively, our results demonstrate that PGRP-SD promotes Imd pathway activation in response to DAP-type bacteria, which is consistent with its DAP-type peptidoglycan-binding activity (Leone et al., 2008).

## PGRP-SD Functions Upstream of PGRP-LC

Our observation that PGRP-SD, a secreted PGRP with an affinity for DAP-type peptidoglycan, promotes Imd pathway activation supports the notion that PGRP-SD functions as a PRR upstream of the Imd pathway. An attractive hypothesis would be that PGRP-SD binds to extracellular peptidoglycan to facilitate its interaction with PGRP-LC. This would be similar to the function of mammalian secreted CD14, which binds to lipopolysaccharides (LPSs) in circulation to bring it in close proximity to the LPS receptor complex composed of TLR4 and MD2 (Park and Lee, 2013). To test such a model, we investigated how PGRP-SD interacts with PGRP-LC and peptidoglycan. Figure 4A shows that overexpression of *PGRP-SD* in an otherwise wild-type background did not activate the Imd pathway in the absence of infection. Overexpression of *PGRP-SD*, however, enhanced Imd pathway activation upon injection of live *E. carotovora* but only when a low dose of the bacterium was used (Figures 4A and 4B). In line with this, *PGRP-SD* overexpression also enhanced Imd signaling after injection of polymeric *E. coli* peptidoglycan and TCT (Figures S4A and S4B). This indicates that PGRP-SD is not essential for Imd pathway activation but enhances Imd pathway function, notably at a low immune elicitor concentration. The Imd stimulatory effect of PGRP-SD at low bacterial doses was abolished in *PGRP-LC* but not in *PGRP-LE* mutants, confirming that PGRP-SD functions upstream of PGRP-LC (Figure 4C). We observed that the loss of *PGRP-SD* provoked a stronger immune deficiency than the loss of *PGRP-LE* in response to systemic infection with Gram-negative bacteria (Figure S3K).

## PGRP-SD Re-localizes Peptidoglycan to the Cell Surface

To analyze whether PGRP-SD interacts with membrane-bound PGRP-LC, we performed an immuno-precipitation assay by incubating PGRP-SD-V5 produced in S2 cells and PGRP-LCx-GFP protein purified from flies. Whereas no direct interaction between the two proteins was observed, PGRP-LCx co-purified with PGRP-SD in the presence of *E. coli* peptidoglycan (Figure 4D). No interaction was observed in the presence of monomeric peptidoglycan (TCT). These results suggest that PGRP-SD might interact with PGRP-LCx but only in the presence of polymeric peptidoglycan. We then analyzed the impact of PGRP-SD on Imd pathway activation in cell culture. PGRP-SD-V5 expressed from a copper-inducible promoter in S2 cells was found in both the cellular fraction (Figure 4E) and the conditioned media of S2 cells (Figure 4F), confirming that PGRP-SD is a secreted protein. Additionally, consistent with the result obtained in vivo (Figure 4A), PGRP-SD by itself did not induce *Dpt* expression in S2 cells. Instead, it strongly enhanced peptidoglycan-mediated induction of *Dpt* (Figure 4G). This effect was abolished when *PGRP-LCx*, but not *PGRP-LCa* or *PGRP-LCy*, was silenced by RNAi (Figure 4H; Figure S5A). A similar albeit weaker effect was observed when cells were treated with TCT (Figure 4G). In the case of TCT, both *PGRP-LCx* and *PGRP-LCa* were required for *Dpt* and *Attacin A1* induction (Figure 4H; Figure S5A). Next, to test whether PGRP-SD promotes peptidoglycan re-localization to the cells, we imaged biotin-labeled *E. coli* peptidoglycan incubated with S2 cells with or without PGRP-SD expression. Figure 4I shows a marked increase in peptidoglycan localization on S2 cells in the presence of PGRP-SD (Figure 4I; Figure S5B), which required PGRP-LC (Figures S5C and S5D). Importantly, we also observed peptidoglycan re-localization to the cell surface when PGRP-SD was added exogenously to the cells that did not express *PGRP-SD* (Figure S5E), indicating that PGRP-SD functions extracellularly. Consistent with our hypothesis, the addition of *E. coli* peptidoglycan also led to an enrichment of PGRP-SD on S2 cells (Figures 4J; Figure S5F). Thus, PGRP-SD is a secreted protein that interacts with peptidoglycan and facilitates their interaction with the cell membrane, where PGRP-LC is localized.

## The Antagonistic Activities of PGRP-SD and PGRP-LB Shape the Immune Response

Enzymatic PGRPs function extracellularly to scavenge peptidoglycan, thereby reducing Imd pathway activity (Costechareyre et al., 2016; Paredes et al., 2011; Zaidman-Rémy et al., 2006). Among them, PGRP-LB has enzymatic activity against DAP-type peptidoglycan and is regulated at the transcriptional level by the Imd pathway to provide a negative feedback loop on the pathway (Zaidman-Rémy et al., 2006). Although PGRP-SD and PGRP-LB have opposite functions, they share many similarities because they are both secreted, are upregulated upon infection by the Imd pathway, and display binding specificity to DAP-type peptidoglycan. We generated *PGRP-SD*$^{sk1}$*,LB*$^{\Delta}$ double-mutant flies to analyze the interaction between these two extracellular regulators. As expected, *PGRP-LB* mutants showed a much stronger *Dpt* expression than wild-type flies after *E. carotovora* systemic infection (Figure 5A). Interestingly, wild-type levels of *Dpt* expression were observed in the *PGRP-SD*$^{sk1}$*,LB*$^{\Delta}$ double-mutant flies (Figure 5A). This indicates that the absence of PGRP-SD can counteract the excessive activation of the Imd pathway in *PGRP-LB*$^{\Delta}$ flies. The same result was observed with *E. coli* (Figures 5B and 5C). *PGRP-LB*-deficient flies showed a lower survival rate in response to oral bacterial infection and lower longevity as a result of chronic activation of the Imd pathway (Paredes et al., 2011). Consistent with this, the *PGRP-SD*$^{sk1}$ mutation suppressed the *PGRP-LB*$^{\Delta}$ precocious lethality to *E. carotovora* oral infection (Figure 5D). Moreover, *PGRP-SD*$^{sk1}$*,LB*$^{\Delta}$ double-mutant flies exhibited a wild-type lifespan at 29°C, whereas *PGRP-LB*$^{\Delta}$ flies were short lived (Figure 5E). Collectively, our results show that two secreted PGRPs with antagonistic activities function upstream of PGRP-LC to modulate the level of Imd signaling.

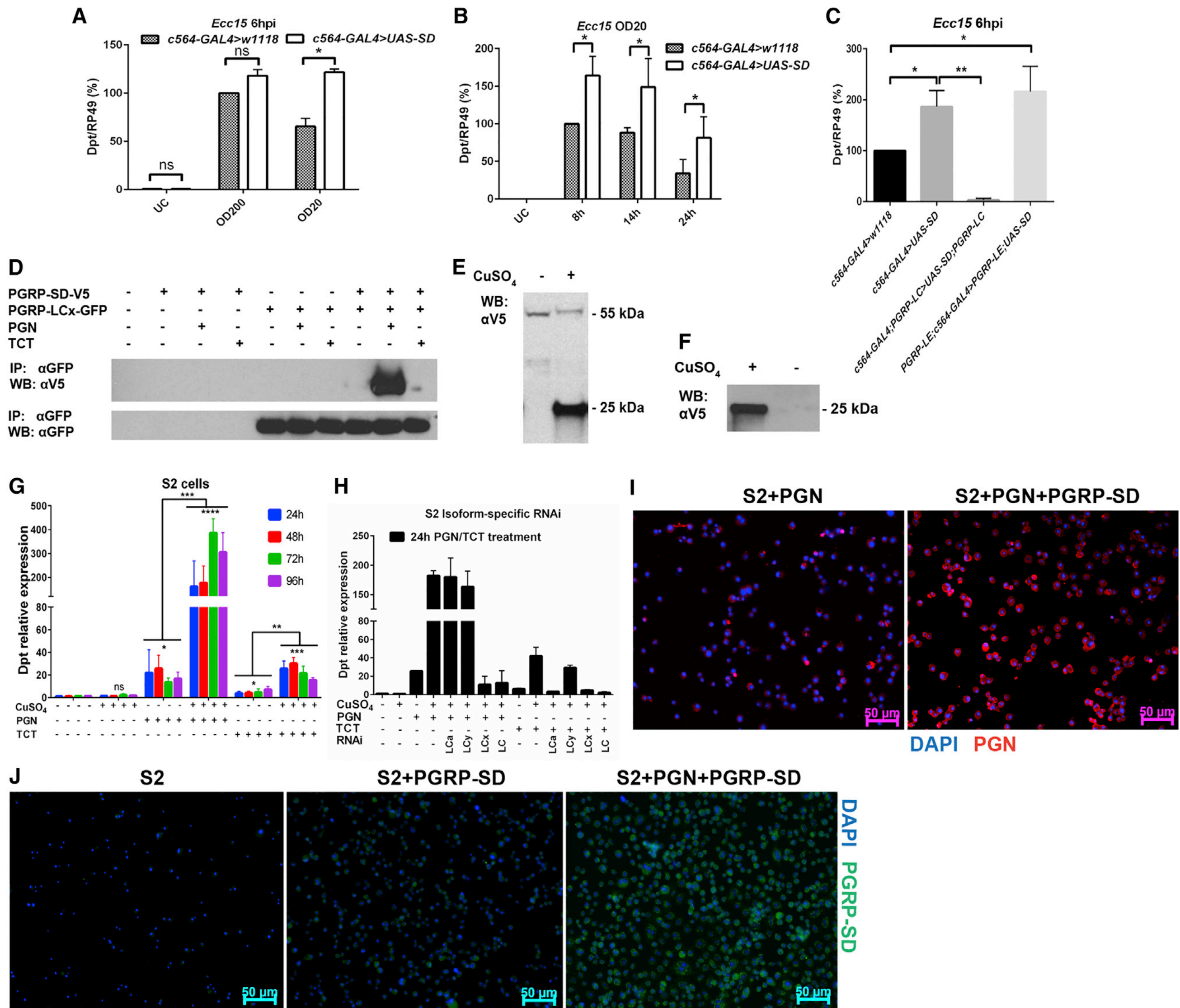


**Figure 4. PGRP-SD Acts Upstream of PGRP-LC to Facilitate Peptidoglycan Sensing and Imd Pathway Activation**

(A) Effect of *PGRP-SD* overexpression in the fat body on *Dpt* induction after systemic infection with *E. carotovora*.

(B) *Dpt* induction after systemic infection with *E. carotovora* in a *PGRP-SD*-overexpressing line and a control line over time.

(C) Effect of *PGRP-SD* overexpression in the fat bodies of Imd pathway mutants on *Dpt* induction after systemic infection with *E. carotovora*. Results are shown as mean ± SD from ten female flies per genotype from at least three independent experiments.

(D) PGRP-SD co-immunoprecipitated with PGRP-LCx in the presence of PGN. Affinity-purified PGRP-LCx-GFP pulled down PGRP-SD-V5 from S2 cellular extracts in the presence of PGN. One representative blot is shown out of two repeats.

(E and F) Detection of PGRP-SD-V5 (~25 kDa) by anti-V5 immunoblot of lysates (E) and culture media (F) of S2 cells expressing V5-tagged PGRP-SD. A non-specific band at 55 kDa was used as a loading control (E). A representative blot out of two independent assays is shown.

(G) *Dpt* induction by peptidoglycan and TCT in S2 cells overexpressing PGRP-SD. S2 cells transfected with copper-inducible *PGRP-SD*-encoding plasmid were induced with copper sulfate (0.5 mM) for 24 hr and treated with *E. coli* peptidoglycan (10 μg/mL) or TCT (1 μM) for the indicated time points. *Dpt* expression in unchallenged cells at 24 hr was set to 1, and all other values were calculated in relation to this value. Asterisks indicate significance levels in comparison to those of unchallenged cells. Results are shown as mean ± SD of three independent experiments.

(H) Effect of isoform-specific *PGRP-LC* knockdown by RNAi on *PGRP-SD*-mediated activation of Imd pathway by *E. coli* peptidoglycan and TCT. Results are shown as mean ± SD of three independent experiments.

(I) Effect of *PGRP-SD* overexpression on peptidoglycan localization in S2 cells. PGRP-SD-expressing (S2+PGN+PGRP-SD) and non-expressing (S2+PGN) cells were treated with peptidoglycan and stained with DAPI (blue) and anti-biotin (to detect biotinylated peptidoglycan, stained in red). Shown is a representative experiment out of two.

(J) Effect of peptidoglycan on PGRP-SD localization in S2 cells. Untreated cells (S2), cells that expressed PGRP-SD-V5 (S2+PGRP-SD), and cells that expressed PGRP-SD-V5 in the presence of *E. coli* peptidoglycan (S2+PGN+PGRP-SD) were stained with anti-V5 to detect PGRP-SD (shown in green) and DAPI (blue). Shown is a representative experiment out of two.

One-way ANOVA with Tukey's post-test was used for data analysis in (C), two-way ANOVA and Tukey's post-test were used for data analysis in (G); and Student's t tests were used for data analysis in (A) and (B). $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$; ns (non-significant), $p > 0.05$. See also Figure S4 and S5.

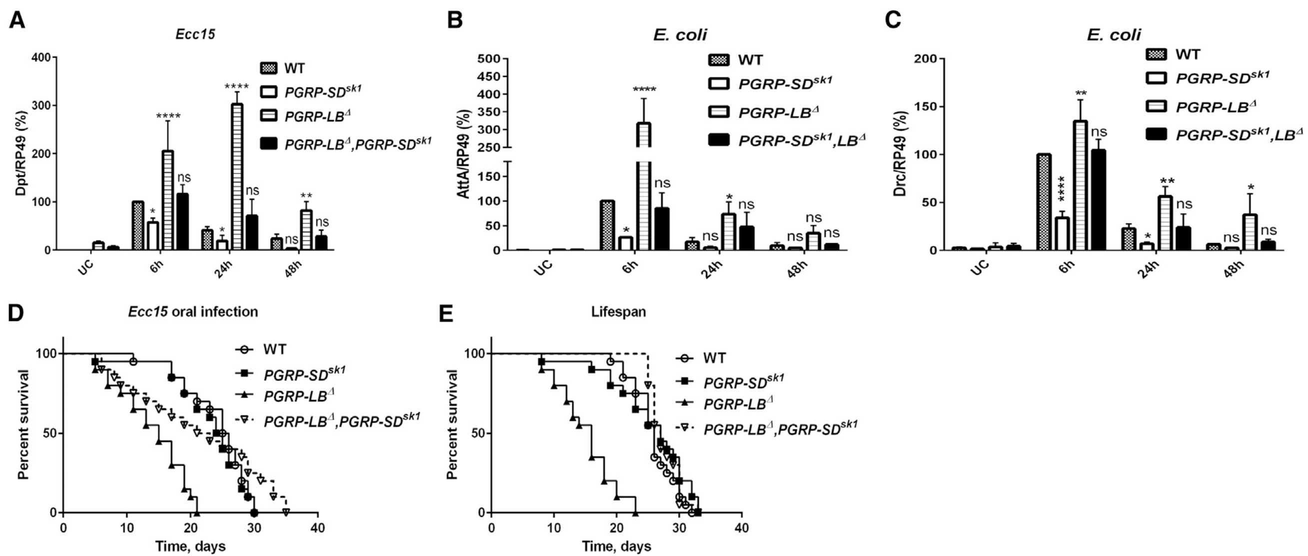


**Figure 5. The *PGRP-SD* Mutation Counterbalances the Excessive Immune Activation in *PGRP-LB* Mutant Flies**

(A) Effect of *PGRP-SD* mutation on *Dpt* expression level in *PGRP-LB* mutants after *E. carotovora* systemic infection.

(B and C) Effect of *PGRP-SD* mutation on *Attacin A1* (B) and *Drosocin* (C) expression in *PGRP-LB* mutants after *E. coli* systemic infection.

(D) Survival rates of *PGRP-LB* flies alone or in combination with *PGRP-SD*$^{sk1}$ after *E. carotovora* oral infection ($p > 0.4$, WT versus *LB,SD*; $p < 0.002$, *LB* versus *LB,SD*).

(E) Lifespan of *PGRP-LB,SD* double mutants and *PGRP-LB* and *PGRP-SD*$^{sk1}$ single mutants ($p > 0.2$, WT versus *LB,SD*; $p < 0.0001$, *LB* versus *LB,SD*).

For qRT-PCR results, *Dpt* mRNA levels in challenged WT flies (6 hr) were set to 100%, and all other values were expressed as a percentage of this value. qRT-PCR results are shown as mean ± SD from ten female flies per genotype from three independent experiments. Two-way ANOVA and Tukey's post-test were for data analysis in (A)–(C). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; ns (non-significant), $p > 0.05$.

## DISCUSSION

For a long time, PGRP-SD was considered to be a PRR for Gram-positive bacteria and to function redundantly with PGRP-SA and GNBP1 upstream of the Toll pathway (Bischoff et al., 2004). Little attention had been devoted to conflicting structural and in vitro binding data showing that PGRP-SD binds DAP-type and not Lys-type peptidoglycans (Leone et al., 2008) or to gene-expression profiling showing that *PGRP-SD* is a bona fide Imd pathway target (De Gregorio et al., 2002). Using newly generated *PGRP-SD* mutations, we have addressed this inconsistency and conclusively demonstrated that PGRP-SD is a PRR for DAP-type bacteria and has an essential function in Imd pathway activation. This conclusion is supported by the fact that *PGRP-SD* mutants exhibit reduced Imd pathway activation after challenge with a number of DAP-type bacteria, as well as both polymeric and monomeric peptidoglycans. We have shown that PGRP-SD co-immunoprecipitates with PGRP-LCx in the presence of peptidoglycan and enhances peptidoglycan-mediated activation of the Imd pathway in a PGRP-LC-dependent manner. Additional biochemical and cell-culture experiments revealed that PGRP-SD promotes peptidoglycan concentration at the proximity of the cell membrane in a PGRP-LC-dependent manner. This suggests a model in which PGRP-SD binds to peptidoglycan in the extracellular compartment and facilitates peptidoglycan concentration at the cell surface, where PGRP-SD-peptidoglycan complexes interact with the cell-surface receptor PGRP-LC to activate the Imd pathway. Contrary to previous studies using the "*PGRP-SD*$^{\Delta 3}$" fly stock (Bischoff et al., 2004; Leone et al., 2008; Wang et al., 2008), our data do not reveal a role for PGRP-SD, alone or in combination with PGRP-SA and GNBP1, in activation of the Toll pathway by Lys- or DAP-type bacteria. Given that none of the *PGRP-SD*$^{\Delta 3}$ labeled stocks we obtained appeared to carry the expected *PGRP-SD* mutation (Figure S1D), we hypothesize that the original *PGRP-SD*$^{\Delta 3}$ fly stock was either initially mischaracterized or rapidly contaminated by a wild-type stock. Our results indicate that the role previously allocated to PGRP-SD as the PGRP-SA-GNBP1 co-receptor either is executed by another protein or has to be explained by another process.

The mode of action of PGRP-SD is strongly evocative of that of soluble CD14, which plays an important role upstream of TLR4 to capture extracellular LPS and bring it to the proximity of a signaling receptor complex composed of MD2 and TLR4 (Park and Lee, 2013; Pugin et al., 1993). CD14 has been shown to underlie the high sensitivity of some mammalian species to LPS (Lee et al., 1992). Similarly, the presence of PGRP-SD contributes to the high sensitivity of *Drosophila* to DAP-type peptidoglycan, but in contrast to PGRP-LC, it is not mandatory for Imd pathway activation when a high dose of immune elicitor is present. Such a high sensitivity of the Imd pathway can be critical in sensing Gram-negative bacteria, which release only low amounts of peptidoglycan, because it is hidden under the LPS layer. An interesting observation was that PGRP-SD also sensitized the Imd pathway to monomeric peptidoglycan, although the stimulatory effect was less marked than with polymeric peptidoglycan. Although we did observe a stimulatory effect of PGRP-SD on TCT sensing in both flies and S2 cells, we did not

detect any biochemical interaction with PGRP-LCx. It is possible that the assay was not sensitive enough to detect such an interaction or that PGRP-SD interacts with another PGRP-LC isoform. An attractive hypothesis is that PGRP-SD oligomerizes upon TCT binding, as previously shown for the intracellular sensor PGRP-LE (Lim et al., 2006), and that PGRP-SD-TCT complexes then interact with PGRP-LCx/a. Future research should address the mechanism by which the peptidoglycan-PGRP-SD complex leads to dimerization of PGRP-LC and subsequent activation of Imd signaling and clarify how PGRP-SD interacts with TCT.

PGRP-SD is not the first PRR reported to act extracellularly upstream of PGRP-LC. A previous study has revealed the existence of an extracellular form of PGRP-LE, named PGRP-LE$^{pg}$, which can potentiate PGRP-LC activation by TCT (Kaneko et al., 2006). The absence of a signal peptide sequence in PGRP-LE and the observation that a *PGRP-LE-GFP* reporter localizes strictly intracellularly (Bosco-Drayon et al., 2012) question the existence of this extracellular PGRP-LE isoform and its precise role. Compared to that of *PGRP-LE*, inactivation of *PGRP-SD* induced a stronger immune defect, indicating that PGRP-SD has an important role in the activation of PGRP-LC during Gram-negative bacterial infection. It cannot be excluded that the main role of PGRP-SD is to enhance PGRP-LC activation by DAP-type polymeric peptidoglycan, whereas the role of extracellular PGRP-LE$^{pg}$ is restricted to TCT sensing. This would explain why PGRP-SD has a modest effect on TCT sensing. Future studies should determine whether PGRP-LE indeed functions extracellularly and analyze its interaction with PGRP-SD. Another interesting line of research would be to investigate the role of PGRP-SD in the gut immune response, given that it is also expressed in the intestinal epithelium.

Finally, our study underlines how tightly the Imd pathway is regulated by both negative and positive regulators extracellularly in the hemolymph. Although our data suggest that the main role of PGRP-SD is to present peptidoglycan to PGRP-LC at the cell membrane, it is possible that PGRP-SD also protects peptidoglycan from being scavenged by enzymatic PGRPs. The observation that *PGRP-SD$^{sk1}$,LB$^{\Delta}$* flies have an apparent wild-type immune response is at first puzzling and raises the question of the necessity of two such antagonistic activities. The existence of these regulators might provide robustness to the immune system to generate a range of immune responses. This could also provide regulatory flexibility by adjusting the immune reactivity in a stage- and tissue-specific manner through the modulation of *PGRP-LB* and *PGRP-SD* expression rather than by changing cellular core components of the pathway. Consistent with this hypothesis, the presence of PGRP-SD in the hemolymph of unchallenged larvae as a result of high basal transcriptional expression might explain the faster immune reactivity of larvae than of adults, where induced PGRP-SD needs to be accumulated to enhance Imd-immune activation.

Over the last two decades, it has been recognized that microbial recognition by the innate immune system is much more complex than the simple molecular contact between a host PRR and a microbial molecule. Pattern recognition is increasingly viewed as a multistep process with additional host factors involved upstream of recognition, including the degradation of bacterial cell-wall compounds and/or the transport of microbial ligands to the signaling receptors. By adding PGRP-SD to the list of PGRPs involved in the sensing of DAP-type peptidoglycan upstream of the Imd pathway, our study uncovers the complexity of pattern recognition in *Drosophila* while revealing general principles of innate immune sensing.

## EXPERIMENTAL PROCEDURES

Standard methods of molecular biology, microbiology, and biochemistry can be found in the Supplemental Experimental Procedures.

### Fly Stocks and Mutant Generation

DrosDel *w$^{1118}$ iso* isogenic flies described previously (Ryder et al., 2004) and *y,w* flies were used as wild-type controls. The *spz$^{rm7}$*, *GNBP1$^{osi}$*, *PGRP-SA$^{seml}$*, *rel$^{E20}$*, *PGRP-LC$^{E12}$*, *PGRP-LE$^{112}$*, *PGRP-LB$^{\Delta}$*, and *UAS-PGRP-LCx-GFP* flies have been described previously (Gobert et al., 2003; Gottar et al., 2002; Neyen et al., 2012; Paredes et al., 2011; Schmidt et al., 2008; Takehana et al., 2004). *UAS-PGRP-SD-IR* (56952) and *Df(3L)BSC388* (24412) were obtained from the Bloomington Stock Center. For the generation of *UAS-PGRP-SD* transgenic flies, a full-length cDNA of *PGRP-SD* was cloned into the pENTR-D-TOPO Gateway vector (Invitrogen) and finally swapped into the pTW transgenesis vector. The transgenic flies were obtained by *P*-element transgenesis. *PGRP-SD$^{sk1}$* and *PGRP-SD$^{sk2}$* flies were generated by the CRISR-CAS9 method, as described previously (Kondo and Ueda, 2013). *PGRP-SD$^{sk1}$* and *PGRP-LB$^{\Delta}$* mutations were introduced into the *w$^{1118}$* DrosDel *iso* isogenic background by chromosomes replacement and backcrossing as described in Ryder et al. (2004). Isogenic *spz$^{rm7}$*, *PGRP-LC$^{E12}$*, *rel$^{E20}$*, and DrosDel *w$^{1118}$ iso* lines were kindly provided by Luis Teixeira (Portugal). *Drosophila* stocks were maintained at 25°C on standard cornmeal-agar fly medium.

### qRT-PCR

For quantification of mRNA, whole flies were collected at the indicated time points. Total fly RNA was isolated from 10–15 adult flies by TRIzol reagent and dissolved in RNase-free water. A total of 500 ng RNA was then reverse transcribed in 10 μL reaction volume with PrimeScript RT (TAKARA) and random hexamer primers. qPCR was performed on a LightCycler 480 (Roche) in 96-well plates with Applied Biosystems SYBR Green PCR Master Mix. Primer sequences were published elsewhere (Neyen et al., 2014).

### PGRP-SD Expression in S2 Cells

S2 cells were cultured in Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated FBS and penicillin (10 U/mL) and streptomycin (100 mg/mL) at 25°C. For immune stimulation, *E. coli* peptidoglycan at a final concentration of 10 μg/mL and TCT at a final concentration of 1 μM were used. RNA extraction, reverse transcription, and qPCR were performed as described for fly tissues.

For protein expression, PGRP-SD cDNA was cloned into a pMT-V5-His vector (Invitrogen). Obtained pMT-PGRP-SD-V5-His plasmid was transfected into S2 cells with Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. PGRP-SD expression was induced by the addition of 0.5 mM $CuSO_4$. Protein expression was verified by western blot with the use of mouse monoclonal anti-V5 (Dianova) antibodies (1:1,000) and anti-mouse HRP-conjugated (Jackson Immunoreseach) secondary antibodies (1:10,000). Western blots were visualized with ECL western blotting detection reagents (GE Healthcare) and exposure to an X-ray film (Kodak). The time-dependent effect of PGRP-SD expression on peptidoglycan- and TCT-induced *Dpt* levels in S2 cells was evaluated as follows. S2 cells were transiently transfected with pMT-PGRP-SD-V5-His plasmid, and 24 hr post-transfection PGRP-SD expression was induced by $CuSO_4$. *E. coli* peptidoglycan (10 μg/mL final concentration) or TCT (1 μM) was added 24 hr after induction. Cells were harvested every 24 hr during a 4 day period for the gene-expression analysis. *PGRP-LC* knockdown by RNAi was achieved by transfection of S2 cells with double-stranded RNA (dsRNA). dsRNA was synthesized with the MEGAscript T7 Kit (Invitrogen).

## Statistical Analysis

All analyses were performed with GraphPad Prism software version 6.05. Survival curves were compared by log-rank tests; p values were Bonferroni corrected where multiple comparisons were necessary. All other data were analyzed by Student's t test or one-way or two-way ANOVA, as well as appropriate Dunnett, Tukey, Bonferroni, or Sidak post hoc tests.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.10.029.

## AUTHOR CONTRIBUTIONS

I.I. and B.L. designed the study and wrote the manuscript. I.I. performed experiments. S.K. and D.M.-L. provided materials and reagents.

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