The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition

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The *Drosophila* immune system discriminates between different classes of infectious microbes and responds with pathogen-specific defense reactions through selective activation of the Toll and the immune deficiency (Imd) signaling pathways. The Toll pathway mediates most defenses against Gram-positive bacteria and fungi, whereas the Imd pathway is required to resist infection by Gram-negative bacteria. The bacterial components recognized by these pathways remain to be defined. Here we report that Gram-negative diaminopimelic acid–type peptidoglycan is the most potent inducer of the Imd pathway and that the Toll pathway is predominantly activated by Gram-positive lysine-type peptidoglycan. Thus, the ability of *Drosophila* to discriminate between Gram-positive and Gram-negative bacteria relies on the recognition of specific forms of peptidoglycan.

The innate immune response is activated by receptors that recognize microbial surface determinants that are conserved among microbes but are absent in the host, such as lipopolysaccharide (LPS), peptidoglycan and mannan[1]. On recognition, these receptors activate signaling cascades that regulate the transcription of target genes encoding regulator and effector molecules. A transcription profile specific to one class of pathogen can be achieved when recognition receptors are linked to distinct signaling pathways.

Drosophila lacks an adaptive immune system and relies solely on innate immune reactions for its defense[2,3]. A hallmark of the *Drosophila* immune response is the synthesis by the fat body of several antimicrobial peptides with distinct but overlapping specificity. These peptides are secreted into the hemolymph, where they directly kill invading microorganisms. Genetic analyses show that antimicrobial peptide genes are regulated by the Toll and Imd pathways. These two pathways share many common features with the mammalian Toll-like receptor (TLR) and tumor necrosis factor-α (TNF-α) receptor signaling cascades that regulate NF-κB transcription factors[3,4]. Each of these pathways leads to the activation of specific NF-κB transactivators, which in turn activate specific programs of transcription[5,6].

The Toll pathway is triggered by the proteolytic cleavage of the Toll ligand Spätzle (Spz) and leads to activation of two Rel proteins, Dorsal immune-related factor and Dorsal. This pathway is activated mainly by Gram-positive bacteria and fungi, and it largely controls the expression of genes encoding antimicrobial peptides (such as *Drs*, which encodes drosomycin) that are active against fungi[7–11]. In contrast, the Imd pathway responds mainly to Gram-negative bacterial infection, and it controls the expression of genes encoding antibacterial peptides (such as *Dpt*, encoding diptericin) through activation of another Rel protein, Relish (encoded by *Rel*)[8,12]. Thus, the control of antimicrobial peptide genes by the Toll and Imd pathways provides a good model of the regulation of innate immune responses in animals and shows how two distinct signaling pathways can generate complex transcriptional patterns in response to different pathogens.

Microbial recognition acting upstream of the Toll and Imd pathways is achieved, at least in part, through peptidoglycan recognition proteins (PGRPs). These proteins bind to peptidoglycan, a component of the bacterial envelope, and are found in many species including insects and mammals[13,14]. Thirteen PGRPs have been identified in *Drosophila*[15], of which three are currently implicated in the immune response. PGRP-SA, an extracellular recognition factor, activates the Toll pathway in response to Gram-positive bacterial but not fungal infection[16]. PGRP-LC, a putative transmembrane protein, acts upstream of the Imd pathway[17–19]. PGRP-LE, a secreted PGRP, can activate the Imd pathway when overexpressed in flies, although determination of its exact function in the *Drosophila* immune response awaits loss-of-function analysis[20].

Despite the identification of some of the recognition proteins acting upstream of the Toll and Imd pathways, the microbial compounds that are recognized by the *Drosophila* immune system during the course of an infection have not been characterized. All of the genetic studies showing that the Imd pathway responds to Gram-negative bacterial infection and that the Toll cascade defends against Gram-positive bacteria and fungi[7–11] have been done with live bacteria.

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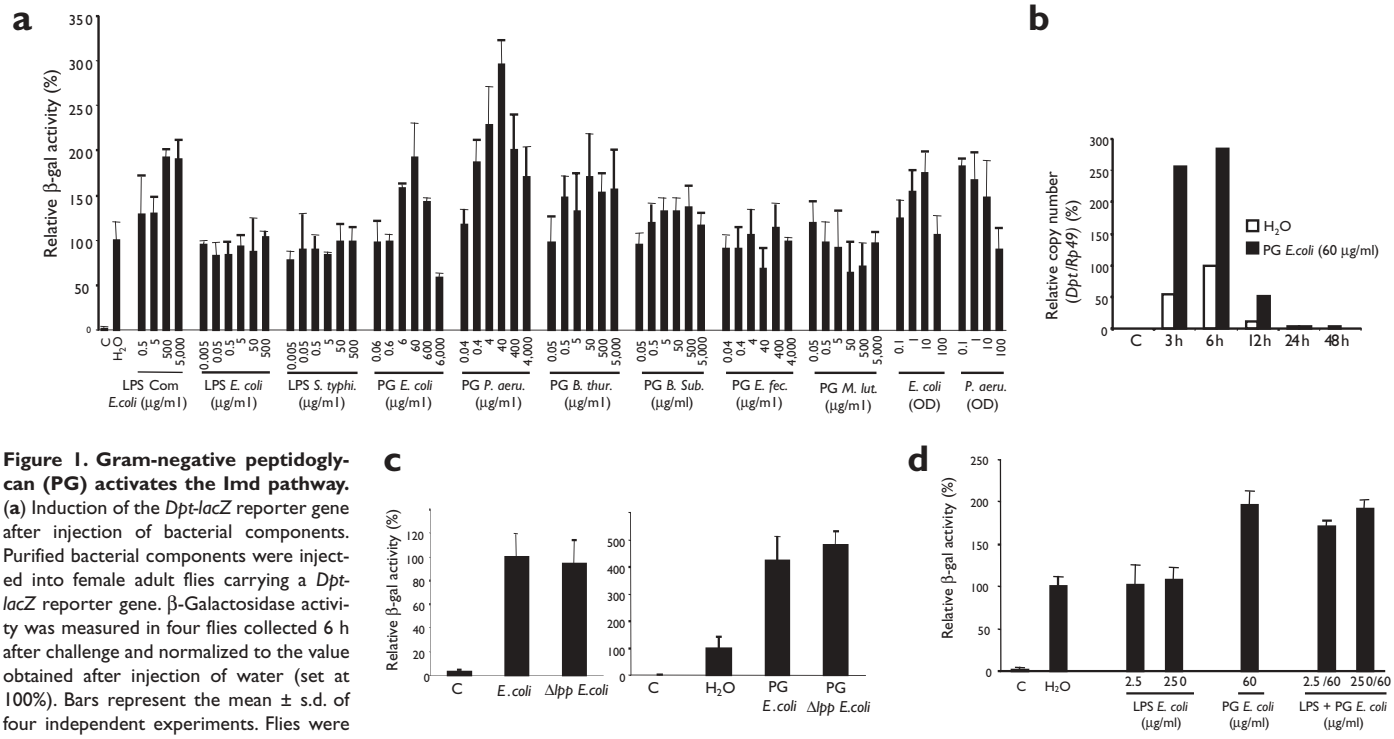

Figure 1. Gram-negative peptidoglycan (PG) activates the Imd pathway. (**a**) Induction of the *Dpt-lacZ* reporter gene after injection of bacterial components. Purified bacterial components were injected into female adult flies carrying a *Dpt-lacZ* reporter gene. β-Galactosidase activity was measured in four flies collected 6 h after challenge and normalized to the value obtained after injection of water (set at 100%). Bars represent the mean ± s.d. of four independent experiments. Flies were injected with commercial LPS, purified LPS extracted from *E. coli* or *S. typhimurium*, or purified PG from *E. coli* (G^-), *P. aeruginosa* PAO1 (G^-), *B. thuringiensis* (G^+), *B. subtilis* (G^+), *E. faecalis* (G^+) or *M. luteus* (G^+). As a positive control, flies were injected with increasing concentrations (measured by optical density; OD) of an overnight suspension of *E. coli* (1106) or *P. aeruginosa* (PAO1). This experiment was repeated twice with similar results. (**b**) Time course of *Dpt* gene expression after injecting Gram-negative PG. Quantitative real-time PCR analysis was done on total RNA extracts from Oregon wild-type female flies collected at the indicated intervals after injecting water or 60 µg/ml of *E. coli* (1106) PG. This experiment was repeated twice with similar results. (**c**) Effect of the *Δlpp* mutation on the ability of *E. coli* to induce *Dpt*. β-Galactosidase activity was measured in females carrying the *Dpt-lacZ* gene collected 6 h after challenge with either a needle dipped in a concentrated pellet (OD 100) of *E. coli* (BW25113) or *Δlpp E. coli* (left) or with 60 µg/ml of PG extracted from *E. coli* (BW25113) or *Δlpp E. coli* (right). C, unchallenged flies. (**d**) LPS and Gram-negative PG do not activate *Dpt* synergistically. Flies were injected with purified LPS, purified LPS plus Gram-negative PG, or commercial LPS from *E. coli*. PG was extracted and purified from *E. coli* (1106), and LPS was extracted and purified from *E. coli* (0111B4).

The presence of lipopolysaccharide (LPS) exclusively on the surface of Gram-negative bacteria has led to the assumption that LPS recognition by the fly immune system is a key step in discriminating between Gram-negative and Gram-positive bacteria. To test this assumption, we have purified components of various bacterial extracts and assayed their ability to induce the Toll and Imd pathways. Here we report that the ability of *Drosophila* to discriminate between Gram-positive and Gram-negative bacteria is not based on the detection of LPS but rather on the recognition of specific forms of peptidoglycan.

Results

LPS does not induce *Dpt* expression *in vivo*

The *Dpt* gene encodes an antibacterial peptide secreted by the fat body in response to bacterial infection. This gene is tightly regulated by the Imd pathway, and its expression profile provides an accurate readout of Imd pathway activity. To elucidate the microbial determinants that activate the Imd pathway, we purified components of various bacterial extracts and used a glass needle to inject them into the body cavities of adult flies carrying a *Dpt-lacZ* transgene. β-Galactosidase activity generated by the reporter gene was measured in whole flies collected 6 h after challenge. Because the injection procedure creates an injury that by itself triggers significant induction of the *Dpt* gene, we compared all β-galactosidase activities to those obtained after control injections of water (Methods).

One of the most potent activators of vertebrate innate immune responses is LPS, a principal component of the Gram-negative bacterial outer envelope[21,22]. Several previous studies have shown that commercial LPS preparations induce the expression of antibacterial peptide genes in *Drosophila*[23,24]; however, our injections of highly purified LPS from two Gram-negative bacteria, *Salmonella typhimurium* and *Escherichia coli*, did not stimulate the expression of *Dpt-lacZ* as compared with control water injections (**Fig. 1a**).

In contrast, the same LPS preparations efficiently activated mouse macrophages through a TLR4-dependant pathway (data not shown).

Table 1. Induction of *Dpt-lacZ* after injecting DAP-type peptidoglycan preparations

Solution	Control[1]	Peptidoglycan[1]	*P* value[2]
E. coli peptidoglycan	10.2 (38, 3.8)	23.5 (59, 6.5)	$<10^{-9}$
P. aeruginosa peptidoglycan	10.4 (16, 3.6)	20.7 (28, 4.7)	$<10^{-3}$
B. thuringiensis peptidoglycan	10.6 (6, 5.1)	18.0 (24, 6.1)	$<10^{-2}$

[1]Results are the mean (number of repeats, s.d.) of *Dpt-lacZ* activity obtained from adult females injected with water (control) or 50 µg/ml of the peptidoglycan preparation listed on the left. The result obtained with unchallenged adults was 0.9 (22, 1). [2]Value obtained with a *t*-test. Statistical analysis was done on raw data (without normalization).

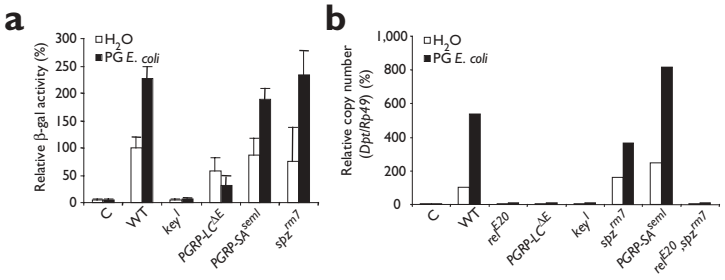

Figure 2. Gram-negative peptidoglycan (PG) induces *Dpt* expression *via* the Imd pathway. (**a**) β-Galactosidase activity in wild-type and mutant flies carrying the *Dpt-lacZ* reporter gene after injection with water or 60 μg/ml of *E. coli* (1106) PG. (**b**) Quantitative real-time PCR analysis was done with total RNA extracts from wild-type and mutant females after injection with water or 60 μg/ml of *E. coli* (1106) PG. Flies with mutations affecting the Toll pathway (spz^{rm7} and $PGRP\text{-}SA^{seml}$), the Imd pathway (key^{1}, $PGRP\text{-}LC^{\Delta E}$ and $relish^{E20}$) or both pathways ($rel^{E20};spz^{rm7}$) were collected for analysis 6 h after injection.

Experiments with LPS extracted from a nonenterobacterial Gram-negative bacteria (*Bordetella pertussis*) produced similar results (data not shown). In agreement with previous results[24], however, injection of commercial LPS activated the *Dpt* reporter gene (**Fig. 1a**). These results suggest that LPS is not the main determinant for recognition of Gram-negative bacteria in flies and that, as previously reported[25], commercial LPS preparations are contaminated by additional bacterial components (see below).

Dpt is induced by Gram-negative peptidoglycan

As PGRP-LC is reported to be a putative transmembrane receptor that functions upstream of the Imd pathway[17–19], we tested the effect of purified peptidoglycan on expression of *Dpt-lacZ*. We extracted and purified peptidoglycan from two Gram-negative bacteria (*E. coli* 1106 and *Pseudomonas aeruginosa* PAO1).

Injection of these Gram-negative bacteria–derived peptidoglycans strongly induced expression of the *Dpt-lacZ* gene (**Fig. 1a**): the stimulatory effect was detected in flies injected with a solution of 5 μg/ml of peptidoglycan and reached a maximum at about 50 μg/ml of peptidoglycan. The expression of *Dpt-lacZ* induced by these peptidoglycan injections was 2–5 times higher than that induced by control water injections (**Fig. 1** and **Table 1**). The difference in the induction from one experiment to another was due to the injection procedure (Methods). But the stimulatory effects of Gram-negative peptidoglycans were statistically significant (**Table 1**) and were similar to the induction observed with live *E. coli* or *P. aeruginosa* under the same injection conditions (**Fig. 1a**). Injecting more than 500 μg/ml of peptidoglycan induced less expression of *Dpt-lacZ*, a trend that we also observed after injecting increasing amounts of a concentrated pellet of *E. coli* and *P. aeruginosa* (**Fig. 1a**).

We corroborated our results from the *Dpt-lacZ* reporter gene assays by quantitative real-time polymerase chain reaction (PCR) analysis of *Dpt* expression. *Dpt* expression increased three- to fivefold after injection of Gram-negative peptidoglycan as compared with injection of water (**Fig. 1b**). The induction of *Dpt* gene was similar to the expression kinetics observed after injections with live *E. coli*[7], although *E. coli* induced higher expression of *Dpt* at later time points (**Fig. 1b**).

Lipoproteins are also major components of the Gram-negative bacterial cell wall. In *E. coli* only one lipoprotein, Lpp, is known to be covalently bound to peptidoglycan[26]. The Gram-negative peptidoglycan preparations that we used were extracted and purified by a procedure that removes all of the proteins associated with peptidoglycan except for two amino acids, lysine and arginine, at the carboxy terminus of the *E. coli* Lpp lipoprotein that remain bound to some diaminopimelic (DAP) residues of the purified peptidoglycan backbone[27]. To determine whether the *Dpt* induction that we observed after peptidoglycan injections was caused by Lpp contamination, we generated an *E. coli* strain lacking the *lpp* gene (*Δlpp*). The *Dpt-lacZ* reporter gene was induced to the same extent after injections of live wild-type *E. coli* and the live *Δlpp* mutant (**Fig. 1c**). Purified peptidoglycans extracted from both of these strains also induced similar expression of *Dpt-lacZ*, confirming that the stimulatory effect of our preparation was due only to peptidoglycan (**Fig. 1c**).

Although purified LPS alone did not induce *Dpt-lacZ* expression, we considered that LPS and Gram-negative bacterial peptidoglycan might interact synergistically to induce *Dpt*. In addition, it has been proposed that PGRP-LC might bind to both peptidoglycan and LPS as part of a recognition complex[17]. To test this possibility, we monitored *Dpt-lacZ* expression after injecting solutions containing both Gram-negative bacterial peptidoglycan and increasing amounts of highly purified LPS. The addition of LPS did not increase the peptidoglycan-induced expression of *Dpt-lacZ* (**Fig. 1d**).

Gram-negative peptidoglycan activates the Imd pathway

To determine whether the induction of *Dpt* expression by Gram-negative peptidoglycan is mediated by the Imd pathway, we injected Gram-negative peptidoglycan into flies carrying mutations affecting components of this pathway: key^{1} is a modification in *kenny*, which encodes the IKKγ subunit of the Drosophila IKK complex, and $PGRP\text{-}LC^{\Delta E}$ is a mutation in PGRP-LC, which encodes a putative receptor in the Imd cascade[10,17–19]. Induction of *Dpt-lacZ* expression by Gram-negative peptidoglycan was completely abolished in both key^{1} and $PGRP\text{-}LC^{\Delta E}$ flies (**Fig. 2a**).

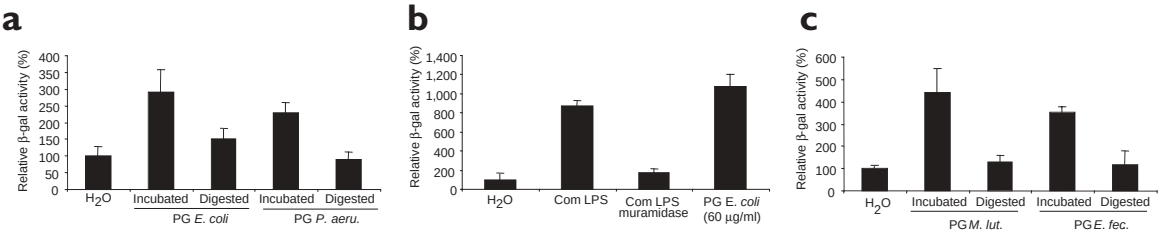

Figure 3. Muramidase-treated peptidoglycan (PG) does not activate antimicrobial peptide genes. (**a**,**b**) β-galactosidase activity was measured in adult female flies carrying a *Dpt-lacZ* reporter gene collected 6 h after injecting 60 μg/ml of *E. coli* or *P. aeruginosa* PGs (**a**) or commercial LPS (**b**). (**c**) β-galactosidase activity was measured in adult females carrying a *Drs-lacZ* reporter gene collected 18 h after injection of 5 mg/ml of *M. luteus* or *E. faecalis* PG. Before injection, the PGs and LPS were incubated overnight at 37 °C in 20 mM sodium phosphate buffer (pH 6.0) in the absence (incubated) or presence (digested) of muramidase.

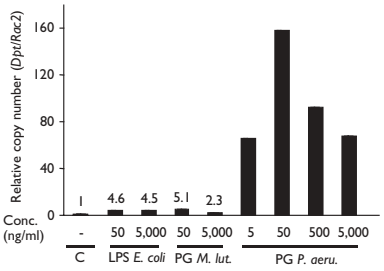

Figure 4. Gram-negative peptidoglycan (PG) is the most potent activator of *Dpt* in cultured cells. *Drosophila mbn-2* cells were incubated with the indicated concentrations of bacterial components (highly purified *E. coli* LPS, *P. aeruginosa* and *M. luteus* PG) for 6 h. Total RNA was extracted from cells and *Dpt* expression was monitored with quantitative fluorescence real-time PCR. Similar results were obtained after injecting *E. coli* PG.

In contrast, *spz*^{rm7}, a mutation in *spz*, which encodes the putative ligand of the Toll receptor, and *PGRP-SA*^{seml}, a mutation in PGRP-SA, which encodes a recognition protein that activates the Toll pathway in response to Gram-positive bacterial infection[16], did not affect *Dpt-lacZ* induction by Gram-negative PG (**Fig. 2a**). We obtained the same results when we used quantitative real-time PCR to assay the endogenous *Dpt* transcript (**Fig. 2b**). In addition, the *Dpt* gene was not induced by Gram-negative peptidoglycan in *rel*^{E20} or *rel*^{E20};*spz*^{rm7} flies. These experiments confirm that *Dpt* induction by Gram-negative peptidoglycan is mediated exclusively by the Imd pathway.

Dpt is induced by peptidoglycan from *Bacillus* species

The only Gram-positive bacterial species that are known to induce *Dpt* expression in *Drosophila* are members of the *Bacillus* genus[7]. To determine whether the differential activation of the Imd pathway by Gram-positive species is determined by differences in their peptidoglycan composition, we injected *Dpt-lacZ* flies with peptidoglycan purified from various Gram-positive bacteria.

Injections of peptidoglycan extracted from either *Micrococcus luteus* or *Enterococcus faecalis* did not induce expression of *Dpt-lacZ* as compared with water injection (**Fig. 1a**). In contrast, peptidoglycan from *Bacillus subtilis* or *Bacillus thuringiensis* induced the expression of *Dpt-lacZ* to almost the same level induced by Gram-negative peptidoglycans (**Fig. 1a, Table 1**).

These results confirm previous observations that *Bacillus* species induce *Dpt* expression[7] and indicate that this induction is due to a characteristic property of *Bacillus* peptidoglycan.

Muramidase-treated peptidoglycan does not induce *Dpt*

The above results indicate that the Imd pathway is activated by a molecular determinant present in Gram-negative bacteria and *Bacillus* peptidoglycans but absent in other Gram-positive peptidoglycans. Peptidoglycan is a polymer consisting of glycan strands of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) that are crosslinked to each other by short peptide bridges[26]. Peptidoglycans from Gram-negative bacteria and *Bacillus* differ from other Gram-positive peptidoglycans by the replacement of lysine with a diaminopimelic acid (DAP) at the third amino acid in the peptide chain. Thus, our results show that the Imd pathway is induced by the DAP-type peptidoglycan found in *Bacillus* and Gram-negative bacterial species but not by the lysine-type peptidoglycan found in *M. luteus* and *E. faecalis*.

It has been shown in mammals that Nod2, a protein involved in intracellular bacterial sensing, recognizes peptidoglycan by detecting a small peptidoglycan motif, the muramyl-dipeptide (MDP)[29,30]. To investigate whether the Imd pathway is also activated through a small peptidoglycan fragment, we monitored the expression of *Dpt-lacZ* after injections of *E. coli* and *P. aeruginosa* peptidoglycans that had been digested to completion with muramidase. The digested peptidoglycan showed a severely reduced ability to induce the *Dpt* reporter gene (**Fig. 3**).

We also used high performance liquid chromatography (HPLC) to isolate the main compounds (muropeptides) released by the complete digestion of Gram-negative peptidoglycans with muramidase (monomers or dimers of disaccharide peptides with tetra- and tripeptide chains with or without 1,6-anhydro-MurNAc) and determined that none of them induced *Dpt-lacZ* expression (data not shown). These results suggest that polymer chain size, and possibly the three-dimensional organization of the peptidoglycan molecule, has a crucial role in recognition by the Imd pathway. Commercial LPS treated with muramidase lost its capacity to induce the *Dpt-lacZ* gene, indicating that the activation properties of commercial LPS preparations are indeed due to peptidoglycan contamination (**Fig. 3b**).

Taken together, our data indicate that the selective activation of the Imd pathway by some bacterial strains is linked to the presence of DAP-type peptidoglycan and, unlike recognition by Nod2 in mammals, is not mediated through the detection of a small peptidoglycan motif.

Gram-negative peptidoglycan induces *Dpt* in *mbn-2* cells

The *mbn-2* cell line derived from larval hemocytes has been used extensively to study the *Drosophila* immune response; therefore, we tested

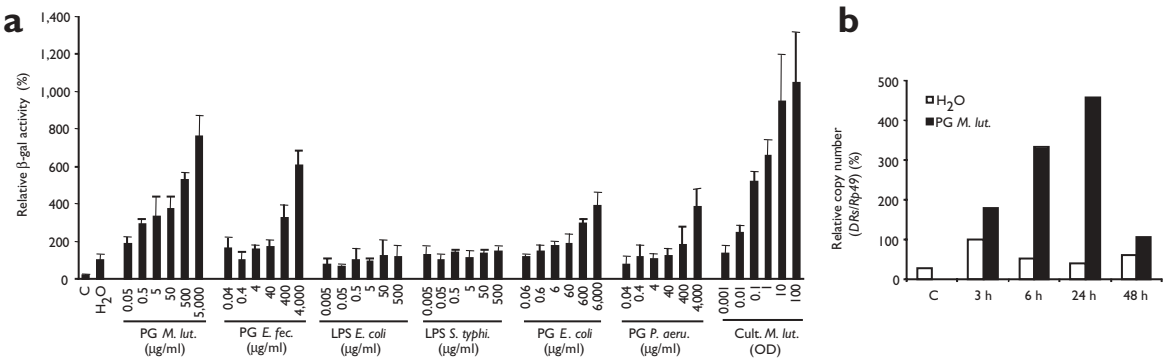

Figure 5. Gram-positive and Gram-negative peptidoglycans (PGs) activate the Toll pathway. (**a**) Induction of the *Drs-lacZ* reporter gene after injection of bacterial components. β-Galactosidase activity was measured in adult females carrying a *Drs-lacZ* reporter gene collected 18 h after challenge. Flies were injected with increasing concentrations of purified PG extracted from *M. luteus* (G$^+$), *E. faecalis* (G$^+$), *E. coli* (G$^-$) or *P. aeruginosa* PAO1 (G$^-$), or purified LPS extracted from *E. coli* or *S. typhimurium*. As a positive control, flies were injected with increasing concentrations of a suspension of *M. luteus* (G$^+$). This experiment was repeated twice with similar results. (**b**) Time course of *Dpt* gene expression after Gram-positive PG injection. Quantitative real-time PCR analysis of total RNA extracted from wild-type Oregon female flies at the indicated intervals after injecting water or 5 mg/ml of *M. luteus* PG.

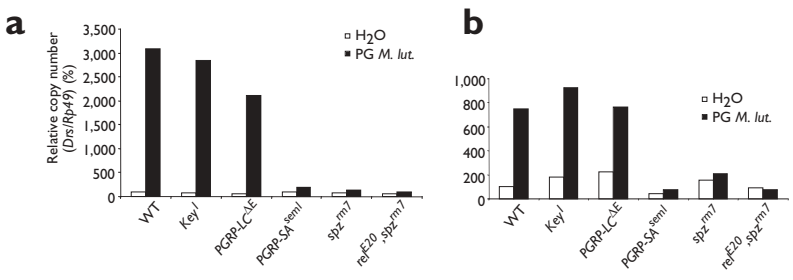

Figure 6. Gram-positive and Gram-negative peptidoglycans (PGs) induce *Drs* expression *via* the Toll pathway. Quantitative real-time PCR analysis of total RNA extracted from wild-type and mutant females injected with 5 mg/ml of Gram-positive lysine-type PG extracted from *M. luteus* (**a**), or 6 mg/ml of Gram-negative PG extracted from *E. coli* (**b**). Flies carrying mutations affecting the Toll pathway (*spz*^{rm7} and *PGRP-SA*^{seml}), the Imd pathway (*key*^{1}, *PGRP-LC*$^{\Delta E}$, *rel*^{E20}) or both pathways (*rel*^{E20};*spz*^{rm7}) were collected for analysis 24 h after challenge.

whether our bacterial extracts could induce *Dpt* expression in this cell line. Highly purified LPS stimulated the *Dpt* gene, thereby confirming previous studies reporting that *mbn-2* cells respond to LPS[23] (**Fig. 4**). Also in agreement with previous studies[17,23], Gram-positive peptidoglycan weakly stimulated the expression of *Dpt* in these cells.

However, *mbn-2* cells strongly expressed the *Dpt* gene when treated with Gram-negative peptidoglycan: the expression induced by Gram-negative peptidoglycan was 40 times higher than that induced by LPS or lysine-type peptidoglycan. These results correlate well with our *in vivo* data and confirm that Gram-negative peptidoglycan is the strongest inducer of the *Dpt* gene in *mbn-2* cells.

Lysine-type peptidoglycans activate the Toll pathway

To determine which bacterial compounds activate the Toll pathway, we used the same approach described for the Imd pathway but, instead of using *Dpt-lacZ*, we monitored the expression of a *lacZ* reporter construct for the antifungal peptide gene *Drs*, which is a target of the Toll pathway[28]. We also assayed β-galactosidase activity in the *Drs-lacZ* flies18 h after injection because *Drs* expression peaks later than *Dpt* expression.

Expression of *Drs-lacZ* was strongly induced by injections of peptidoglycan extracted from *M. luteus* and *E. faecalis*, and this induction increased with the amount of peptidoglycan injected (**Fig. 5a**). This result agrees with both previous reports that infections by Gram-positive bacteria–containing lysine-type peptidoglycans induce *Drs* expression[7–9] and our current observation that *Drs-lacZ* expression was proportional to the amount of a concentrated pellet of whole *M. luteus* bacteria injected into flies (**Fig. 5a**). A time-course study with quantitative real-time PCR also confirmed that the endogenous *Drs* gene was induced more highly after Gram-positive peptidoglycan injection than after control water injection, and that *Drs* was induced in an acute-phase profile that peaked at 24 h (**Fig. 5b**). As observed for *Dpt*, the time course of *Drs* expression was similar to the kinetics of expression observed after injections of live *M. luteus*[7–9], although live bacteria induced higher expression of *Drs* at later time points, as they did for *Dpt*. Gram-positive peptidoglycans also did not induce *Drs* after they had been digested to completion with muramidase (**Fig. 3c**).

Drs induction by lysine-type peptidoglycans was dependant on the *spz* and *PGRP-SA* genes but not on the *PGRP-LC* and *key* genes (**Fig. 6a**). This analysis shows that lysine-type peptidoglycan induces expression of *Drs* exclusively through the PGRP-SA–Toll pathway. Our results are consistent with previous data showing that the Toll pathway is strongly activated in response to Gram-positive bacterial infection and that PGRP-SA binds *M. luteus* peptidoglycan *in vitro*[15].

Gram-negative peptidoglycan weakly activates Toll

The Imd pathway is known to have a crucial role in the response to infections of Gram-negative bacteria, but we were curious to test whether microbial compounds from Gram-negative bacteria might also activate the Toll pathway. The injection of purified LPS injection did not induce *Drs-lacZ* expression (**Fig. 5a**); however, this reporter gene was induced significantly after the injection of Gram-negative peptidoglycans, albeit to lower levels than those induced by lysine-type peptidoglycans.

In contrast to *Dpt*, which is regulated exclusively by the Imd pathway, *Drs* is mainly regulated by the Toll pathway but is also partially induced by the Imd pathway after infections of Gram-negative bacteria[8,12]. To determine whether Gram-negative peptidoglycan can activate the Toll pathway, we monitored *Drs* expression in *key*^{1} and *PGRP-LC*$^{\Delta E}$ mutants that have defects in the Imd pathway. Flies were injected with a solution of 6 mg/ml of Gram-negative peptidoglycan, a dose that would not stimulate the Imd pathway (**Fig. 1a**). This procedure allowed us to monitor the expression of *Drs* without any input of the Imd pathway. Quantitative real-time PCR measurements showed that Gram-negative peptidoglycan induced the *Drs* gene in *PGRP-LC*$^{\Delta E}$ and *key*^{1} mutants and that this induction required the *spz* and *PGRP-SA* genes, indicating that the Toll pathway is activated by Gram-negative peptidoglycan (**Fig. 6b**).

Discussion

By using highly purified products, we have identified the bacterial compounds recognized by the Toll and Imd pathways. In contrast to vertebrates and the invertebrate horseshoe crab, our study suggests that LPS is not the main determinant for Gram-negative bacterial recognition in flies. We observed that enterobacterial and nonenterobacterial purified LPS samples showed no stimulatory effect on expression of the *Dpt* gene by the fat body in *Drosophila* adults.

Purified LPS did induce a weak immune response in the *mbn-2* cell line. The LPS response observed in *mbn-2* cells was modest, however, in comparison with the stimulatory effect of Gram-negative peptidoglycans. Although we cannot exclude the possibility that the LPS also has a weak stimulatory effect *in vivo* that was not detected in our assay, our results indicate that peptidoglycan is the most active determinant of Gram-negative bacteria. This finding extends the results of previous studies showing that peptidoglycan but not LPS activates the prophenoloxidase cascade in the silkworm *Bombyx mori*[31,32].

Our study shows that the Imd pathway is activated by specific recognition of Gram-negative and *Bacillus* peptidoglycans, whereas the Toll pathway is more responsive to lysine-type peptidoglycans found in most Gram-positive bacteria. Purified peptidoglycans recapitulate all of the induction properties of live Gram-negative and Gram-positive bacteria, indicating that peptidoglycan is the main bacterial product recognized by the Imd and Toll pathways. Taken together, our results show that the capacity of the *Drosophila* immune system to discriminate between distinct classes of bacteria by the Toll and Imd pathways is mediated through specific peptidoglycan recognition.

Our data are in keeping with the identification of PGRP-LC and PGRP-SA as putative receptors of the Imd and Toll pathway[16–19]. They suggest that PGRP-LC senses a specific structure that is present in Gram-negative and *Bacillus* peptidoglycans but absent in other Gram-positive

peptidoglycans, whereas PGRP-SA binds with higher affinity to lysine-type peptidoglycans than to DAP-type peptidoglycans. These differences between PGRP-LC and PGRP-SA provide an explanation of why the Toll pathway is more activated by infections of Gram-positive bacteria than by infections of Gram-negative bacteria.

Peptidoglycans from *Bacillus* and Gram-negative bacteria are crosslinked with a peptide containing a *meso*-DAP residue, whereas a lysine is found in the same position on other Gram-positive bacteria[33]. This variation probably results in distinct conformational differences, allowing discriminatory recognition. The observation that *Bacillus* peptidoglycan is a less potent inducer of the Imd pathway than Gram-negative peptidoglycan might be explained by the fact that *Bacillus* peptidoglycan contains a high proportion of amidated-DAP and only 3% of *meso*-DAP[33]. The idea of specific peptidoglycan recognition through PGRP is supported by the observation that another *Drosophila* PGRP, PGRP-LE, binds to DAP-type peptidoglycans but not to lysine-type peptidoglycans *in vitro*[20].

The stronger activation of the Toll pathway by Gram-positive bacteria with lysine-type peptidoglycan than by Gram-negative bacteria is probably accentuated during bacterial infection, because activity of the Toll pathway is proportional to peptidoglycan concentration and Gram-positive bacterial cell walls contain much more peptidoglycan than do Gram-negative bacterial cell walls. It is also interesting to note that, within the range of peptidoglycan concentrations that we tested, maximum activation of the Imd pathway was reached by using at least 100 times less peptidoglycan than the dose required to strongly activate the Toll pathway. This finding may reflect the ability of the insect immune system to recognize Gram-negative bacteria efficiently even though these bacteria contain less peptidoglycan.

It is surprising that flies can detect Gram-negative bacteria on the basis of a microbial component that is present at the surface of the inner membrane and is therefore hidden by the LPS-containing outer membrane. It is possible that the Imd pathway receptor, PGRP-LC, recognizes small amounts of peptidoglycan that are continuously released by Gram-negative bacteria[34]. Alternatively, Gram-negative bacteria may be degraded by humoral or cellular mechanisms that release peptidoglycan and elicit the antimicrobial response. This latter possibility is supported by observations that *P. aeruginosa* cannot initiate the activation of prophenoloxidase in *Galleria mellonela* hemolymph unless the bacterial cells are damaged[35]. Our identification of the bacterial molecules that specifically trigger these two pathways will aid more detailed analyses of how bacterial elicitors are released and recognized during the infectious process and how *Drosophila* mounts specific immune responses adapted to the type of aggressor.

Two studies have shown that mammalian Nod2 functions as a general sensor of peptidoglycans through recognition of MDP, the minimal bioactive peptidoglycan motif that is common to all bacteria[29,30]. In support of these studies, peptidoglycan treated with muramidase, an enzyme that generates small peptidoglycan fragments, induces the expression of Nod2 in cell culture (S. Girardin, personal communication). Our observations that after digestion with muramidase neither Gram-negative nor Gram-positive peptidoglycan activated an immune response in flies indicate that the bacterial sensing that regulates the synthesis of antimicrobial peptides by the *Drosophila* fat body is not mediated through a small peptidoglycan motif, as it is in the Nod2 system. Our study suggests that polymer chain size, and possibly the three-dimensional organization of the molecule, has a crucial role in bacterial sensing, in agreement with a study showing that the minimum structure of peptidoglycan required for inducing antibacterial peptides in the silkworm *B. mori* is two repeating GlcNAc-MurNAc units with peptide chains[36]. We cannot, however, definitively exclude the possibility that other immune-responsive tissues (such as hemocytes) may respond to small peptidoglycan fragments.

Our study and the work on mammalian Nod2 indicate that peptidoglycan is a complex bacterial elicitor and that the innate host defense has developed several ways to detect peptidoglycan. In vertebrates, TLR2 has been also implicated in peptidoglycan sensing[22,37], but the precise nature of the peptidoglycan fragment recognized by TLR2 is not known. It would be worthwhile determining whether vertebrates, like the fruit fly, use distinct receptors to recognize Gram-negative and Gram-positive peptidoglycans.

Methods

Fly stocks. OR^R, DD1 (*y, w, P(ry+, Dpt-lacZ), P(w+, Drs-GFP)*) or 8871A (*w, P(w+, Drs-lacZ))* flies were used as wild-type strains[38]. *Dpt-lacZ* is a *P* transgene inserted on the X chromosome that contains a fusion between 2.2 kilobases (kb) of upstream sequence from the *Dpt* gene and the coding sequences from the gene encoding β-galactosidase[38]. *Drs-lacZ* is a *P* transgene inserted on the X chromosome that contains a fusion between 2.4 kb of upstream sequence from *Drs* gene and the coding sequences from β-galactosidase[38]. The *spz*^{rm7}, *key*^{1}, *PGRP-SA*^{seml}, *PGRP-LC*$^{\Delta E}$ and *rel*^{E20} alleles have been described[8,10,16,18,12,38]. We maintained fly stocks at 25 °C in standard medium.

Injection and *lacZ* measurements. We injected 9.2 nl of solution (water, bacterial extracts or overnight bacteria culture) into the thorax of female adults (aged 3–4 d) with a Nanoject apparatus (Drummond, Broomall, PA). After injection, flies were incubated for 6 h (*Dpt-lacZ*) or 18 h (*Drs-lacZ*) at 25 °C. Bacterial strains and LacZ measurements were done as described[7,38]. Because the injection procedure creates an injury that by itself triggers a significant induction of the *Dpt* and *Drs* reporter genes, we normalized measurements of β-galactosidase activity after injecting microbial compounds or live bacteria to measurements obtained after injecting water under the same conditions (needle, time, experimenter). The values obtained after water injection were arbitrarily set at 100. Subtle changes in the size and sharpness of the needle affected the expression of *Dpt* and *Drs* (a larger needle generally induced higher expression of antimicrobial peptide genes). This effect explains most of the differences in the stimulation that we observed from one experiment to another. The effect of injury was less marked at later time points and consequently had less influence on *Drs* expression.

Bacterial products and bacterial strains. Commercial LPS (Sigma, St. Louis, MO) from *E. coli* 055:B5 was used either directly or after treatment with muramidase. LPS from *E. coli* 0111B4, *S. typhimurium* LT2 and *B. pertussis* was highly purified. The purification steps involved the extraction of phospholipids with chloroform/methanol (1:2) followed by treatment with DNase, RNase and then protease until thin-layer chromatography and ultraviolet spectra showed no detectable contaminants[39]. We check the electrophoretic behavior and mass of all purified samples by SDS-PAGE and mass spectrometry, respectively[40]. Purified LPS from *S. typhimurium* LT2 was used directly, whereas *E. coli* 0111B4 and *B. pertussis* LPS were treated by a procedure that improves LPS solubility by removing divalent cations that form links between molecules[41]. This procedure rules out erroneous results arising from aggregation.

Purified peptidoglycans were obtained from cultures of *E. coli* (1106), *P. aeruginosa* (PAO1), *M. luteus*, *E. faecalis*, *B. thuringiensis* and *B. subtilis* grown overnight at 37 °C in LB medium. Cultures were stopped by rapid chilling to 0–4 °C. Collected cells were washed with a cold 0.85% NaCl solution and centrifuged again. We then rapidly suspended the bacteria with vigorous stirring in 20 ml of a hot (95–100 °C) aqueous solution of 4% SDS for 30 min. After standing overnight at 20 °C, the suspensions were centrifuged for 30 min at 200,000*g* in a Beckman TL100 centrifuge and the pellets were washed several times with water. Final suspensions were made in 2 ml of water and aliquots (100 μl) were hydrolyzed and analyzed with a Hitachi L8800 amino acid analyzer. The peptidoglycan content of the sacculi was expressed in terms of its muramic acid content. We treated the crude preparations successively with pancreatin, pronase and trypsin (Sigma) to eliminate peptidoglycan-associated proteins[27]. Hydrolysis of an aliquot of the peptidoglycan preparations after several washings in water showed that they contained only peptidoglycan constituents, muramic acid, glucosamine, alanine, glutamic acid and diaminopimelic acid or lysine, in the expected molar ratios.

Peptidoglycans were digested by overnight treatment with the muramidase mutanolysin (Sigma) and the reaction was stopped by incubating samples in a boiling water bath for 5 min. We reduced the resulting soluble fragments (muropeptides) with sodium borohydride and separated them by reverse-phase HPLC as described[26]. MDP (MurNAc-L-Ala-D-isoGln) was obtained from Calbiochem (La Jolla, CA) and MurNAc peptides were generated by mild acid hydrolysis of UDP-MurNAc peptides in 0.1 M HCl for 10 min at 95 °C. The *E. coli* mutant strain BW25113 Δ*lpp*:Cm^R, in which the whole *lpp* gene was replaced with an antibiotic (chloramphenicol) resistance gene, was created as described[42]. The oligonucleotides used for PCR amplification of the antibiotic resistance gene flanked by sequences designed for specific disruption of the *lpp* gene were 5′-CGC TAC ATG GAG ATT AAC TCA ATC TAG AGG GTA TTA ATA ATG AAA GCT ACA TAT GAA TAT CCT CCT TAG-3′ and 5′-GGC GCA CAA TGT GCG CCA TTT TTC ACT TCA CAG GTA CTA TTA CTT GCG GGT GTA GGC TGG AGC TGC TTC-3′.

Cell culture and immune stimulations. We maintained *Drosophila mbn-2* cells in Schneider medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum. For immune stimulation, cells were incubated with different concentrations of bacterial components (commercial LPS, highly purified LPS from *E. coli* and highly purified peptidoglycan from *M. luteus* and *P. aeruginosa*) for 6 h.

Quantitative real-time PCR. Total RNA was extracted with RNAzol reagent (Invitrogen, Carlsbad, CA). Complementary DNA was synthesized by using the First cDNA synthesis kit (Roche, Basel, Switzerland) in accordance with manufacturer's instructions. For quantitative analysis of *Dpt* expression, fluorescence real-time PCR was done with the double-stranded DNA dye SYBR Green (Perkin Elmer, Boston MA). Primer pairs for *Dpt* (sense, 5′-GGC TTA TCC GAT GCC CGA CG-3′; antisense, 5′-TCT GTA GGT GTA GGT GCT TCC C-3′) and control *Rac2* (sense, 5′-CAG ACG ATC GAG AAG CTG AAG G-3′; antisense, 5′-GTG CCG CTT GGG TCC TCG AAC G-3′) were used to detect target gene transcripts. We analyzed SYBR Green with an ABI PRISM 7700 system (Perkin Elmer) in accordance with the manufacturer's instructions. All samples were analyzed in triplicate, and the amount of messenger RNA detected was normalized to control *Rac2* mRNA values. We used normalized data to quantity the relative levels of a given mRNA according to cycling threshold (ΔCt) analysis.

To quantify *Drs* and *Dpt* mRNA in whole animals, we extracted RNA with RNA TRIzol (Invitrogen). cDNAs were synthesized with SuperScript II (Invitrogen) and PCR was done with the double-stranded DNA dye SYBR Green I (Roche) Primer pairs for *Dpt* (sense, 5′-GCT GCG CAA TCG CTT CTA CT-3′; antisense, 5′-TGG TGG AGT GGG CTT CAT G -3′), *Drs* (sense, 5′-CGT GAG AAC CTT TTC CAA TAT GAT G-3′; antisense, 5′-TCC CAG GAC CAC CAG CAT-3′) and control *Rp49* (sense, 5′-GAC GCT TCA AGG GAC AGT ATC TG-3′; antisense, 5′-AAA CGC GGT TCT GCA TGA G-3′) were used to detect target gene transcripts. SYBR Green was analyzed on a Lightcycler (Roche).

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Competing interests statement

The authors declare that they have no competing financial interests.

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