Barrier epithelia that are persistently exposed to microbes have evolved potent immune tools to eliminate such pathogens. If mechanisms that control Drosophila systemic responses are well-characterized, the epithelial immune responses remain poorly understood. Here, we performed a genetic dissection of the cascades activated during the immune response of the Drosophila airway epithelium i.e. trachea. We present evidence that bacteria induced-antimicrobial peptide (AMP) production in the trachea is controlled by two signalling cascades. AMP gene transcription is activated by the inducible IMD pathway that acts non-cell autonomously in trachea. This IMD-dependent AMP activation is antagonized by a constitutively active signalling module involving the receptor Toll-8/Tollo, the ligand Spätzle2/DNT1 and Ect-4, the Drosophila ortholog of the human Sterile alpha and HEAT/ARMadillo motif (SARM). Our data show that, in addition to Toll-1 whose function is essential during the systemic immune response, Drosophila relies on another Toll family member to control the immune response in the respiratory epithelium.
Author Summary

Invertebrates solely rely on innate immune responses for defense against microbial infections. Taking advantage of its powerful genetics, the fly *Drosophila melanogaster* has been extensively used as a model system to dissect the molecular mechanisms that control innate immunity. This work led to the discovery of the essential role of the Toll-1 receptor in triggering the systemic immune response in flies, and paved the way for the discovery of the function of members of the Toll-like receptor (TLR) family in mammalian immunity. Whereas all TLRs are implicated in the mammalian immune response, Toll-1 was, so far, the only *Drosophila* Toll family member to be involved in the regulation of the immune response. In the present study, we show that another Toll family member, Toll-8 (Tollo), plays an important role in controlling the respiratory epithelium immune response. Our data indicate that, by antagonizing the IMD pathway, Tollo is preventing over-activation of the antibacterial response in the airway epithelium.

**Results**

**AMP activation followed a stereotypical pattern in the *Drosophila* tracheal network.

The tracheal system is a relatively simple model system that has provided an important insight into the biology of branching morphogenesis [20–25]. In mammalian immunity, the fat body cell-autonomously controls immune response, the IMD pathway can be activated non-cell autonomously in the tracheal network. In *Drosophila* respiratory epithelium, We show that, in contrast to systemic fat body immune response, the IMD pathway can be activated non-cell autonomously in the tracheal network. We present evidence that IMD pathway activation is tightly regulated in the cells of the respiratory epithelium. We demonstrate that the molecular mechanisms underlying IMD down-regulation following infection, are different from those previously reported in the gut and in the respiratory epithelium. We show that, in contrast to systemic fat body (but can be activated by ectopic IMD pathway triggering, see later), it is strictly IMD-dependent in the trachea [5,26,28]. Indeed, over-activation of the IMD (UAS-PGRP-LCa, UAS-IMD), but not of the Toll pathway (UAS-toll, Tt), is sufficient to induce tracheal expression of Drs-GFP in non-infected larvae (Figure S1A and S1B). Concomitantly, loss-of-function mutations in IMD pathway components (Relish, PGRP-LC, IMD) prevent Drs-GFP tracheal activation in infected larvae, whereas Toll signalling mutants such as PGN do show a wild-type tracheal response upon infection. To analyze whether all tracheal cells were competent to trigger AMP production upon IMD pathway activation, we induced UAS-IMD-expressing clones in tracheal cells, using fat body clones as controls. Overexpression of IMD led to a strictly cell-autonomous and fully penetrant activation of both *Drosomycin* and *Dipt-Cherry* in the fat body (Figure 2B and 2C). In the trachea, although most IMD-expressing cells showed Drs-GFP expression, a fraction did not (Figure 2A). In addition, Drs-GFP activation was not always associated with the expression of the UAS-IMD transgene (Figure 2A), suggesting that IMD pathway activation in trachea is not strictly cell autonomous. These results were confirmed by using a UAS-PGRP-LCa transgene that activated Drs-GFP both autonomously and non-autonomously in tracheal cells (Figure 2E) but strictly cell-autonomously in the fat body (Figure 2F). We next addressed whether PGRP-LC function was required cell-autonomously for IMD pathway activation in the trachea upon infection. Analysis of MARCM loss-of-function clones for PGRP-LC indicates that tracheal cells mutant for PGRP-LC were totally impaired in their ability to trigger *Drosomycin* expression, following *Ecc* infection (Figure 2D and H). These results indicate that, although PGRP-LC is essential in tracheal cells for IMD pathway triggering, IMD pathway activation in one tracheal cell can spread to neighboring cells. This contrasts with the strictly cell-autonomous IMD-dependent immune response observed in fat body cells.

**Tollo is expressed apically in the *Drosophila* trachea cells.

In order to get a further insight into the mechanisms that control AMP induction in trachea, we looked for putative immune
genes expressed in this tissue. A recent report identified the repertoire of all immune genes expressed in the trachea [29]. One of the striking data of this study, confirmed by FlyAtlas (http://flyatlas.org/), was that, in addition to Toll itself, two other Toll family members, 18-Wheeler (Toll-2) and Tollo (Toll-8), are strongly expressed in trachea. 18-wheeler being implicated in developmental processes with indirect impacts the immune response [30], we focused our study on the putative function of the Tollo transmembrane protein in the tracheal immune response. Using Lac-Z reporter lines (data not shown) and q-

Figure 1. Spatio-temporal expression of tracheal Drosomycin-GFP. (A) Dorsal view of Drs-GFP third instar larvae. In non-infected larvae, sporadic GFP expression is visible in posterior spiracles (PS) and/or in visceral branches (VB). Upon Ecc infection, larvae display a highly reproducible pattern of GFP expression, classified as follow: Class I, PS + posterior VB only; Class II, PS + all VB; Class III, VB + dorsal trunk (DT), with Drs-GFP first in the anterior half (limit marked by arrows) and only later, in the entire trunk. FB: fat body. (B) Quantification of tracheal Drs-GFP-positive larvae and distribution of classes upon Ecc infection. Each histogram corresponds to the mean value of 5 experiments. A total number of 100 larvae were counted for each experiment. Statistics apply for the “no signal” and the class III categories only. Values indicated by identical symbols (* or **) are not significantly different (P>0.05) from one another. All other differences are statistically significant (P<0.05). (C) Quantification of tracheal Drs-GFP-positive larvae at 4h, 8h, 12h and 24h post-infection by Ecc. Each histogram corresponds to the mean value of 3 experiments. A total number of 90 larvae were counted for each experiment.

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RT-PCR (Figure 3A), we confirmed that Tollo mRNA is highly enriched in the tracheal epithelium, and expressed at lower levels in other tissues. To investigate the subcellular localization of the Tollo protein, we genetically associated a UAS-Tollo::Myc construct with the trachea-specific Breathless-Gal4 driver (Btl-Gal4). Anti-Myc antibody staining suggested that Tollo was localized apically at the cell membrane facing the airway lumen (Figure 3B). Double staining experiments showed that Tollo::Myc partially co-localized with the apical marker Cadherin::GFP, but was mutually exclusive with Viking::GFP, a basal membrane-associated protein (Figure 3B). These results indicate that Tollo is a protein enriched in the tracheal epithelium with an apical subcellular localization.

AMPs are specifically over-produced in infected Tollo mutant trachea

The rather restricted expression pattern of Tollo mRNA in the trachea and the apical subcellular distribution of Tollo protein prompted us to investigate its putative function in the immune response. For that purpose, we used two previously characterized hypomorphic alleles (Tollo<sup>145</sup> and Tollo<sup>R5A</sup>) together with a complete loss-of-function allele (Tollo<sup>C5</sup>) that we generated by P-element mediated homologous recombination [31–32] (Figure S2A). All Tollo mutants were viable with no obvious developmental defects and gave rise to phenotypically normal pharate adults indicating that Tollo has no essential role in Drosophila development. We tested the ability of these Tollo mutant larvae to mount an immune response. In the absence of infection, approximately 5% of wild-type larvae showed Drs-GFP expression in VB and/or PS (Figure S3). Similar Figures were obtained with Tollo mutants suggesting that Tollo is not required to set up the basal level of AMP production in the absence of infection (Figure S3). After bacterial infection, however, the immune response was much stronger in Tollo mutants than in wild-type sibling larvae (Figure 4A and 4B and Figure S3). While we could identify the three previously described classes of Drs-GFP positive larvae in both control and Tollo mutants, the relative proportion of these was significantly different between genotypes (Figure S3). The percentage of larvae showing no GFP expression was reduced to 5–10% in Tollo mutant (compared to 40% in controls), whereas Class III larvae, which represented 15% of controls, reached up to 50% in the Tollo mutant larvae (Figure S3). Similar results were obtained with three independent Tollo alleles and in trans-heterozygous allelic combination demonstrating that this phenotype was, indeed, due to Tollo inactivation and not to other mutations on the chromosome (data not shown). The effects were not only qualitative but also quantitative. In most infected Tollo

Figure 2. IMD pathway activation is not strictly cell-autonomous in trachea. IMD overexpressing clones in trachea (A) and fat body (B, C) cells are marked by RFP (A, B) or GFP (C) expression. (A) IMD overexpression activates Drs-GFP cell-autonomously (66%, n = 68) (arrow) and non-autonomously (12%, n = 68) (dashed arrow) in the trachea. Note that 34% (n = 68) of IMD-expressing cells are unable to activate Drs-GFP (arrow head). On the contrary, 98% (n = 38, for Drs-GFP) and 97% (n = 57, for Dpt-Cherry) of IMD-expressing cells activate Drs-GFP (B) Dpt-Cherry (C) in a strictly cell-autonomous fashion in the fat body. Clones overexpressing PGRP-LCa in trachea (E) and fat body (F, G) are marked by RFP (E, F) or GFP (G) expression. Tracheal cells expressing PGRP-LCa activate Drs-GFP autonomously (74%, n = 46) (arrow) and non-autonomously (39%, n = 46) (arrow head) (E). Fat body cells expressing PGRP-LCa (99%, n = 48 for Drs-GFP) and (98%, n = 63 for Dpt-Cherry) activate Drs-GFP (F) and Dpt-Cherry (G) autonomously. RFP expressing clones never activates Drs-GFP cell-autonomously (0%, n = 52) nor cell non-autonomously (0%, n = 52) in the trachea. (D, H) Visceral branches of Ecc-infected larvae containing PGRP-LC mutant clones, marked by RFP expression (MARCM see Methods). Cells lacking PGRP-LC (red) are unable to activate Drs-GFP expression, while surrounding cells do (green). Nuclei are stained with Dapi (blue). Scale bar is 100 µm.

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mutant larvae, Drs-GFP expression was intense, whereas it was rarely the case in controls (Figure 4A). q-RT-PCR experiments indicate that Drosomycin, Drosocin and Attacin mRNA levels were respectively increased by 6, 7 and 2.6 fold after infection in Tollo mutants compared to wild-type trachea (Figure 4B). To ensure that this effect was indeed a consequence of Tollo inactivation in the tracheal network itself, we combined the Btl-Gal4 driver with a UAS-TolloIR RNA interference construct. As shown in Figure 4A and Figure S3, larvae, in which Tollo was eliminated specifically in trachea, also showed increased Drs-GFP expression in this tissue after infection.

In addition to regulating Drosomycin expression in the trachea, the IMD pathway also controls Diptericin transcription locally in the gut and systemically in the fat body [13,26]. To test whether
Tollo acts as a general negative regulator of IMD-dependent mechanisms in other immune tissues, we analyzed the effects of inactivating Tollo on IMD pathway activation in the gut and the fat body. Using the Dpt-Cherry reporter construct for the larval stage (Figure 4D) and q-RT-PCR for both larval and adult stages (Figure 4E and 4F), we showed that Tollo was not implicated in IMD negative regulation in either tissue. This was the case for both immune responses induced by septic injury or by oral ingestion. In addition, we showed that Tollo mutants were unaffected in their ability to activate the Toll pathway during a Gram-positive bacteria-mediated systemic immune response (Figure 4F). Altogether these results demonstrate that the Tollo receptor is specifically required to dampen IMD pathway-dependent responses in the tracheal network after infection.

AMP over-production in Tollo mutants is not secondary to trachea defects

Since AMPs are induced upon cellular stress, we tested whether Drosomycin expression in Tollo mutants was a secondary consequence of a possible implication of Tollo in tracheal formation. The following reasons led us to believe that it was not the case. 1) Tollo mutant embryos gave rise to viable adults, suggesting that Tollo mutant trachea are fully functional in larvae and adults. 2) Tracheal cell morphology of Tollo and control larvae appeared similar when observed under transmission electron microscopy (Figure 5A). 3) No constitutive AMP transcription was detected in non-infected Tollo mutant larvae (Figure 4B and Figure S3). We then wondered whether Drosomycin over-activation could be linked to the presence of higher levels of potential immune elicitors in Tollo mutant and RNAi trachea. This could be due to the presence of higher bacterial load in the trachea. However, as shown in Figure 5B and Figure S4B, the number of Ecc-GFP in Tollo mutant trachea was identical in Tollo mutants and in controls. Alternatively, Tollo mutant trachea could be more permeable to contaminated external fluid. To test this hypothesis, wild type and Tollo mutant larvae were incubated in the presence of a fluorescent dye, bromophenol blue. External fluid penetration inside the trachea lumen was not different in wild-type and Tollo mutant larvae (Figure 5C and 5D). This indicates that over-activation of Drosomycin in both Tollo mutant and RNAi trachea cannot be attributed to an increase in fluid penetration, and therefore putative immune elicitor load within the tracheal lumen. Altogether, these results demonstrate that the infection-dependent Drosomycin over-activation observed in Tollo mutant, is not secondary to defective trachea but rather suggests a direct implication of the Tollo protein in the regulation of IMD-dependent Drosomycin expression.
Figure 5. Tracheal morphology, putative immune elicitors and fluid penetration in trachea are not affected by Tollo mutations. (A) Electron microscopic pictures of tracheal transversal sections of control (Oregon R), Tollo mutant (Tollo<sup>C5</sup>/Tollo<sup>R5A</sup>), Ect4 mutant (Ect4<sup>EY04273</sup>/Df(3L)ED4408) or DNT1 mutant (DNT1<sup>502</sup>/DNT1<sup>502</sup>) third instar larvae. No obvious morphological differences could be observed between control and mutant trachea. (e) epicuticle, (t) taenidium, (p) procuticle and (bm) basement membrane. Scale bar is 2 μm. (B) Midgut and tracheal load of Ecc-GFP in control (Tollo<sup>C5</sup>+/+) and Tollo mutant (Tollo<sup>C5</sup>/Tollo<sup>R5A</sup>) third instar larvae, 4h and 24 h post-infection. Note that bacterial load for Ecc-GFP is much
phenotype requires functional PGRP-LC receptor and intracytoplasmic adaptor IMD, since double mutant Tollo ; PGRP-LC and imd ; Tollo trachea did not show any signs of Drs-GFP activation after infection (Figure 4A). This epistatic relationship was confirmed by q-RT-PCR on Drosomycin mRNA (Figure 4C). In genetic terms, Tollo is hypostatic, or acts in parallel to PGRP-LC and imd. Consistently, Relish nuclear translocation monitored with an anti-Relish antibody was higher in Tollo RNAi-infected tracheal cells than in controls (Figure S6). These results suggest that Tollo is not directly involved in IMD pathway activation per se but that, in its absence, IMD pathway activation is more efficient upon infection.

Discussion

Epithelial responses are first and foremost local responses to prevent the epithelium from unnecessary immune reactions. Since the recognition steps in Drosophila respiratory epithelia involve the transmembrane receptor PGRP-LC and occur within the extracellular space, it is expected that molecular mechanisms must be at work to prevent constitutive or excessive immune response in this tissue, particularly essential for animal growth and viability. In this report, we present data demonstrating that the transmembrane receptor Tollo is part of a signalling network, whose function is to specifically down-regulate AMP production in the trachea. We show that Tollo antagonizes IMD pathway activation in the respiratory epithelium, and that DNT1/Spz2 and Ect4/SARM are putative Tollo ligand and transducer, respectively, in this process. Our data demonstrate that, in addition to the family founder Toll-1, another member of the Leucine-Rich-Repeat family of Toll proteins, is regulating the Drosophila innate immune response. Although it has been abundantly documented that every single mammalian TLR has an immune function [4], the putative implication of Toll family members, other than Toll-1 itself, in the Drosophila immune response has been a subject of controversy [41]. Data showing that Drosophila Toll-9 over-expression was sufficient to induce AMPs expression in vivo has prompted the idea that Toll-9 could maintain significant levels of anti-microbial molecules, thus providing basal protection against microbes [42]. However, our recent analysis of a complete Toll-9 loss-of-function allele has shown that this receptor is neither implicated in basal anti-microbial response nor required to mount an immune response to bacterial infection [43]. The present data are also fully consistent with a recent report showing that Toll-6, Toll-7 and Toll-8 are not implicated in systemic AMP production in flies [44], and demonstrate that a Toll family member, Tollo, is a negative regulator of local airway epithelial immune response upon bacterial infection. In contrast to Toll-1, whose activation is inducible in the fat body, Tollo pathway activation seems to be constitutive in the trachea. Despite these differences, both receptors use a member of the Spz family as ligand. Interestingly, sequence similarities, intron’s size and conservation of key structural residues, indicate that Spz2/DNT1 is phylogenetically the closest family member to the Toll ligand Spz [45]. Furthermore, both Spz and Spz2/DNT1 have been shown to have neurotrophic functions in flies [46]. It would be of great interest to test whether Tollo also mediates Spz2 function in the nervous system.

Both during embryonic development and immune response, Spz is activated by proteolytic cleavage [10,47–48]. This step depends upon the Easter protease that is implicated in D/V axis specification and on SPE for Toll pathway activation by microbes. Since Spz orthologs are also produced as longer precursors, they are likely to be activated by proteolysis. The fact that Tollo and Spz2 loss-of-function phenotypes correspond to excessive AMP production, suggests that in wild-type conditions, the Tollo pathway is constitutively activated by an active form of the Spz2 ligand. This situation is reminiscent to that observed in the embryonic ventral follicle cells, in which a Pipe-mediated signal induces a constitutive activation of the Easter cascade leading to Spz cleavage, Toll activation and, in turn, ventral fate acquisition [49]. It should be noted that Easter and one Pipe isoform are very strongly expressed in the tracheal cells (Flyatlas), and are candidate proteins in mediating Tollo activity in the respiratory epithelia.

The fact that Ect4, but not dMyd88 mutant, loss-of-function mutant phenocopies Tollo mutant suggest that Ect4 could be the TIR domain adaptor transducing Tollo signal in the tracheal cells. Alternatively, Ect4/SARM could mediate Tollo function by interfering with IMD pathway signalling. In mammals, SARM is under the transcriptional control of TLR and negatively regulates TLR3 signalling by directly interfering with the association between the RHIM domain-containing proteins TRIF and RIP [50]. Since PGRP-LC contains a RHIM domain as TRIF, and IMD is the Drosophila counterpart of RIP, one can envisage that Drosophila SARM could act by interfering with the PGRP-LC/IMD association required for IMD pathway signalling. Similarly to its function as a negative regulator in fly immunity, SARM is the only TIR domain-containing adaptor that acts as a suppressor of TLR signalling [36,50].

One obvious question relates to the mode of action of Tollo on IMD pathway downregulation. Two mechanisms have been recently described that result in the down-regulation of the IMD pathway. The first one regulates PGRP-LC membrane localization, and is dependent on the PIRK protein [51–53]. Upon infection, the intracellular PIRK protein is up-regulated and, in turn, represses PGRP-LC plasma membrane localization leading to the shutdown of the IMD signalling [53]. In infected pirk mutants, IMD-dependent AMPs are overproduced in both the gut and the fat body. In our conditions, however, inactivation of PIRK specifically in the trachea did not influence Drosomycin activation in trachea (Figure S7A). To verify whether Tollo is acting via membrane this PIRK similar to PIRK, we looked at PGRP-LC membrane localization using a UAS-PGRP-LC::GFP construct. PGRP-LC membrane localization was identical in wild-type and Tollo mutant tracheal cells (Figure S7B). The second mechanism that modulates IMD activation, acts directly on the promoters of IMD target genes. Ha et al. (2005) have shown that the Caudal transcription factor sits on some of the IMD target promotors preventing their activation by Relish [54]. We thus tested the putative implication of Caudal in Tollo signalling by using Drs-GFP reporter transgenes containing either wild-type Caudal Responsive Elements (CDREs) or mutated versions unresponsive to Caudal activity [55]. Upon
infection, Drs-GFP with mutated CDREs was activated in fat body but not in gut or trachea (Figure S7C). In conclusion, Caudal acts as a transcriptional activator, rather than a repressor, for the Drs-GFP reporter in trachea. These results indicate that Tollo does not regulate the IMD pathway via PGRP- LC membrane localization or through promoter targeting of Caudal. One challenging task for the future will be to identify the mechanism used by Tollo to counter-balance tracheal PGRP-LC activation. It has been reported that the loss of Tollo function in ectodermal cells during embryogenesis alters glycosylation in nearby differentiating neurons [31,56-57]. Since the pattern of oligosaccharides expressed in a cell can influence its interactions with others and with pathogens, Tollo could function by modifying glycosylation pattern in response to microbes. It could be envisaged that Tollo mediates PGRP-LC glycosylation, and thereby reduces its ability to respond to bacterial elicitors. Further work will be required to address the above hypothesis, whereby Tollo activity and glycosylation modification could be linked in order to regulate the IMD pathway activation in trachea.

Material and Methods

Bacterial strains

The following microorganisms were used: Erwinia carotovora carotovora 15 2141 (Ecc), Erwinia carotovora carotovora 15 POM1-GFP spectronycinR (Ecc-GFP), Escherichia coli 1106 (EcoGi) and Micrococcus luteus CIPA270 (M. luteus).

Bacterial load analysis

Bacterial load of surface sterilized individuals was quantified by plating appropriate serial dilutions of lysates obtained from 6 dissected guts or trachea (from larvae) on nutrient agar plates (Luria Bertani + spectinomycin 100 μg/ml). Biological triplicates were collected for each experimental condition at 4h and 24h after Ecc-GFP infection. Homogenization of tissues was performed using the Precellys 24 tissue homogenizer (Bertin technologies, France) and 0.75-1mm glass beads in 500 μL of LB + spectinomycin.

Drosophila melanogaster strains and maintenance

PGRP-LCDE12 is a complete deletion of the PGRP-LC locus [16]. Flies carrying this mutation are unable to activate the IMD pathway. spzR1 is a null allele which prevents Toll pathway activation [5], yuc, Drs-GFP [27], Dpt-Cherry [38], TolloC5 [32], TolloL45 [59], UAS-TolloR (VDRC #9431), UAS-Tollo-Myc [31], DNT141 [46], UAS-spezR (VDRC #26115), Ect4B8026 BL#15733, Df(3L)ED4408 BL#8065, T7 BL#3238 (a dominant gain-of-function allele of Tl, Bl-Gal4 BL#8807, UAS-myrRFP BL#7118, act>CD2>Gal4 BL#4780, cad-EGFP BL#30875, Vg-GFP (a gift from Michel Sméria), hs-Gal4 BL#20773, RelishZ [60], mid [5], UAS-spez act [30], dMyd88c03881 [34], UAS-PGRP-LC::GFP (a gift from François Leulier) and Df [61]. Generation of the TolloC5 allele was performed as described in [62] using the two following inserted elements: d01565 and PBag0021 [63]. Complete deletion of the Tollo gene was confirmed by sequencing genomic DNA extracted from TolloC5 mutants (molecular details upon request). Fly stocks were raised on standard cornmeal-agar medium at 25°C.

Natural infection of larvae and adults

Cells from overnight bacterial cultures were recovered by centrifugation at 4,000 g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in fresh LB media. Cell suspensions were serially diluted in PBS, and the concentration of cells was determined by optical-density (OD) measurement. 200 μl of an overnight bacterial culture of Ecc (OD = 200) were directly added on top of feeding third instar larvae into a standard cornmeal-agar medium at 25°C. A similar volume of LB broth was used in control experiments. Larvae were monitored for Drosomycin and Diptericin transcription by fluorescence analysis using Drs-GFP and Dpt-cherries respectively, and by qRT-PCR, 24 h after infection. Septic injuries were performed by prickng adult males with a thin needle contaminated with M. luteus or E. coli, 200 μl of Bromophenol Blue (SIGMA # B8026) at 10 g/l were directly added on top of feeding third instar larvae.

Flip-out clones and MARCM

For Drs-GFP study, Drs-GFP; UAS-myrRFP; act>CD2>Gel4 females were crossed to either yashfy; UAS-IMD or to yashfy; UAS-PGRP-LC males. For Dpt-Cherry study, yashfy; UAS-GFP; act>CD2>Gel4 females were crossed to Dpt-Cherry; UAS-IMD or to Dpt-Cherry, UAS-PGRP-LC males. In both cases, larvae of the progeny were heat shocked at early-mid L3 stage (72h-96h after egg deposition, AED) and observed 24 h later. Generation of MARCM clones in trachea was performed by crossing MARCM virgin females of genotype yashfy; Tub-Gal80 FRT2A en masse to the Drs-GFP, Bl-Gal4, UAS-myrRFP, PGRP-LCDE12 FRT2A line. Resulting embryos were submitted to a heat shock 4-6 h AED for 1 h at 38°C in a circulating water bath, and kept at 25°C until larvae reached early-mid third instar (72h-96h AED), when they were infected by Ecc and observed 24 h later.

Immunostaining on larvae

Larval tissue were dissected in PBS and fixed for 20 min in 4% paraformaldehyde on ice. After several rinses in PBT (PBS + 0.1% Triton X-100), they were blocked for 1 hr in PBT-3% BSA at 4°C and then incubated with antibody at the appropriate dilution in PBT-TSA 3% overnight at 4°C. Primary antibodies were: rabbit Anti-Relish (1:500) or Mouse Anti-Relish (9E10 Santa Cruz at 1:500). Several washes in PBT were followed by a 2 hr incubation with secondary antibody to RT (Alexa Fluor 540 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG diluted 1:500, Molecular Probes), then 5 washes in PBS. The tissues were finally mounted in Vectashield (Vector Laboratories) fluorescent mount-
ing medium, with DAPI. Images were captured with a LSM 510 Zeiss confocal microscope.

Quantitative real-time PCR
Quantitative real-time PCR and SYBR Green analysis were performed as previously described [50]. Primer information can be obtained upon request. The amount of mRNA detected was normalized to control rp49 mRNA values. Normalized data was used to quantify the relative levels of a given mRNA according to cycling threshold analysis (ΔCt).

Electronic microscopy
For electron microscopic sections, third instar larval trachea were dissected and fixed at RT in 4% PFA and 2% glutaraldehyde in 0.12 M sodium cacodylate buffer at pH 7.4 for 1 h. The trachea were then washed 3 x 10 min in 0.12 M sodium cacodylate buffer, post-fixed in 2% OsO4 in 0.12 M sodium cacodylate buffer for 1 h and washed again 3 x 10 min. Samples were subsequently dehydrated through series of ethanol gradients and infiltrated with propylene oxide, embedded in epoxy resin (Fluka, Sigma) and polymerized at 80°C. Ultrathin (30 nm) plastic sections were cut using a Leica UltraCut microtome with a diamond Diatome knife and post-stained with 2% uranyl acetate, followed by treatment with Reynolds’ lead citrate, and stabilized for transmission EM by carbon coating. Examination was performed with a Zeiss Leo 912 microscope at 100 kV. Images were captured using a Gatan 792 Bioscan camera using Digital Micrograph software.

Supporting Information
Figure S1 Ecc-mediated Drs-GFP activation in the trachea is IMD-dependent and Toll-independent. (A) Dorsal views of Drs-GFP larvae of the following genotypes: UAS-IMD (Drs-GFPhs-Gal4/+;UAS-imd/+), UAS-PGRP-LC (Drs-GFPhs-Gal4/+;UAS-PGRP-LC/+), UAS-esp act (Drs-GFPhs-Gal4/UAS-esp act), Tl3 (Drs-GFP;Tl3/+), PGRP-LCDK12 (Drs-GFP; PGRP-LCDE12/PGRP-LCDK12), imd− (Drs-GFPimd−/imd−) and RelishE20 (Drs-GFP; RelishE20/RelishE20). In non-infected larvae, gain-of-function mutations of IMD pathway components, but not of Toll pathway components, are sufficient to promote intense expression of Drs-GFP in trachea. Upon Ecc infection, Drs-GFP expression is lost in PGRP-LC, imd− or Relish mutants. Images were taken 24 h after heat-shock or Ecc infection. PS: posterior spiracles, VB: visceral branch, DT: dorsal trunk, FB: fat body. (B) Quantification of Drs-GFP expressing larvae in various genotypes. Statistics apply for the “no signal” and the class III categories only. Each histogram corresponds to the mean value of 3 experiments. A total number of 80 larvae were counted for each experiment. Values indicated by identical symbols (*) or ** or *** are significantly different (P<0.05) from one another. All other differences are statistically significant (P<0.05). (EPS)

Figure S2 Down regulation of Tollo mRNA in Tollo mutants and Tollo RNAi larvae and down regulation of Ect4 mRNA in Ect4 mutant. (A) Tollo mRNA detection by q-RT-PCR is shown for total third instar larvae or dissected trachea. Tollo mRNA measured in wild-type (OregonR) larval tissue was set to 100, and values obtained with other tissues were expressed as a percentage of this value. Histograms correspond to the mean +/- SD of 3 experiments. Values indicated by identical symbols (*) or ** are not significantly different (P>0.05) from one another. All other differences are statistically significant (P<0.05). (B) Ect4 mRNA detection by q-RT-PCR is shown for dissected trachea. Ect4 mRNA measured in wild-type (OregonR) larval tissue was set to 100, and values obtained with other tissues were expressed as a percentage of this value. Histograms correspond to the mean +/- SD of 3 experiments. Values indicated by different symbols (*) and ** are significantly different from one another (P<0.05). (EPS)

Figure S3 Negative regulation of tracheal immune response by Tollo. Quantification of Drs-GFP expressing larvae in various genotypes. Tracheal expression of Drs-GFP in Ecc-infected larvae is enhanced by either Tollo mutations or Tollo RNAi-mediated inactivation. Statistics apply for the “no signal” and the class III categories only. Each histogram corresponds to the mean value of 3 experiments. A total number of 120 larvae were counted for each experiment. Values indicated by identical symbols (*) are not significantly different (P>0.05) from one another. All other differences are statistically significant (P<0.05). (EPS)

Figure S4 Bacterial penetration into larval trachea upon Ecc infection. (A) Ecc-GFP localization in trachea of wild-type larvae. Ecc-GFP can be found either in posterior spiracle (PS), visceral branches (VB) or dorsal trunk (DT). Pictures were taken 24 h after infection. (B) Histograms show quantification of larvae with Ecc-GFP positive trachea in control (TolloC3/+ and Btl-Gal4) or Tollo mutants (TolloC3/TolloE3A) and Tollo RNAi (Btl-Gal4; UAS-TolloB6) third instar larvae. Each histogram corresponds to the mean value of 5 experiments. A total number of 100 larvae were counted for each experiment. Values indicated by identical symbols (*) are not significantly different (P>0.05) from one another. (EPS)

Figure S5 The TIR domain-containing protein Ect4 (SARM) and the cytokine Spz2 (DNT1) negatively regulate tracheal immune response. (A, B) Quantification of Drs-GFP expressing larvae in various genotypes. Statistics apply for the “no signal” and the class III categories only. Each histogram corresponds to the mean value of 3 experiments. A total number of 80 larvae were counted for each experiment. Values indicated by identical symbols (*) or ** or *** are not significantly different (P>0.05) from one another. All other differences are statistically significant (P<0.05). (EPS)

Figure S6 Nuclear localization of Relish is increased in Tollo RNAi trachea. Confocal images representative of control or Tollo RNAi trachea from Ecc-infected larva and stained with anti-Relish antibody (Red). Nuclei are stained with Dapi (blue). Scale bar is 50 μm. (EPS)

Figure S7 Negative regulation by Tollo does not involve PIRK,PGRP-LC membrane localization or Caudal Responsive Elements. (A) Quantification of Drs-GFP expressing larvae in various genotypes. Tracheal expression of Drs-GFP in Ecc-infected larvae is unaffected by pirk RNAi-mediated inactivation. Statistics apply for the “no signal” and the class III categories only. Each histogram corresponds to the mean value of 3 experiments. A total number of 80 larvae were counted for each experiment. Values indicated by identical symbols (*) or ** are not significantly different (P>0.05) from one another. All other differences are statistically significant (P<0.05). (B) Tollo is not affecting PGRP-LC::GFP membrane localization in trachea. Confocal images representative of control or Tollo mutant trachea expressing a PGRP-LC::GFP fusion protein, in third instar larvae. The apical localization of PGRP-LC::GFP is unaffected by Tollo mutations. The following genotypes are shown: Tub-Gal4, Tub-
providing flies stocks and reagents: Alicia Hidalgo (DNT14 mutants), Won-Jae Lee (D/CBEmut)-GFP flies and anti-Relish antibody), Pat Simpson (Tollo24 mutants), Amy H. Tang (US-PGRP-4C-GFP flies), Michael Tiemeyer (UAS-Tollo::Myc flies), Jeongbin Yim (Tollo14 mutants). We thank Berra Erkosar, François Leulier, Karine Narbonne-Revau and Thomas Rival for critical comments on the manuscript.

Author Contributions
Conceived and designed the experiments: IA BC JR. Performed the experiments: IA CT BC. Analyzed the data: IA CT BC JR. Wrote the paper: IA BC JR.

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Author Contributions
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