Negative regulation of IMD contributes to disease tolerance during systemic bacterial infection in *Drosophila*

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Abstract

Disease tolerance is an infection phenotype where hosts show relatively high health despite harbouring elevated pathogen loads. Compared to the mechanisms of immune clearance our knowledge of the mechanisms underlying increased tolerance remains incomplete. Variation in the ability to reduce immunopathology may explain why some hosts can tolerate higher pathogen burdens with reduced pathology. Negative immune regulation would therefore appear to be a clear candidate for a mechanism underlying disease tolerance but this has not been tested directly for bacterial infections. Here, we examined how the negative regulation of the immune deficiency (IMD) pathway affects disease tolerance in *Drosophila melanogaster* when infected with the gram-negative bacterial pathogen *Pseudomonas entomophila*. We find that UAS^{RNAi}-mediated reduced expression of the negative regulators of IMD (*pirk* and *caudal*) severely reduced the ability to tolerate infection in both males and females across a wide range of infectious doses. While flies unable to regulate the IMD response exhibited higher expression of antimicrobial peptides and lower bacterial loads as expected, this was not accompanied by a proportional reduction in mortality. Instead, tolerance (measured as fly survival relative to its microbe load) was drastically reduced, likely due to the combination of increased immunopathology and cytotoxicity of elevated AMP expression. Our results therefore highlight that in addition to regulating an efficient pathogen clearance response, negative regulators of IMD also contribute to disease tolerance.

Introduction

To survive infection hosts must not only identify and eliminate the pathogen but must also reduce infection-induced tissue damage. Tissue damage may arise either directly from pathogen growth and secreted virulence factors, or as a negative side-effect of the immune response in the form of immunopathology (Graham, Allen, and Read 2005; Sears et al. 2011; Badinloo et al. 2018). For example, the upregulation of antimicrobial peptides (AMPs) after pathogenic infection (Badinloo et al. 2018; De Gregorio et al. 2002; Zerofsky et al. 2005), the accumulation of toxic/damage molecules such as reactive oxygen species (ROS) (Han and Ulevitch 2005; Schneider 2007; Lambeth 2007), and the phenoloxidase (PO) response (a fast-acting immune effector in insects) are all known to cause tissue damage (Sadd and Siva-Jothy 2006; Cerenius, Lee, and Söderhäll 2008; Khan, Agashe, and Rolff 2017). Overall, the fine balance between clearing pathogens and avoiding tissue damage requires tightly regulated induction and resolution of immune responses (Sears et al. 2011; Soares, Gozzelino, and Weis 2014).

Understanding the distinct pathogen- or host-derived causes of tissue damage is important not only from a therapeutic perspective (Vale, Fenton, and Brown 2014; Vale et al. 2016; Louie et al. 2016), it also offers important mechanistic explanations for the widely variable infection phenotypes observed between individuals (Hill 1998; Råberg, Graham, and Read 2009; Kutzer and Armitage 2016). The field of host-pathogen evolutionary ecology typically describes individuals with low pathogen loads and relatively high health during infection as ‘resistant’ because they are able to clear - or otherwise quantitatively reduce - the establishment and growth of pathogens within the host (Hill 1998; Råberg, Graham, and Read 2009; Lazzaro, Scurman, and Clark 2004; Malo and Skamene 1994). Other infection phenotypes are more difficult to explain as the consequence of immune clearance, such as the observation that some individuals exhibit low levels of pathology (measured as either mortality or physiological performance), despite harbouring relatively high pathogen loads (Råberg, Graham, and Read 2009; Kutzer and Armitage 2016; Raberg, Sim, and Read 2007; Ayres and Schneider 2012). Instead, tolerating higher pathogen loads is more likely to be under the control of mechanisms that prevent, limit or repair tissue damage (Soares, Gozzelino, and Weis 2014; Vale, Fenton, and Brown 2014; Soares, Teixeira, and Moita 2017; R. Martins et al. 2019). However, compared to the detailed knowledge of immune clearance mechanisms we associate with resistance, our knowledge of the specific mechanisms underlying increased tolerance remains limited (Kutzer and Armitage 2016). Previous work in *Drosophila* has employed GWAS and transcriptomics to uncover several candidate genes associated with disease

tolerance, including many that do not have an obvious immune related function such as *CrebA* (Troha et al. 2018), *grainyhead* and *debris buster* (Howick and Lazzaro 2014), *dFOXO* (Dionne et al. 2006; Lissner and Schneider 2018). Curiously, genes related to canonical immune pathways are conspicuously absent from these genome-wide screens of tolerance, even though we would expect the regulation of inflammation to be a key mechanism underlying disease tolerance (Sears et al. 2011; Vale, Fenton, and Brown 2014; Ayres and Schneider 2012; Merklings et al. 2015).

In the present work, we take a more targeted approach to specifically investigate if the negative regulation of immune responses has any measurable effect on the phenotype of disease tolerance. We focused on the immune deficiency (IMD) pathway, one the best described immune signalling pathways in *Drosophila* (Leulier et al. 2003; Bruno Lemaître and Hoffmann 2007; Myllymäki, Valanne, and Rämetsä 2014). Following infection, DAP-type peptidoglycans from gram-negative bacteria are recognised by the pathogen-sensing receptors *PGRPs* (*LC* and *LE*) (Vodovar et al. 2005; Diepöis et al. 2015). These receptors then recruit the adaptor molecule IMD and activate the intracellular signalling cascade that leads to the activation of a *NF-κB* *relish* (see **Fig. 1**) also reviewed in (Myllymäki, Valanne, and Rämetsä 2014; Kuraishi, Hori, and Kurata 2013). Upon activation, *relish* translocates to the nucleus and induces transcription of a set of IMD-responsive AMPs. Minutes after pathogen recognition, a cocktail of AMPs such as *Diptericins*, *Attacins* and *Cecropins* is secreted by the fat body (Bruno Lemaître and Hoffmann 2007; B. Lemaître, Reichhart, and Hoffmann 1997). *Relish* also induces the transcription of negative regulators of IMD (*pirk*, *caudal*, *PGRPs-LB*, *LC*, *LE*,) which ensures an appropriate level of immune response, while avoiding immunopathology (Lee and Ferrandon 2011; Kleino and Silverman 2014). *Pirk*, for example, interferes with the interaction of *PGRP-LC* and *-LE* with the molecule IMD, and limits the activation of the IMD pathway (see **Fig. 1**) (Aggarwal et al. 2008; Kleino et al. 2008; Basbous et al. 2011). Similarly, the homeobox protein *caudal* downregulates the expression of AMPs in flies (Ryu et al. 2008). Taken together, the negative regulation of IMD-pathway ensures an appropriate level of immune response and enables individuals to reduce immune activity in response to reductions in bacterial numbers (Lee and Ferrandon 2011; Kleino et al. 2008; Paredes et al. 2011).

We measured disease tolerance during systemic infection with the gram-negative bacterium *Pseudomonas entomophila* using *Drosophila* lines where individual components of the IMD-pathway were knocked down using UAS^{RNAi} including (i) the transcription factor- *relish*, its negative

regulators (ii) *pirk* and (iii) *caudal*, and (iv) a major IMD-responsive AMP *Diptericin-B*, because of substantial evidence that *Diptericins* are very effective during defense against Gram-negative bacteria (Bruno Lemaître and Hoffmann 2007; Wicker et al. 1990). Given the widespread evidence of sex differences in immunity and response to infection, reviewed in (Klein and Flanagan 2016; Belmonte et al. 2020), we also specifically tested whether female and male flies differ in their ability to tolerate bacterial infection.

Figure 1: A simplified version of *Drosophila* IMD (immune deficiency) pathway activated by the DAP type peptidoglycan following the gram-negative bacterial infection (*Pseudomonas entomophila*). These receptors then recruit the adaptor molecule Imd and activate the intracellular signalling cascade that leads to the activation of a NF- κ B *relish*. Upon activation, relish translocates to the nucleus and induces transcription of a set of cocktail of IMD-responsive AMPs such as *Diptericins*, *Attacins* and *Cecropins*, minutes after pathogen recognition. The negative regulators of IMD such as *pirk*, *caudal*, PGRPs-LB ensure an appropriate level of immune response, while avoiding immunopathology

Materials and methods

Fly strains and husbandry: We used the following fly lines: UAS-*relish*^{RNAi} (Bloomington stock# 33661), UAS-*caudal*^{RNAi} (Bloomington stock# 57546), UAS-*pirk*^{RNAi} (Bloomington stock# 67011) and UAS-*DiptericinB*^{RNAi} (Bloomington stock# 28975) also see Supplementary Information **Table S1** for details about fly genotypes. All fly lines were obtained from the Bloomington *Drosophila* stock centre at

the Indiana University. Each of the transgenic fly line contains a homologous hairpin under the control of the Upstream Activating Sequence (UAS^{RNAi}) to a Gal4 temperature-sensitive transcriptional activator (*Bloomington stock# 67065*). This fly line drives the Gal4 expression in the entire body of fly and contains a temperature sensitive Gal80, which inhibits Gal4 at the permissive temperature (25°C). All fly stocks were reared in plastic vials (12 ml) on a standard Lewis medium at 25±2°C with a 12 hour light: dark on 14 days cycle (by transferring flies into new food vials). All the crosses were made at 25°C and after eclosion, offspring were transferred to agar vials (12 flies per vial) at the non-permissive temperature (29°C) for two days to facilitate silencing of the candidate genes until experimental day.

Bacterial culture preparation: The gram-negative bacterium *Pseudomonas entomophila* has a broad host range, infecting insects, nematodes, plants, and higher vertebrates (Dieppois et al. 2015). In flies, *P. entomophila* infection results in pathology to the gut epithelium during systemic infections, eventually causing death (Vodovar et al. 2005; Dieppois et al. 2015; Troha and Buchon 2019). To obtain pure cultures, frozen bacterial stock cultures (-70°C) were streaked onto fresh LB agar plates and single colonies were inoculated into 15ml LB broth and incubated overnight at 37°C with shaking at 120rpm (revolutions per minute). The overnight cultures were diluted 1: 100 into fresh LB broth of 15ml and incubated again at 37°C with shaking at 120rpm. At the mid-log phase (optical density OD₆₀₀ = 0.75), we harvested the bacterial cells by centrifugation at 5000 rpm for 5 min at 4°C and re-suspended the bacterial pellet in 1XPBS (phosphate buffer saline) solution. The final inoculum was adjusted to OD₆₀₀ = 1.0, and this was the starting bacterial inoculum used for all flies for systemic infection.

Gene expression: We quantified the expression of major IMD-responsive AMPs namely, *diptericin* (*Dpt*), *attacin-C* (*AttC*), *cecropin-A1* (*CecA1*), by quantitative real-time polymerase chain reaction (qRT-PCR). We randomly selected a subset of IMD-pathway mutants and functional flies both males and females at 8 hours following infection for RNA extraction, we included 15 flies i.e., 5-7 replicates of 3 flies pooled together for each treatment (Knockdown and functional flies) and sexes. We first homogenised whole flies (3 in group) using sterile micro-pestles into 1.5µl microcentrifuge tubes containing 80µL of TRIzol reagent (Invitrogen, Life Technologies). The tubes containing the fly homogenate were kept frozen at -70°C until RNA extraction. We

performed mRNA extractions using the standard phenol-chloroform method and included a DNase treatment (Ambion, Life Technologies). We confirmed the purity of eluted samples using a Nanodrop 1000 Spectrophotometer (version 3.8.1) before proceeding with RT (reverse transcription). The cDNA was synthesized from 2µL of the eluted RNA using M-MLV reverse transcriptase (Promega) and random hexamer primers, and then diluted 1:1 in nuclease free water. We then performed quantitative RT-PCR (qRT-PCR) on an Applied Biosystems StepOnePlus machine using Fast SYBR Green Master Mix (Invitrogen Ltd.) using a 10µL reaction containing 1.5L of 1:1 diluted cDNA, 5µL of Fast SYBR Green Master Mix a 3.5µL of a primer stock containing both forward and reverse primer at 1µM suspended in nuclease free water (final reaction concentration of each primer being 0.35µM). For each cDNA sample, we performed two technical replicates for each set of primers and the average threshold cycle (Ct) was used for the analysis. We obtained the AMP primers from Sigma-Aldrich Ltd (see supplementary **Table S2** for information about primers). We optimised the annealing temperature (T_a) and the efficiency (Eff) of the *Dpt* primer pair was calculated by 10-fold serial dilution of a target template (each dilution was assayed in duplicate); *Dpt*: T_a = 59 °C, Eff= 102%; *AttC*: T_a = 60 °C, Eff= 94%; *CecA1*: T_a = 59.5 °C, Eff= 109%.

Fly survival following bacterial infection: To test the impact of *Pseudomonas entomophila* infection on fly survival, we challenged experimental flies to 4 different bacterial concentrations or infection doses ranging between (I) ~650 cells (II) ~1400 cells (III) ~2000 cells (IV) ~2900 cells/µl of *P. entomophila* culture, obtained by serial dilution. Flies (n=10-18 fly vials /sex/infection dose/fly line) were infected systemically by intra-thoracic pricking (Khalil et al. 2015) with a needle immersed in bacterial suspension under minimal CO₂ anaesthesia. The effect of injury caused by pricking was controlled by including mock infections performed using a needle dipped in sterile 5% sucrose solution. We observed the mortality for 10 days post-infection every 3 hours (±0.5h) for the first 2 days (between 6 am and 11 pm) and every 12 hours for the following 7 days.

Quantification of bacterial load following bacterial infection: During the survival assay, flies were randomly sampled (10-18 live fly vials/sex/infection dose/fly line) and the bacterial load was quantified around 8 hours following infection. Initially, flies (group of 3) were transferred to a 1.5ml microcentrifuge tubes and surface sterilized by adding 70% ethanol for 30-60 seconds to ensure

we counted only colony forming units (CFUs) inside the flies. Ethanol was then removed by briefly washing flies twice with sterile distilled water. To ensure that this method was efficient in surface cleaning flies, we plated 4µl of the second wash on LB agar (from random flies) ensuring that the flies are free from surface bacteria (i.e., no viable CFUs). We then placed in 1.5ml Eppendorf tubes and added 100µl of LB broth to each tube and the flies were thoroughly homogenized using a motorized pestle. We immediately plated this homogenate in serial dilutions ranging from 100 to 10⁻⁶ using 1xPBS (phosphate buffer) on LB agar plates and incubated at 30°C for about 18 hours after which viable colonies were counted manually (Siva-Jothy et al. 2018).

Statistical analysis

1. Gene expression: We analysed the gene expression data by first calculating the ΔCT value (Livak and Schmittgen 2001) for the expression of a gene of interest relative to the reference house-keeping gene rp49 (Gupta and Vale 2017). We then used analysis of variance (ANOVA) model to analyse normally distributed data, i.e., to test for the effects of the experimental treatment on the ΔCT values of whether the AMPs expression differed significantly between knockdown and fully functional flies of each IMD-pathway fly line. We used the R package ‘ggplot2’ for graphics and data visualization (Wickham 2016; Bunn and Korpela, 2019)
2. Bacterial load: We then quantified differences in bacterial load following bacterial infection by analysing the bacterial measurement (log₁₀) around 8 hours following *P. entomophila* infection. We found that residuals of bacterial load data were normally distributed when tested with Shapiro-Wilks test and hence we used ANOVA and analysed the bacterial load data by fitting ‘knockdown’ (functional flies and IMD-pathway knockdown flies) and ‘sex’ as categorical fixed-effects, while infection dose (4 different infectious doses of *P. entomophila*) and ‘vials’ (replicates) as a random-effects for each of the fly lines (functional flies and IMD-pathway knockdown flies).
3. Survival post-infection: We analysed the survival data after *P. entomophila* bacterial infection for all the IMD-pathway mutants by a mixed effects Cox model using the R package ‘coxme’

(Therneau 2015) for both males and females respectively. In each case, we specified the model as: survival $\sim$ knockdown (functional and knockdown) x sex (male and female) x dose (4 concentrations of *P. entomophila*) (1 | vial (n replicates)), with ‘knockdown’ and ‘sex’ as fixed effects, and vials as a random effect for all the fly lines (IMD-pathway mutants). We fit separate models for each fly lines during *P. entomophila* infections, since they were assayed on different days. Next, to understand how different knockdown flies (i.e., having fully functional vs. knockdown of IMD-pathway) differ in survival to bacterial infections in detail, we also analysed survival data for *P. entomophila* infections for each of the IMD-pathway mutant/fly lines separately using Cox Proportional Hazard survival analysis. We estimated the impact of harbouring IMD-pathway knockdowns as the hazard ratio of IMD-knockdowns vs. fully functional fly lines. A hazard ratio significantly greater than one indicates higher risk of mortality in the IMD-mutants relative to fully functional individuals; hence, a significant impact on fly’s survival.

4. Measuring disease tolerance: Finally, to understand how the negative immune regulation contribute to disease tolerance during bacterial infections we plotted tolerance by calculating (median lifespan/mean bacterial load) for male and female flies (i.e., flies with functional and knockdown IMD pathway regulators) after bacterial exposure with 4 different infection doses. Tolerance is usually measured as a reaction-norm or slope which could be either linear or non-linear, where the pathogen burden is plotted on the x-axis and health or fitness is plotted on the y-axis (Raberg, Sim, and Read 2007; Ayres and Schneider 2012; Medzhitov, Schneider, and Soares 2012; Louie et al. 2016; Gupta and Vale 2017; Oliveira, Bahia, and Vale 2020). This standard reaction norm approach that measures tolerance as the rate of health decrease for increasing microbe loads is only statistically sound when mortality and microbe loads measurements are taken from the same individual or from the same group of replicate individuals. Given the destructive sampling required to measure microbe loads in flies we chose instead to use another commonly used metric of tolerance using the average mortality of flies of a given line relative to the average bacterial load of that line, see for example (Adelman et al. 2013; Knutie et al. 2017; Cornet et al. 2014). This is sometimes referred to as ‘point’ tolerance and is intuitive to interpret: relative to a given microbe load, does one line die more (less tolerant) or less (more tolerant) (Little et al. 2010).

Figure 2: Design of experiments to assay (1) gene expression of the major IMD-regulated AMPs (n=15 flies; 5-7 replicates of 3 flies pooled together for each treatment, sex and fly line combination) (2) internal bacterial load (n=10-18 fly vials /sex/infection dose/fly line) and (3) survival following systemic bacterial infection with *Pseudomonas entomophila* n=10-18 fly vials /sex/infection dose/fly line) to test impact of the negative regulation of IMD pathway on phenotype of disease tolerance (4).

Results and discussion:

1. Loss of IMD negative regulation leads to increased AMP expression and decreased bacterial load

We first began by quantifying major IMD-regulated AMP expression (*AttC*, *CecA1* and *Dpt*) following infection with *P. entomophila* in both male and female flies (**Fig. 3A, Table-S3**). As expected, *relish* knockdown flies had lower AMP expression while loss of negative regulators *caudal* and *pirke* resulted in higher AMP expression (**Fig. 3A, Table-S3**). Next, we wanted to test whether AMP expression correlated with the bacterial replication in flies. Consistent with their AMP expression, both male and female flies with reduced negative regulation (*caudal* and *pirke*) exhibited a reduction in bacterial numbers while flies lacking *relish* exhibited higher levels of bacterial numbers (compare **Fig. 3A** gene expression and **Fig. 3B** bacterial load data), clearly indicating the expected importance of AMPs in defence against this bacterial pathogen (**Fig. 3B, Table-S4**).

Figure 3: (A) Quantification of major IMD-responsive AMPs by qRT-PCR (n=15 flies/sex/treatment) for IMD-pathway mutants and functional flies (males and females) around 8 hours following *P. entomophila* systemic infection (0.05 OD or ~2000 bacterial cells/ μ l). Each data point represents 5-7 replicates of 3 flies pooled together for each infection treatment, fly line and sex **(B)** internal bacterial load after 8 hours following infection (n=5 replicates, 3-pooled flies/sex/treatment). Asterisks inside the panels indicate significant differences in AMP expression and bacterial load between knockdown flies and flies with fully intact IMD functioning ($p < 0.05$).

2. Flies with reduced expression of *pirk* or *caudal* show increased mortality despite having lower bacterial loads

In flies, systemic infection with *P. entomophila* results in severe pathology, eventually causing death (N. E. Martins et al. 2013). Induction of AMPs can occur as early as 2 hours after infection (B. Lemaitre, Reichhart, and Hoffmann 1997). *Relish* knockdown flies are known to be short-lived and also exhibit increased bacterial loads after infection. For instance, even a single *E. cloacae* cell

infection is sufficient to kill the fly (Hedengren et al. 1999). Given our observation that knocking down negative regulators of IMD resulted in an increase in AMP expression, we would expect mortality in these flies to be lower than controls if variation in mortality was mainly explained by pathogen clearance. However, while mortality was higher in *relish* and *DiptericinB* knockdowns as expected, we also observed higher mortality when knocking down the expression of both negative regulators, *pirk* and *caudal* (Fig. 4, Table-S5), despite the increased expression of AMPs and their relatively lower bacterial load (Fig. 3). This increased mortality of flies lacking negative regulators of IMD such as *pirk* and *caudal* has been observed previously (Kleino et al. 2008; Ryu et al. 2008; Morris et al. 2016) and suggests that mechanisms other than pathogen clearance actively contribute to the variation in mortality during systemic bacterial infection.

Figure 4: (A) Survival curves for male and female *UAS^{RNAi}* flies (n=16-24 fly vial/treatment/sex/fly line) (a) *UAS-Relish^{RNAi}* (b) *UAS-Caudal^{RNAi}* (c) *UAS-Pirk^{RNAi}* (d) *UAS-DptB^{RNAi}* after systemic infection of *P. entomophila* (dose ~2000 cells/μl). **(B)** hazard ratio calculated from the survival curves. Asterisks in panel B indicate increased susceptibility of knockdown flies compared to functional flies (p<0.05).

3. Knocking down of *caudal* or *pirk* expression in flies results in reduced disease tolerance

By affecting the mortality of flies independently of their microbe loads, negative regulators of IMD would fit the functional definition of a tolerance mechanisms (Kutzer and Armitage 2016; Ayres and Schneider 2012; Lissner and Schneider 2018). To quantify the tolerance response of each fly

line more precisely, we measured the tolerance phenotype (measured as fly survival relative to its bacterial load) of male and female flies differing in functional components of IMD-pathway (*relish*, *caudal*, *pirk* and *DptB*). Overall, we found that flies without the negative IMD regulators *caudal* and *pirk* showed reduced tolerance to systemic *P. entomophila* infection compared to flies with fully intact immune system (Fig. 5, Table-S6). Flies without the transcription factor *relish* or the AMP *DptB* also showed reduced tolerance, which may reflect feedbacks between increased pathogen growth and decreased ability to limit damage when microbe loads get too high (that is, the per-pathogen damage becomes higher in flies unable to clear infection in the first place) (Råberg 2014).

Figure 5: Disease tolerance in IMD-pathway mutants and fully functional flies. The relationship between host (fly) survival and bacterial load (single infection dose) analysing using point tolerance around 8 hours post *P. entomophila* infection for male and female flies. Each data represents ‘point tolerance’, the ratio estimates calculated from median lifespan and average bacterial load for IMD-pathway knockdown and flies with fully intact IMD functioning. Asterisks inside the panel indicates significant difference in disease tolerance between knockdown flies and flies with fully intact IMD functioning ($p < 0.05$).

To further characterise the tolerance response of each IMD fly line across a wide range of pathogen exposure, we infected the IMD-pathway mutants with different doses of *P. entomophila* (ranging from ~650 bacterial cells infection dose to leading up to ~2900 cells). When IMD mutant flies were exposed to very high bacterial infection, all fly lines showed reduced tolerance (Fig. 6, Table-S6). We found similar effect for both females and males suggesting that overall tolerance effects

both sexes similarly when exposed to systemic *P. entomophila* bacteria. Altogether, our data show that the negative regulators of IMD pathway play a major role in antibacterial protection and mutant flies without negative regulators of IMD are less tolerant to infection.

Figure 6: Disease tolerance in IMD-pathway mutants and fully functional flies. The relationship between host (fly) survival and bacterial load of 4 infection doses [i.e., (I) ~650 cells (II) ~1400 cells (III) ~2000 cells (IV) ~2900 cells] of *P. entomophila*, analysing using point tolerance around 8 hours following *P. entomophila* infection for (A) male and (B) female flies. Each data represents ‘point tolerance’, the ratio estimates calculated from median lifespan and average bacterial load for IMD-pathway knockdown and flies with fully functional IMD. Asterisks inside the panel indicates significant difference in disease tolerance between knockdown flies and with fully intact IMD functioning ($p < 0.05$).

One likely cause of flies lacking negative IMD regulation still succumb to death despite reducing pathogen burden is immunopathology due to continued immune activation. There is a precedent for the effects of negative regulators on disease tolerance. For example, *G9a*, a negative regulator of Jak/Stat, was identified as being important in tolerating *Drosophila C Virus* infections by reducing immunopathology during the antiviral immunity (Merkling et al. 2015). During bacterial infections, *PGRP-LB* functions as a negative regulator of the *Imd* pathway by regulating the IMD-responsive AMP *Diptericin* (Zaidman-Rémy et al. 2006). More recently it has been shown that *PGRP-LB* mutant flies experience sex-differences in survival despite undetectable differences in their bacterial loads (Vincent and Dionne 2021). Though this study did not directly measure disease tolerance (i.e., survival relative to microbe load), its findings are consistent with the phenotype of

disease tolerance. Here, we have explicitly analysed the tolerance response in the IMD mutants and found a clear effect of its negative regulators on disease tolerance, though this was comparable in both males and females. How these negative regulators contribute to disease tolerance is likely linked to their role in reducing immunopathology. In the case of *caudal* and *pirk*, there is a possible source of tissue damage through the cytotoxic effects of AMPs, because we observed higher AMP expression in these mutants. AMPs are known to be harmful to both prokaryotic and eukaryotic cells and tissues (Song et al. 2005; Paredes-Gamero et al. 2012) and overexpression of AMPs in flies leads to cytotoxicity, causing neurodegeneration and age-related mortality (Badinloo et al. 2018; Cao et al. 2013). This would support the idea that elevated levels of several AMPs are sufficient to cause cytotoxicity to cells/tissues, contributing to immunopathological consequences.

Concluding remarks

In the present work, we used a highly virulent fly pathogen (*P. entomophila*) and flies lacking various components of IMD-signalling. Our data show that flies with reduced expression of the negative regulators of IMD *pirk* and *caudal* experienced reduced tolerance despite having higher AMP expression and reducing the bacterial numbers. We hypothesise that this is likely due to immunopathology arising through the accumulation of cytotoxicity (AMPs and ROS). Our results highlight that the negative regulators *caudal* and *pirk* are important not only in regulating pathogen clearance by the IMD pathway, but also play a role in disease tolerance (**Fig. 7**). Uncovering the role of these regulators in disease tolerance phenotype opens up the possibility of using them as therapeutic targets where the aim is to enhance host disease tolerance (R. Martins et al. 2019; Vale et al. 2016; Vale, Fenton, and Brown 2014; Schneider and Ayres 2008). Future work may also investigate if natural genetic variation in disease tolerance can be explained by variation in negative immune regulators.

Figure 7: Summary highlighting that in addition to regulating an efficient pathogen clearance response, negative regulators of IMD also contribute to the phenotype of disease tolerance. Flies with reduced expression of the negative regulators *pirk* and *caudal* show reduced tolerance (overall health state) despite having higher AMP expression and reducing the bacterial numbers. Flies with reduced *relish* and *DptB* expression also exhibited reduced tolerance and survival mostly due to pathogen-induced mortality because of lower AMPs to target the bacterial replication.

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Author contribution

A.P and P.F.V designed the experiments; A.P carried out experiments; A.P and K.M.M performed qPCR experiments; A.P and P.F.V analysed the data and wrote the manuscript.

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Competing interests

None.

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Supplementary information

Figure S1. Survival curves of female flies of different IMD-pathway mutants (n=16-24 flies/treatment/sex/fly line) **(a)** *UAS-Relish*^{RNAi} **(b)** *UAS-Caudal*^{RNAi} **(c)** *UAS-Pirk*^{RNAi} **(d)** *UAS-DipB*^{RNAi} during 4 concentrations of systemic *Pseudomonas entomophila* infection ranging between (1) ~650 cells (2) ~1400 cells (3) ~2000 cells (4) ~2900 cells/μl of *P. entomophila* culture. Since all 4 infection concentrations were performed simultaneously for each of the fly line, we used a single mock-controls (SI) across all 4 different infection doses.

Figure S2. Survival curves of male flies of different IMD-pathway mutants (n=16-24 flies/treatment/sex/fly line) **(a)** *UAS-Relish^{RNAi}* **(b)** *UAS-Caudal^{RNAi}* **(c)** *UAS-Pirk^{RNAi}* **(d)** *UAS-DptB^{RNAi}* during 4 concentrations of systemic *Pseudomonas entomophila* infection ranging between (1) ~650 cells (2) ~1400 cells (3) ~2000 cells (4) ~2900 cells/ μ l of *P. entomophila* culture. Since all 4 infection concentrations were performed simultaneously for each of the fly line, we used a single mock-controls (SI) across all 4 different infection doses.

Figure S3. The estimated hazard ratios calculated from survival curves (**Fig. S1** and **S2**) for flies with fully functional immune system and IMD-pathway mutants for both Sexes. The different IMD-pathway mutants include **(a)** *UAS-Relish^{RNAi}* **(b)** *UAS-Caudal^{RNAi}* **(c)** *UAS-Pirk^{RNAi}* **(d)** *UAS-DptB^{RNAi}*. We used 4 different concentrations of systemic *P. entomophila* infection. A greater hazard ratio >1 shown as horizontal dashed grey lines indicates higher susceptibility of IMD-mutant flies than the functional flies to *P. entomophila* infection.

Figure S4. Bacterial loads around 24 hours after different concentrations of systemic *P. entomophila* infection for **(A)** female and **(B)** male flies for IMD-pathway mutants **(i)** *UAS-Relish*^{RNAi} **(ii)** *UAS-Caudal*^{RNAi} **(iii)** *UAS-Pirk*^{RNAi} **(iv)** *UAS-DipB*^{RNAi}.

Table S1: Fly strains used in this study

<i>Fly line</i>	<i>Bloomington stock #</i>	<i>Genotype</i>
UAS- <i>relish</i> ^{RN^{Δi}}	33661	<i>y[1]sc[*]v[1]; P{y[+t7.7]v[+t1.8]=TRiP.HMS00070}attP2</i>
UAS- <i>caudal</i> ^{RN^{Δi}}	57546	<i>y[1]sc[*]v[1]; P{y[+t7.7]v[+t1.8]=TRiP.HMC04863}attP40</i> ,
UAS- <i>pirk</i> ^{RN^{Δi}}	67011	<i>y[1]sc[*]v[1]; P{y[+t7.7]v[+t1.8]=TRiP.HMS05477}attP40</i>
UAS- <i>DiptericinB</i> ^{RN^{Δi}}	28975	<i>y[1]v[1]; P{y[+t7.7]v[+t1.8]=TRiP.HM05186}attP2</i>
(UAS ^{RN^{Δi}}) ubiquitous driver	67065	<i>w[*]; P{UAS-3xFLAG.dCas9.VPR}attP40, P{tubP-GAL80^u}10; P{tubP-GAL4}LL7/TM6B, Tb^l</i>

Table S2: Primers used in this study

<i>Obtained from</i>	<i>AMPs</i>	<i>Sequence</i>
1. (Troha et al. 2018)	<i>Dpt_F</i> Forward:	5' GACGCCACGAGATTGGACTG 3'
	<i>Dpt_R</i> Reverse	5' CCCACTTTCCAGCTCGGTTC 3'
	<i>AttC_F</i> Forward:	5' TGCCCGATTGGACCTAAGC 3'
	<i>AttC_R</i> Reverse:	5' GCGTATGGGTTTTGGTCAGTTC 3'
2. (Loch et al. 2017)	<i>CecA1_F</i> Forward	5' TCITCGTTTTTCGTCGCTCTCA 3',
	<i>CecA1_R</i> Reverse	5' ATTCCCAGTCCCTGGATT GTG 3'.
3. (Gupta and Vale 2017)	<i>Rp49_F</i> Forward:	5' ATGCTAAGCTGTGCGACAAATG 3'
	<i>Rp49_R</i> Reverse:	5' GTTCGATCCGTAACCGATGT 3'

Table S3: Gene expression of major IMD-pathway regulated AMPs. Gene expression quantified relative to the internal control gene rp49 was quantified in 5-6 replicate individuals (3 pooled flies) of males and females exposed to *P. entomophila* systemic bacterial infection (dose 0.05 OD or ~2000 bac. cells). Analysed using ANOVA and specified the model as gene expression ~ knockdown (*functional and knockdown*) x sex (*male and female*) x AMPs (*major IMD-regulated*) for each of the fly line (IMD-mutants).

<i>Genotype</i>	<i>Source</i>	<i>df</i>	<i>Sum Sq</i>	<i>F value</i>	<i>p</i>
<i>UAS-Relish^{RN/Ai}</i>	Knockdown (<i>knockdown and functional flies</i>)	1	1160.3	148.38	<0.001
	AMPs (<i>major IMD-responsive AMPs</i>)	2	11.2	0.717	0.49
	Sex	1	0.4	0.051	0.82
	Knockdown x AMPs	2	49.1	3.139	0.05
	Knockdown x Sex	1	0.3	0.033	0.85
	AMPs x Sex	2	1.4	0.087	0.91
	Knockdown x AMPs x Sex	2	11.7	0.748	0.47
<i>UAS-Caudal^{RN/Ai}</i>	Knockdown	1	460.4	159.2	<0.001
	AMPs	2	10.6	1.835	0.17
	Sex	1	23.5	8.137	<0.001
	Knockdown x AMPs	2	82.3	14.24	<0.001
	Knockdown x Sex	1	1.1	0.394	0.53
	AMPs x Sex	2	4.4	0.770	0.46
	Knockdown x AMPs x Sex	2	25.2	4.365	<0.001
<i>UAS-Pirke^{RN/Ai}</i>	Knockdown	1	538.3	338.9	<0.001
	AMPs	2	34.2	10.77	<0.001
	Sex	1	0.0	0.000	0.99
	Knockdown x AMPs	2	47.1	14.82	<0.001
	Knockdown x Sex	1	8.5	5.360	<0.001
	AMPs x Sex	2	35.6	11.22	<0.001
	Knockdown x AMPs x Sex	2	7.2	2.255	0.11

Table S4: Summary of log₁₀ transformed bacterial load data after exposure to 4 different concentrations (doses) of *P. entomophila* systemic infection, analysed using ANOVA by fitting 'Knockdown', 'Sex' and 'dose' as categorical fixed-effects and replicate 'vials' as a random-effects for each of the genotype.

<i>Genotype</i>	<i>Source</i>	<i>df</i>	<i>Sum Sq</i>	<i>F value</i>	<i>p</i>
<i>UAS-Relish^{RN/Ai}</i>	Knockdown	3	351.07	45.4374	<0.001
	Sex	1	39.32	15.2654	<0.001
	Dose	3	518.53	67.1097	<0.001
	Knockdown x Sex	1	15.53	6.0297	<0.001
	Knockdown x dose	3	14.43	1.8681	0.13
	Sex x dose	3	1.64	0.2120	0.88
	Knockdown x Sex x dose	1	3.72	1.4450	0.23
	<i>Random effect</i>	<i>Std err</i>			
	<i>Vials</i>	<i>1.605</i>			
<i>UAS-Caudal^{RN/Ai}</i>	Knockdown	3	157.03	20.6574	<0.001
	Sex	1	115.72	45.6679	<0.001
	Dose	3	528.70	69.5504	<0.001
	Knockdown x Sex	1	0.38	0.1497	0.69
	Knockdown x dose	3	27.01	3.5529	0.014
	Sex x dose	3	3.55	0.4665	0.70
	Knockdown x Sex x dose	1	1.10	0.4360	0.50
	<i>Random effect</i>	<i>Std err</i>			
	<i>vials</i>	<i>1.592</i>			
<i>UAS-Pirke^{RN/Ai}</i>	Knockdown	3	164.02	18.3271	<0.001
	Sex	1	98.90	33.1544	<0.001
	Dose	3	407.46	45.5296	<0.001
	Knockdown x Sex	1	2.57	0.8601	0.35

<i>UAS-DptB^{RN<i>Δ</i>i}</i>	Knockdown x dose	3	3.17	0.3541	0.78
	Sex x dose	3	2.17	0.2428	0.86
	Knockdown x Sex x dose	1	0.06	0.0215	0.88
	<i>Random effect</i>	<i>Std err</i>			
	<i>vials</i>	1.727			
	Knockdown	3	128.94	13.2207	<0.001
	Sex	1	87.43	26.8915	<0.001
	Dose	3	615.60	63.1179	<0.001
	Knockdown x Sex	1	0.44	0.1354	0.71
	Knockdown x dose	3	27.20	2.7885	0.04
	Sex x dose	3	7.48	0.7674	0.51
	Knockdown x Sex x dose	1	1.43	0.4411	0.50
	<i>Random effect</i>	<i>Std err</i>			
	<i>vials</i>	1.803			

Table S5: Summary of mixed effects Cox model, fitting the model to estimate response to *P. entomophila* infection in flies with fully intact immune system and flies with deficiencies in IMD-pathway. We used data from the individuals of 3-5-day adult males and females infected with 4 concentrations (doses) of *P. entomophila* for each fly lines (IMD-pathway mutants) and specified the model as: survival ~ Knockdown * Sex * dose (1 | vial), with ‘Knockdown’ and ‘Sex’ as fixed effects, and ‘vials’ as a random effect. The table shows model output (ANOVA) for survival post-infection for flies with fully function immune system and defects in IMD functioning.

<i>Genotype</i>	<i>Source</i>	<i>Loglik</i>	<i>Chisq</i>	<i>df</i>	<i>p</i>
<i>UAS-Relish^{RN<i>Δ</i>i}</i>	Knockdown	1344.5	33.429	1	<0.001
	Sex	1344.5	0.0003	1	0.98
	Dose	1273.7	141.55	4	<0.001
	Knockdown x Sex	1273.5	0.4192	1	0.51
	Knockdown x dose	1270.0	7.0930	4	0.13
	Sex x dose	1269.5	0.9557	4	0.91
	Knockdown x Sex x dose	1266.8	5.4275	4	0.24
	<i>Random effect</i>	<i>Std dev</i>			
<i>UAS-Caudal^{RN<i>Δ</i>i}</i>	<i>Vials</i>	0.244			
	Knockdown	1337.6	28.624	1	<0.001
	Sex	1337.5	0.0156	1	0.9
	Dose	1285.3	104.43	4	<0.001
	Knockdown x Sex	1285.3	0.0005	1	0.98
	Knockdown x dose	1283.6	3.4040	4	0.49
	Sex x dose	1280.6	6.0031	4	0.19
	Knockdown x Sex x dose	1279.2	2.8850	4	0.57
<i>UAS-Pirke^{RN<i>Δ</i>i}</i>	<i>Random effect</i>	<i>Std dev</i>			
	<i>vials</i>	0.02			
	Knockdown	1412.1	21.126	1	<0.001
	Sex	1412.1	0.0001	1	0.99
	Dose	1357.4	109.30	4	<0.001
	Knockdown x Sex	1356.6	1.6275	1	0.20
	Knockdown x dose	1355.3	2.5330	4	0.63
	Sex x dose	1353.9	2.9391	4	0.56
<i>UAS-DptB^{RN<i>Δ</i>i}</i>	Knockdown x Sex x dose	1352.3	3.2265	4	0.52
	<i>Random effect</i>	<i>Std dev</i>			
	<i>Vials</i>	0.14			
	Knockdown	1386.1	29.146	1	<0.001
	Sex	1386.0	0.2848	1	0.59
	Dose	1331.1	109.81	4	<0.001
	Knockdown x Sex	1331.0	0.1187	1	0.73
	Knockdown x dose	1330.8	0.3249	4	0.98
<i>UAS-DptB^{RN<i>Δ</i>i}</i>	Sex x dose	1329.1	3.5419	4	0.47
	Knockdown x Sex x dose	1328.0	2.1198	4	0.71
	<i>Random effect</i>	<i>Std dev</i>			
	<i>Vials</i>				

Table S6: ‘Point tolerance’ for all doses - Summary of ANCOVA. To assess differences bacterial loads following infection with 4 concentrations (dose) of *P. entomophila*, we analysed the average bacterial load, measured around 8 hours following infection using ANCOVA and fitted ‘Knockdown’ and ‘Sex’ as categorical fixed effects and ‘bacterial load’ as continuous co-variate for each fly line.

Genotype	Source	df	F ratio	p
<i>UAS-Relish^{RN<i>Δ</i>i}</i>	Knockdown	1	17.584	0.003
	Sex	1	2.2509	0.17
	Dose	3	5.2387	0.02
	Bacterial load	1	0.0728	0.79
	Knockdown * Bac. load	1	13.549	0.006
<i>UAS-Canda^{RN<i>Δ</i>i}</i>	Knockdown	1	9.1734	0.01
	Sex	1	0.5894	0.46
	Dose	3	3.7512	0.059
	Bacterial load	1	0.0386	0.84
	Knockdown * Bac. load	1	5.9732	0.04
<i>UAS-Pirke^{RN<i>Δ</i>i}</i>	Knockdown	1	25.071	0.001
	Sex	1	0.9570	0.35
	Dose	3	2.8131	0.10
	Bacterial load	1	8.6071	0.01
	Knockdown * Bac. load	1	0.1030	0.75
<i>UAS-DptB^{RN<i>Δ</i>i}</i>	Knockdown	1	25.486	0.001
	Sex	1	0.0266	0.87
	Dose	3	10.069	0.004
	Bacterial load	1	4.2575	0.07
	Knockdown * Bac. load	1	17.282	0.003