Models of infectious diseases in the fruit fly
Drosophila melanogaster

Marc S. Dionne¹ and David S. Schneider²,*

We examined the immune response of a fly as physicians might, by looking at the genesis of diseases caused by microorganisms. Fly infections are complex and there are few simple rules that can predict how an infected fly might fare. As we observed the finer details of the infections, we found that almost every microbe caused a different type of pathology in the fly. Two pattern recognition pathways, Toll and immune deficiency (Imd), were found to detect, and respond to, infections. The physiological response of the fly was modified further by Eiger, insulin, Wnt inhibitor of dorsal (WntD) and nitric oxide (NO) signaling. As in humans, some of the damage that occurred during the fly immune response was caused by an over-aggressive response rather than by the microbes themselves. When looking at the matrix of signaling pathways and the microbes being tested, it was immediately obvious that most of the pathways would need to be studied in more detail before defining the rules that govern their role in pathogenesis. This detailed analysis of signaling and pathogenesis has the potential to allow the fly to be used as a model patient instead of as simply an innate immune system model.

After proving the link between NFκB and Toll in Drosophila, the Toll signaling pathway developed into a touchstone of innate immunity (Lemaitre et al., 1996; Rosetto et al., 1995). The yearly publications on Toll-like receptors (TLRs) in vertebrates now outnumber those on the Drosophila world by at least 10 to 1, illustrating the conservation of fundamental immune signaling pathways and the importance of initial findings in Drosophila to vertebrate organisms.

As our understanding of Toll signaling deepened, it became apparent that some things work differently in flies versus humans. For example, TLRs in vertebrates appear to bind many of their microbial ligands directly; however, in the fly, pattern recognition occurs upstream through peptidoglycan binding proteins. The variability in some of the fine mechanics of signaling between flies and humans highlights the need to identify pathway components that are organism specific. However, the conservation of key mechanisms regulating central physiological functions demonstrates the usefulness of the fly as a research tool.

The utility of the fly extends to the study of infectious disease; Drosophila is used to dissect host interactions with known insect pathogens and is a tractable model system for human disease. Studies with insect pathogens teach us general lessons about the pathology of infection that are useful even if the microbes are not primary human pathogens. Although fly models of human disease cannot replicate the entire disease process that occurs in the human, they possess rapid and simple genetics enabling studies to be carried out that would not be possible in larger animals.

Fly immunity is a multilayered system
The fly has a multilayered immune system consisting of at least seven defensive mechanisms that might be encountered by a pathogen as it tries to enter the body of the fly (Fig. 1). Moving from the outside of the body to the inside these are: (1) regulation of the native microbiota in the gut through antimicrobial peptides (AMPs) and reactive oxygen species (Ryu et al., 2008); (2) the barrier epithelial response, which can recognize infections and wounds, produce local AMPs and send signals to the rest of the body (Tzou et al., 2000); (3) the clotting response, which not only seals wounds and prevents bleeding, but can physically trap bacteria (Scherfer et al., 2006); (4) the phenoloxidase response, which deposits melanin at the site of an immune reaction, releasing potentially antimicrobial reactive oxygen species (Sugumaran, 2002); (5) the phagocytic response, through which phagocytes can kill microbes directly by either encapsulation or phagocytosis, or indirectly by releasing systemic signals (Agaisse et al., 2003; Dijkers and O’Farrell, 2007; Elrod-Erickson et al., 2000; Kocks et al., 2005); (6) the systemic AMP response, which involves the release of massive quantities of AMPs from the fat body (the liver analog) into the circulation (Meister et al., 1997); and (7) the RNAi

¹Departments of Craniofacial Development and Microbiology, King’s College London, London, SE1 9RT, UK
²Department of Microbiology & Immunology, 299 Campus Drive, Stanford University, Stanford, CA 94305-5124, USA
*Author for correspondence (e-mail: dschneider@stanford.edu)
response, which is required to fight viral infections (Galiana-Arnoux et al., 2006; Wang et al., 2006; Zambon et al., 2005). The majority of the work in flies concentrates on the immune response in the fat body. The transcriptional response of the fat body is so large that 1000-fold inductions can be measured even when whole fly homogenates are assayed. This transcriptional response is primarily under the control of three Rel-related transcription factors, Dif, Dorsal and Relish; these transcription factors are themselves regulated by two cross-reacting pathways, Toll and immune deficiency (Imd). Many recent reviews describe various aspects of this biology (Brennan and Anderson, 2004; Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007; Royet and Dziarski, 2007; Schneider, 2007).

In order to demonstrate how basic Drosophila studies on infection and immunity apply to medicine, we have written this review from the viewpoint of a ‘doctor of fly medicine’ treating ‘fly patients’ with primary defects in the immune system. As ‘fly physicians’, our primary concern is correlating genetic defects with susceptibility to pathogens. For example, fly patients may want to know, ‘If I have a mutation in the Imd signaling pathway, will I be more susceptible to Streptococcus pneumoniae?’ The mechanism-based answer is that there is no evidence that Imd is involved in this process because S. pneumoniae doesn’t induce transcriptional changes that are typically dependent on Imd. However, this overlooks important information. The patient would benefit if it learned that the loss of Imd would make them more susceptible to S. pneumoniae, even though the exact mechanism is not yet recognized. Therefore, this review concentrates on the survival outcomes of infections of various mutant fly lines with pathogens rather than the transcriptional responses induced by microbial elicitors.

The information in Fig. 2 focuses on infection experiments in which microbes were injected into the circulation of adult flies, because this is where the largest number of microbes have been tested to date, and it highlights the largest variety of microbes while covering the smallest number of responses. The one exception to this, is the inclusion of nitric oxide (NO) signaling as a host response to infection. In this case, a pathogenic infection of the gut leads to the production of NO, which acts on hemocytes (fly phagocytes) (Dijkers and O’Farrell, 2007). The hemocytes produce an unknown signal, which then induces AMP production in the fat body. This was included as an intriguing example of the integration of responses in multiple organs, involving other pathways that are already discussed in the literature (Dijkers and O’Farrell, 2007).

The essential message from this analysis is that there is no simple set of rules that can be used to describe infections in the fly. The table in Fig. 2 shows that there are many classes of pathogens and each causes a different type of infection. Since this is consistently true in all other systems that have been examined in depth, from plants to humans, it is not particularly surprising that it holds true in the fly.

**Are there simple rules to control Toll and Imd signaling?**

The Toll pathway is activated when the Toll receptor binds its ligand Spaetzle. Spaetzle is secreted as a pro-protein and must be proteolytically activated through the action of a protease cascade before it can activate Toll. Microbial elicitors such as Lys-type peptidoglycan from some Gram-positive bacteria and glucan from fungi can induce this pathway. In addition, a fungal protease is sufficient, although not necessary, to activate Toll (Gottar et al., 2006). This leaves open the possibilities that either the fly is monitoring the presence of fungal virulence factors, as described in the guard model for plant immunity, or that the fungus is intentionally activating Toll. The final output of Toll is the activation of two transcription factors, Dif and Dorsal. In summary, the majority of the literature states that Toll responds to Lys-type peptidoglycan and fungal glucan, and can be activated by a fungal
The Drosophila immune system

**COMMENTARY**

**Challenge**
- S. marcesens
- Fungus
- Gram+ bacterium
- Gram– bacterium
- Virus
- Intracellular
- Extracellular

**Signaling Pathways**
- JAK/STAT
- Eiger
- WntD
- Insulin
- Toll
- Imd
- Insulin
- Calcineurin
- Nitric oxide
- WntD transcription is increased by Toll activity. WntD reduces Toll signaling by preventing the nuclear translocation of the transcription factor Dorsal.

**Pathogens reveal complexity.** As more microbes are added to this list and the microbes are categorized depending upon their pathogenicity, rather than their ability to induce a set of transcripts, a new picture emerges. Toll and Imd signaling remain critical for fighting microbes; however, no simple rules emerge.

**Simple rules?** A subset of microbes gives the impression that Toll and Imd signaling are highly microbe-specific. This subset suggests that Toll is required to fight fungi and Gram-positive bacteria, whereas Imd is required to fight Gram-negative bacteria.

**Insulin signaling alters resistance to infection.** Constitutive insulin signaling caused by the loss of FoxO makes flies resistant to M. marinum infections, not by enabling the flies to fight the microbe, but by causing them to resist pathological wasting. Loss of insulin signaling leads to resistance of E. faecalis or P. aeruginosa by an unknown mechanism.

**Too much immune activation can be a bad thing.** WntD is a negative feedback regulator of Toll. WntD transcription is increased by Toll activity. WntD reduces Toll signaling by preventing the nuclear translocation of the transcription factor Dorsal. WntD mutants hyperactivate Toll during an infection and this increases sensitivity to Listeria infections.

**JAK/STAT signaling is required to fight a virus.** This pathway is regulated by the Unpaired ligands and the receptor Domeless, and may inhibit the transcriptional response of the Imd pathway. Mutations in this pathway have not been widely tested for their effects on infection.

**Eiger, a TNF family member, is required to fight extracellular microbes but causes harm when fighting some intracellular microbes.** Loss of Eiger function increases the sensitivity of flies to extracellular infections by reducing the ability to control microbial growth. In contrast, Eiger mutations make flies resistant to S. typhimurium by allowing the fly to tolerate the microbe without affecting microbial growth.

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Fig. 2. A summary of the interactions between the fly immune system and a variety of pathogens.
The IMD pathway is activated by peptidoglycan binding proteins that are found both on the membrane and within cells. Biochemical work supports the hypothesis that this pathway is activated by DAP-type peptidoglycan. Recent work shows that tissue damage may also be a method of activating IMD through the cleavage of a transmembrane peptidoglycan recognition protein (PGRP) PGRP-LC (Schmidt et al., 2008). The final output of this pathway is the activation of transcription factor Relish. In summary, signaling papers conclude that the IMD pathway is activated by DAP-type peptidoglycan and proteolytic cleavage (Brennan and Anderson, 2004; Ferrandon et al., 2007; Lemaître and Hoffmann, 2007; Royet and Dziarski, 2007; Schneider, 2007).

**Exceptions to the rules for Toll and IMD signaling**

Many experiments measuring AMP induction support the status quo for Toll and IMD signaling: Toll is activated by glucan and Lys-type peptidoglycan, whereas IMD is activated by DAP-type peptidoglycan. However, the literature contains a growing number of apparent violations of these simple rules. These experiments consistently demonstrate that flies carrying mutations blocking either the Toll or IMD pathways are unexpectedly sensitive to microbial infection. Mutations in Toll pathway components can alter resistance to *Listeria monocytogenes*, *Pseudomonas aeruginosa* and *Serratia marcescens* – all of which produce DAP-type peptidoglycan (Lau et al., 2003; Lazzaro et al., 2004; Mansfield et al., 2003). In addition, IMD pathway mutants die faster than wild-type flies when infected with a variety of fungi and Lys-type peptidoglycan-containing bacteria (De Gregorio et al., 2002; Hedengren-Olcott et al., 2004; Pham et al., 2007). Given the recent work on tolerance and the uncoupling of resistance from survival, as shown below, it is important to consider these outliers because they can point to previously overlooked physiologies. Of course, another explanation is that all of these outlier experiments are simply wrong; we took the view that, as they are all peer-reviewed publications, these observations warrant discussion and might teach us something new.

Flies with mutations in the Toll pathway are unexpectedly sensitive to *L. monocytogenes* and *P. aeruginosa*, as measured by survival (Lau et al., 2003; Mansfield et al., 2003). Although we typically measure the output of Toll signaling by looking at the expression of the AMP drosomycin from the fat body, it is also clear that Toll somehow regulates hemocyte activation (Zettervall et al., 2004). Given that *L. monocytogenes* infects these cells, it would not be surprising if Toll had an effect on Listeria infections. Further evidence for the participation of Toll in ‘off target’ infections comes from ecological immunity studies (Lazzaro et al., 2004). The analysis of genetically diverse strains of *Drosophila* has demonstrated that their abilities to resist infection, as measured by bacterial growth, can differ greatly. Candidate polymorphic traits were followed and the authors found that polymorphisms in the Toll pathway were correlated with altered resistance to the DAP-containing bacteria *S. marcescens*. A recent structural study suggests that one of the pattern recognition molecules upstream of Toll binds to DAP-type peptidoglycan, providing a biochemical explanation for how Toll mutants might be sensitive to DAP-containing microbes (Leone et al., 2008).

In *S. marcescens*, Toll and IMD signaling appears to be complex: one strain of *S. marcescens* kills both wild-type flies and flies with mutations in IMD or Toll extremely rapidly (Nehme et al., 2007). One interpretation of this is that the microbe is completely resistant to the fly’s immune response. A second interpretation is that the flies die too rapidly to determine whether the mutations have an effect on survival. We marked *S. marcescens* as showing no change in killing rates between wild-type and Toll or IMD mutant flies. As mentioned above, there is evidence that Toll, as well as IMD, signaling is used to suppress the growth of another strain of *S. marcescens* in a similar injection model.

Flies lacking the *imd* gene are unexpectedly sensitive to the Gram-positive bacterium *S. pneumoniae* and the fungus *Beauveria bassiana* (De Gregorio et al., 2002; Hedengren-Olcott et al., 2004; Pham et al., 2007). These microbes cause pathology in the fly, suggesting that a PGRP-LC-mediated response to tissue damage may be an important mediator of antimicrobial defenses in the fly.

Further exceptions can be found by looking at mycobacteria and at viruses. Injection of *Mycobacterium marinum* into the fly produces an early transcriptional response similar to medium alone (Dionne et al., 2003; Dionne et al., 2006). Neither the IMD nor the Toll pathway appears able to detect this particular microbe, even though its peptidoglycan is similar to the DAP type that is an optimal agonist for the IMD pathway. Toll mutant flies are also more sensitive to the virus *Drosophila X Virus* (DXV) (Zambon et al., 2005). In this case, flies carrying an activated allele of Toll are sensitive to the virus and have an increased titer. The same is true for loss-of-function mutants in the transcription factor Dif, which blocks Toll signaling. As discussed below, there are now several examples where the immune response of the fly is finely balanced and that over- or under-activation of the pathway can lead to pathology during an infection. The mechanisms behind this increased sensitivity are unknown.

The point of all of this is not to undercut the importance of the original papers that demonstrated the importance and the discriminatory power of the IMD and Toll pathways. We believe these papers are rightly regarded as the cornerstones of modern *Drosophila* immunology; however, from the standpoint of a ‘fly physician’, it is important for us to recognize where these rules break down so as to better understand the diseases and physiology of the patient. Perhaps the way to deal with this in the future is to consider immunity to be a quantitative trait and to report the statistically significant changes in survival, rather than simplifying phenotypes so that they appear to fall into only two classes.

**Why does an infected fly die?**

As fly doctors, we need to be concerned with the cause of death in our patients. The most commonly studied fly deaths caused by infections are cases where there is a mutation in the Toll or IMD signaling pathway and the mutant flies die faster than wild-type flies. Studies have found that when the mutant flies have died faster there is an enormous increase in the amount of bacterial growth in these mutants. Given that these pathways are responsible for inducing AMP transcription and that mutation of these pathways leads to microbial growth and host death, AMP transcription is...
often used as a proxy for resistance to infection. The assumption is that these AMPs are the main weapons used to fight infection and that these block bacterial reproduction.

Several recent papers have uncoupled bacterial growth and, by extension, AMP transcription, from survival. Wild-type and genetically diverse strains of flies infected with *P. aeruginosa* all die, but there is no correlation between the number of bacteria growing in these flies and their mean time of death (Corby-Harris et al., 2007). A screen for mutant flies with increased sensitivity to *L. monocytogenes* revealed that one-third of the 12 mutants isolated died rapidly upon infection, but showed no significant change in bacterial growth as compared with wild-type flies (Ayres et al., 2008). Work on Eiger and insulin signaling (shown below) produced similar results (Dionne et al., 2006; Schneider et al., 2007). These results show the limitation of using AMPs as a proxy for the defense against infection; expression studies teach us important lessons about the requirements for AMP induction but miss important immune responses.

Evolutionary ecologists suggest that when a host’s fitness is limited by infection, there are two evolutionary routes to fight this (Raberg et al., 2007). The first is resistance, which directly reduces the number of pathogens preying on the host. The second is tolerance, in which the host evolves mechanisms to limit the damage created by the pathogen. This uncoupling of bacterial growth and survival suggests that tolerance mechanisms play an important role in fighting infections in the fly. The majority of work to date has not measured survival and bacterial growth but has relied on proxies. Fly patients are dying to know the mechanisms behind tolerance.

**Insulin signaling alters pathogenesis**

Insulin signaling status is an important predictor of survival for Drosophila infections. *M. marinum* infections induce a pathological state in which insulin signaling is reduced and the flies appear to die from a wasting condition in which they consume all of their fat and glycogen stores (Dionne et al., 2006). The lifespan of the fly can be extended by forcing the induction of the insulin signaling pathway. This resembles the situation in humans, where patients entering an emergency room and suffering from septic shock do better if their insulin levels are carefully controlled (van den Berghe et al., 2001). In flies, this insulin effect appears to be a tolerance effect and not a resistance effect because changes in insulin signaling do not alter *M. marinum* numbers but do alter survival.

The fly insulin signaling story is complicated; inactivation of the insulin signaling pathway by mutation of the chico gene makes flies resistant to *Enterococcus faecalis* and *P. aeruginosa* infections (Libert et al., 2008). Bacterial loads were not determined in these experiments and thus we cannot distinguish between endurance and tolerance effects.

The message for fly physicians here is that it is important to understand the nature of the infection before manipulating insulin signaling. Not many organisms have been tested here and there is currently no set of rules. Future experiments will involve testing a broad array of organisms and uncovering the molecular mechanism behind insulin’s affects on immunity. The *M. marinum* story provides one explanation for pathology, but insulin has pleiotropic effects, altering autophagy, energy storage and feeding, and could affect different microbes in different ways.

**Too much signaling is a bad thing**

The fly community is just starting to determine the pathological causes of death in infected flies. In humans, it is clear that damage caused by an infection can come from our immune responses and that appropriate negative regulation is critical. Experiments in the fly suggest that the situation is similar and that the community is beginning to determine how misregulation of our immune response can cause harm.

An example of the importance of negative regulation in surviving infections comes from the study of the signaling molecule, Wnt inhibitor of dorsal (WntD) (Ganguly et al., 2005; Gordon et al., 2005). Two papers have identified the Wnt family member, WntD, as a negative regulator of Toll signaling in the maternal control of dorsal-ventral pattern formation. Studies have shown that WntD is induced by Toll signaling and that it blocks the activation of Toll signaling by preventing the activation of the transcription factor Dorsal. WntD flies were found to be significantly more sensitive to infection with *L. monocytogenes* and the hypothesis for this was that this sensitivity was due to increased signaling by Toll through Dorsal (Gordon et al., 2005). To test this hypothesis, the authors measured the survival of flies with 0, 1 or 2 copies of Dorsal in a WntD background. A reduction in the number of Dorsal copies was shown to reduce the severity of the phenotype, thereby supporting the hypothesis. We note that Dorsal is not typically considered to be a major regulator of the massive fat body transcriptional response. The mechanism behind the pathology of induced Dorsal activity is not yet known.

**Eiger, a member of the tumour necrosis factor (TNF) ligand family is involved in the immune response**

The TNF family plays an important role during infections in humans. The fly has a single member of this family, *Eiger*. *Eiger* plays a complicated role in fly immunity, just as TNF does in humans. *Eiger* mutants die faster when infected by extracellular microbes, regardless of whether they are Gram-positive or Gram-negative bacteria, or fungi (Brandt et al., 2004; Schneider et al., 2007). This is a resistance phenotype because the increase in death rate is associated with an increase in microbial load. In contrast to the results with extracellular infections, infection of *Eiger* mutants with intracellular microbes leads to either increased survival or no change in microbial load. Thus *Eiger* has a tolerance effect on intracellular microbes such as *Salmonella typhimurium*.

*Eiger* mutations have been shown to affect three immune responses in the fly. *Eiger* mutants do not show a normal melanization response in larvae (Bidla et al., 2007), but do display a reduction in the phagocytosis of labeled, dead *Staphylococcus aureus* in adult flies. It has yet to be determined whether this is a direct immune phenotype or whether there is a change in the development of the phagocytes (Schneider et al., 2007). Lastly, *Eiger* mutants show statistically significant increases in the transcription of diptericin, an AMP typically used as a proxy for Imd signaling, suggesting that *Eiger* could be an inhibitor of Imd signaling (Schneider et al., 2007). It is unclear why the release of Imd from negative control should lead to increased susceptibility to any microbes.

The implication for fly medicine here, is that manipulation of the *Eiger* pathway in the fly should be done only with knowledge of the type of infection, because an incorrect treatment will kill the fly more rapidly.
JAK/STAT signaling
Flies appear to lack interferons, but they nonetheless have a Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway that affects immunity. This pathway is controlled by a single receptor, Domeless, and by three ligands, Unpaired 1, 2 and 3. Studies of this system in vivo demonstrate that immune activation of hemocytes can induce Unpaired 3 expression in hemocytes (Agaisse et al., 2003). This ligand acts on the fat body and induces the production of a subset of genes that are different from those typically induced by Imd activation. As stated above, JAK/STAT activation is also expected to reduce Imd signaling.

A full analysis of the contribution of this pathway to the survival of infections in adult flies has been performed with only one virus, Drosophila C Virus (DCV) (Dostert et al., 2005). This virus has been shown to kill flies with an altered JAK/STAT signaling pathway more rapidly than wild-type flies. These mutants also carry a higher load of virus, which suggests a defect in resistance. The mechanism governing JAK/STAT-regulated resistance to DCV is unknown. Given that this pathway can negatively regulate the Imd pathway, JAK/STAT signaling may help fight viral infections by downregulating Imd during the response. Alternatively, JAK/STAT signaling can induce its own unique immune response and this could be required for immunity. More microbes should be tested for changes in resistance and tolerance in JAK/STAT mutants.

Simplifications
All diagrams simplify and we omitted many immune processes in making ours. Our purpose in making Fig. 2 and writing this review is to highlight the variety of immune responses generated by different pathogenic microbes in the fly. One big simplification is that we drew our diagram as if all signaling was occurring in a single cell. This is probably not true in all cases and in some cases we know for certain that it is not (Dijkers and O'Farrell, 2007). We focus almost entirely on injection experiments, ignoring interesting and exciting work on gut infections and the interaction of the fly's immune system with its native microbiota. We also omit work carried out using eukaryotic parasites such as Crithidia, Plasmodium, parasitoid wasps and parasitic nematodes. Wounding and the response to damaged tissue were omitted, as was a detailed discussion on the activity of Drosophila phagocytes. A huge story emerging in fly immunity is that the fly's immune system integrates the total physiology of the fly and its environment when responding to an infection. For example, there are bidirectional loops that have been identified between reproduction, circadian rhythm, energy metabolism, aging and past immune responses. None of these was integrated into this model. This research has a large potential impact on models of the fly for disease as it shows how the immune response affects, and is affected by, other physiologies. None of these events is yet understood at a mechanistic level.

We have presented a picture in which the Toll and Imd signaling pathways are the primary sentinels of immunity and where their activity is altered by a variety of feedback loops. It is clear that there is more to surviving an infection than simply resisting the microbe. The community has a reasonable understanding of the methods that are required for resistance, including AMPs, clotting and phagocytosis. In only one case do we have an explanation for changes in tolerance – that of wasting in M. marinum infections. On top of this, we have shown that fly disease is complicated; when enough factors are studied, it appears that every microbe tested provokes a different disease in the fly. This is good news because otherwise the fly would serve as a poor model of human immunity and infection. It also means that we cannot look at one or two microbes and make sweeping conclusions about a large class of microbes. We hope that this review will highlight rich areas for future work in Drosophila infection and immunity, and will reveal new possibilities for translation of this work into the realm of human medicine.

COMPETING INTERESTS
The authors declare no competing financial interests.

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