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Bacterial effectors: learning on the fly

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Introduction

Over the past two decades a number of findings made in *Drosophila melanogaster* have provided important new insights into mammalian innate immunity[1,2]. The power of this system is best exemplified by the discovery that Toll, a receptor used for dorso-ventral patterning in the developing embryo, is reused in the adult fly as a component of a microbial sensing pathway[3]. This seminal discovery led to identification of the Toll-like receptors (TLRs) as the critical innate immune receptors in mammals[4]. Other such examples demonstrate an amazing conservation between how flies and mammals fight infectious agents[1,5]. The majority of the studies to date have used *Drosophila* as a model host to understand the signals downstream of the two key pattern recognition receptors, Toll and Peptidoglycan Recognition Protein (PGRP)-LC[6,7]. Similarly, work in mammals has also focused on dissecting Pattern Recognition Receptor (PRR)-triggered pathways[8]. These receptors recognize critical microbial components such as peptidoglycan or LPS, which are found on commensal and pathogenic microbes[5]. However, a poorly understood aspect of innate immunity is how we differentiate pathogens and non-pathogens. A common defining characteristic of pathogenic bacteria is the expression of effector molecules or so-called ‘virulence factors’, which modify host defense mechanisms[9–11]. These bacterial factors include a variety of proteins, such as toxins that are internalized by receptors and translocate across endosomal membranes to reach the cytosol as well as others that are introduced directly into the cell by means of bacterial secretory apparatuses[12,13]. In this chapter we will discuss how *Drosophila* have been used as a system to study these important microbial effectors, and to understand how they contribute to pathogenicity.

Microbial Effectors

Although the term ‘effector’ is sometimes used only to describe the molecules introduced by the type III secretory apparatus expressed primarily by Gram-negative microbes, for simplicity, we will use this term to more loosely to encompass all secreted toxins. Effectors manipulate a variety of processes, including innate immune signaling pathways, the cytoskelton, protein translation, ubiquitination and the cell cycle[12,14]. Although these molecules make important contributions to the pathogenic potential of a microorganism, systematic study is hindered by a number of issues. Firstly, despite targeting a relatively

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limited number of host cellular functions and processes, they demonstrate a remarkable structural diversity. For this reason it is often difficult to predict their mechanism of action or their cellular targets. Secondly, any particular bacteria can introduce a number of effectors into the host. Importantly, as these effectors are frequently redundant for particular activities, classic mutant/deletion based strategies do not always result in clear phenotypes. Thirdly, they are often toxic to eukaryotic cells especially when ectopically expressed, limiting the work that can be done *in vitro*. Thus, the study of each specific effector has its unique challenges. Aside from the many powerful genetic tools available in the fly system and the well-characterized innate immune pathways, *Drosophila* offers a number of advantages to study these types of molecules. For example, in tissue culture, the tightly regulated *Drosophila* metallothionein promoter is ideal for expression of potentially toxic effector proteins, which may kill cells from the leaky expression found on other promoters. Similarly, it is possible to use the UAS system driven by Gal4, with or without the addition of Gal80 suppressor, to achieve tight *in vivo* tissue-specific or inducible expression. The wide range of tools available lead us to suggest that *Drosophila* may be an attractive system in which to try and better understand effectors and their mechanisms of action. Here we will not attempt to provide a comprehensive review but rather discuss a few examples in which *Drosophila* has already been used to study bacterial effectors and provide the proof of principle for this approach. We will then discuss some potential future directions and applications of this as a model system.

Using Drosophila to study bacterial effectors that regulate Rho GTPases: filling in the GAPs

More than 30 bacterial effectors from Gram-negative or Gram-positive bacteria directly or indirectly target the most studied Rho GTPase members : Rho, Rac and/or Cdc42[12,15]. RhoGTPases are pleiotropic regulators of cellular homeostasis, and are more specifically involved in the regulation of the cytoskeletal rearrangements necessary for migration or phagocytosis[16]. Therefore, Rho GTPases are not only master regulators of the cytoskeleton but also central elements of the host responses against pathogens[17]. For this reason, modification of the host Rho GTPases is a widespread strategy used by bacterial pathogens to manipulate mammalian host defenses, and they are frequently targeted by bacterial virulence factors[12]. RhoGTPases cycle between an active GTP-bound state and an inactive GDP bound state. Their activation requires guanine nucleotide exchange factors (GEF), whereas GTPase-activating proteins (GAP) stimulate GTP hydrolysis to inactivate the RhoGTPases[16]. Bacteria have evolved strategies to target the RhoGTPases family either by direct post-translational modification or by mimicking GEF or GAP activity[1218]. Many of the bacterial effectors isolated from pathogenic bacteria are inhibitors of RhoGTPases. These bacterial proteins are either used to disrupt the RhoGTPase cycle or to block the binding of these molecules to their downstream effectors. As highlighted by P. Boquet and E. Lemichez[12], it is surprising to observe that Rac GTPase seems to be the only common target of this group of bacterial toxins, and the fact that Rac regulates numerous cellular pathogen defense pathways is probably not a coincidence. Among these bacterial RhoGTPase inhibitors is a family of bacterial effectors that triggers GTP hydrolysis to inhibit RhoGTPases, thus mimicking eukaryotic GAP proteins. These bacterial effectors, including SptP from *Salmonella typhimurium*, YopE from *Yersinia spp*. and ExoS from *Pseudomonas aeruginosa*, are prokaryotic GAP proteins. These three bacterial proteins have a GAP domain that shares no sequence similarities but nonetheless all have potent GAP activity[18]. This suggests that these bacterial effectors are the product of a convergent evolution and that many microbes have evolved distinct strategies to inhibit a common target, the RhoGTPases.

Marie Odile Fauvarque's work on ExoS toxin from *Pseudomonas aeruginosa* provides one of the first, and most elegant examples, of how *Drosophila* can be used to investigate the effect of a bacterial toxin[19]. ExoS is a *P. aeruginosa* exotoxin directly translocated into the host cell cytoplasm through the type III secretion system. ExoS contains a GAP domain that prevents cytoskeleton reorganization by the Rho family of GTPases and an ADP-ribosyltransferase domain that modifies RasGTPases[20]. To investigate the role of the GAP domain of ExoS toxin, they took advantage of the genetically tractable fly system to generate a transgenic *Drosophila* expressing the ExoS GAP domain (ExoSGAP) of the toxin. Through transgenic expression, they were able to identify Rac (rather than Rho or Cdc42) as the *in vivo* target of this effector. Moreover, using this system they showed that flies resistance to *P. aeruginosa* infections was altered when ExoSGAP was expressed either ubiquitously or specifically in hemocytes, but not when expressed in the fat body, the major source of anti-microbial peptide production[19]. This suggested that the innate immune response is not dependent on a modified anti-microbial peptide production. Flies expressing ExoSGAP showed increased sensitivity to infection with Gram-positive *Staphylococcus aureus*, which was attributed to the reduced phagocytic capacity of ExoSGAP-expressing hemocytes[19]. This system allowed the authors to decipher *in vivo* the role of the GAP domain of ExoS on phagocytosis, and to suggest a major role of ExoS to inhibit cellular defence during infection with *P. aeruginosa*.

Interestingly, this virulence strategy is not specific to bacteria and has also been utilized by eukaryotic parasites to corrupt the host response[21]. The parasitoid wasp *Leptopilina boulardi* is a natural parasite to *Drosophila* larvae and recently, LbGAP, a GAP parasite-derived protein specific for the Rac GTPases, has been shown to translocate from the parasite into *Drosophila* hemocytes. Similar to the role of bacterial RhoGAP in mammals, this could be a protection mechanism used by the parasitoid wasp to be protected from the innate immune response of *Drosophila* host larvae[21]. From a pathogen point of view, these observations highlight the fact that pathogens that infect insects and mammals use evolutionarily convergent strategies, targeting the same key factors to control the host response of their particular hosts. From the host perspective, these observations indicate a conserved and important role of the Rac GTPase in the innate immunity of flies to humans.

Effector-triggered immunity: PR1 and CNF1, alerting the host to the presence of pathogens

Current models of innate immunity suggest that responses are triggered primarily by pattern recognition receptors (PRRs) that recognize conserved molecular patterns expressed by microbes[4]. Although most PRR ligands are shared between commensal and virulent strains, the host demonstrates a remarkable capacity to tailor the response to the virulence of the invading microorganism. However, how the host can detect specifically the virulence associated with microbes is poorly understood. One possibility, suggested from work in plants, is that effectors themselves can be sensed by the immune system. In resistant plants, such effectors are able to induce protective immune responses, that are referred to in the plant field as 'effector-triggered immunity'[22]. Although suggested from plants, there are very few studies in mammals that have addressed this. However, two studies in flies suggest that effector-triggered immunity is an important mechanism for discerning pathogenic microbes by metazoans.

Drosophila immunity to fungal and Gram-positive pathogens is dependent largely on the Toll signaling pathway[6]. The canonical activation step in this pathway is the cleavage of the secreted protein Spatzle. Once cleaved, Spatzle then acts as the Toll ligand, inducing multimerization and signaling similar to mammalian MyD88-dependent NF-kB activation[23]. To understand how this pathway was activated by virulent microbes, Gottar *et al.*[24] studied the response to an entomopathogenic fungi *Metarhizium anisopliae* in flies. One of the main

virulence strategies used by this fungus is mediated by PR1, a member of the subtilisin family of proteases that perforate the cuticle barrier and allow entry of the fungi into the insect body cavity[25]. To investigate the contribution of the PR1 protease the authors generated PR1 transgenic flies. Surprisingly, ectopic expression of this protease was sufficient to drive an immune response, and these flies had increased expression of Drosomycin in the absence of immune challenge. The mechanism involved the ability of PR1 to initiate a cascade of events resulting in Persephone-dependent Drosomycin expression. From this elegant experiment they propose a model where sensing of this fungus is mediated by a dual detection system; the first triggered by recognition of the fungal cell wall, the second in response to the secreted virulence factor, and both are required to maximally activate the Toll pathway[24].

An interesting extension of the work of Gottar *et al*.[24], is that the immune response induced by bacterial effectors might actually contribute to protective immunity, and as in plants, might help the resistant host limit bacterial replication. In our recent work we have used flies to address this possibility (data not shown). We have focused on Cytotoxic Necrotizing Factor 1 (CNF1), a toxin from uropathogenic *Escherichia coli*. CNF1 is an archetypal example of a RhoGTPase activating toxin and belongs to a family including CNF2 from *E. coli* as well as DNT from *Bordetella* spp. or CNFy from *Yersinia pseudotuberculosis*[26]. CNF1 is a deamidase, which catalyzes the activation of RhoGTPases[27,28]. CNF1 intoxication of mammalian epithelial cells induced activation of Rac. This in turn is involved in the clustering of different components of the SCF ubiquitylation complex, comprising Skp1 and neddylated-Cullin-1, together with IkBα and is associated with NF-kB p65 translocation to the nucleus[29]. More recently, we have used *Drosophila* to identify the innate immune pathway initiated in response to the CNF1 toxin (Boyer et al, submitted). We found that CNF1 toxin is sufficient to initiate defense signals in the absence of other bacterial components, and identified a conserved immune pathway that signals initiation of this response in flies and mammals. Analogous to ‘effector-triggered immunity’ observed in plants[22], we propose that the inappropriate activation of RhoGTPases by CNF1 is effectively monitored by the host, to the detriment of the bacteria. This mechanism of immune surveillance, based on monitoring the activity of virulence factors, provides a framework for a recognition system able to deal with the large number of highly varied microbial toxins targeting RhoGTPases. We anticipate that other targets of microbial virulence determinants will be similarly monitored. This work provides the first example of an evolutionarily conserved means by which pathogenicity is detected through sensing a microbial effector.

Using Drosophila to study effectors that inhibit innate immune responses: *Yersinia pestis* YopJ

Known primarily as a pathogen of historical importance and the causative agent of ‘plague’, *Yersinia pestis* is a highly virulent bacterium. To reach its pathogenic potential, during an infection *Y. pestis* injects a number of bacterial effector proteins directly into host immune cells using a type III secretion system[30].These effector proteins function to inhibit various cellular and immune pathways. Recently the precise function of one of these proteins, YopJ, has been debated. YopJ was first observed to promote apoptosis and inhibit NF-kB signaling pathways, which are essential for innate immune activation[31–33]. Initially YopJ was proposed to act as an ubiquitin-like protein protease, cleaving ubiquitin or ubiquitin-like proteins from their conjugated substrates[34–36]. However, recent evidence indicates that YopJ has a novel function, that of a serine/threonine acetyl-transferase[37,38]. In this role YopJ is proposed to acetylate critical serine and threonine residues of MAP2 kinases such as MKK2, MKK6 and IKK. In order to further understand the molecular role of YopJ, we (Paquette et al, unpublished) have used Drosophila. Similar work using the YopJ related protein AvrA

from Salmonella typhimurium has also been described[39]. Over expression of YopJ in immune stimulated Drosophila S2 cells was found to inhibit the IMD pathway, without affecting the Toll pathway, indicating that YopJ has a specific molecular target. Using, RNAi to probe this phenotype, we identified a new target for YopJ, TAK1, a member of the MAP3 kinase family. Thus mechanisms of effector-mediated immune suppression can also be identified with this approach.

Drosophila as a tool to decipher the role of effectors in chronic infection and inflammation: *Helicobacter pylori* CagA

In addition to the obvious consequence that bacterial effectors have on regulating innate immunity, there are pleotropic consequence during chronic infection. As an example, chronic *H. pylori* infection is the causative agent of gastritis, peptic ulcers and gastric cancer[40]. During infection the bacteria uses a type four secretion system to inject bacterial toxins directly into the host cells. One major virulence factor that associates with *H. pylori* is the cytotoxin-associated gene A (CagA) protein[41]. Once inside a host cells CagA is phosphorylated by Src kinases and acts to disrupt receptor typrosine kinase (RTK) signaling pathways by activating Src homology 2 domain containing tyrosine phosphatase (SHP-2). In tissue culture systems CagA has been shown to interact and activate SHP-2, resulting in cell elongation[42]. As SHP-2 normally binds to Gab proteins, CagA is hypothesized to mimic Gab proteins even though they share no sequence similarity, and thus to function as an oncogene by activating RTK signaling. In order to more fully understand the mechanism of CagA in epithelial tissues Botham *et al.*[43] undertook a study in which they expressed CagA in the eye of developing *Drosopihla*. CagA expression driven by the GMR driver resulted in a severe eye deformation. In order to determine if CagA could mimic Gab, Botham and collegues performed an elegant rescue experiment using the *Drosophila* Gab homolog, DOS. In homozygous *dos* loss of function mutants, pupal development is severely reduced and adult animals are never generated. Using a ubiquitous drive (Hsp-Gal4), expression of CagA rescued the *dos* mutant lethality. Furthermore, using the FLP/FRT to generate *dos/dos* in the eye, it was also shown that CagA could directly rescue *dos/dos* dependent photoreceptor development. These data show that CagA does in fact act to mimic DOS during eye development. Lastly under the assumption that CagA mimics Gab, it was tested if the SHP-2/CSW protein was required downstream for proper eye development. Using *csw* mutant *Drosophila*, it was shown that overexpression of CagA did not rescue the *csw* dependent lack of photoreceptors, indicating that CagA requires SHP-2/CSW for proper function. Taken together this work shows how the *H. pylori* bacterial effector protein CagA functions as a mimic of Gab in an *in-vivo* epithelial model system, and is an elegant example of what is possible using the powerful genetic tools available in *Drosophila* to investigate the function of bacterial effectors.

Future directions

Finding the bad guys: Using Drosophila to identify bacterial effectors

In work that has been pioneered by Dr Svenja Stöven[44,45], it has recently been shown that *Drosophila* might be a powerful system to screen for bacterial effectors involved in virulence. To demonstrate that *D. melanogaster* is a suitable *in vivo* model for the identification of *F.tularensis* virulence determinants, they first targeted the *igl* operon and the regulator *mglA*, bacterial genes known to be required for bacterial intracellular growth and virulence in mice. They injected flies with either the wild type strain or with isogenic Δ*iglB*, Δ*iglC*, Δ*iglD* and Δ*mglA* mutants and found that flies injected with Δ*iglB*, Δ*iglC*, Δ*iglD* or Δ*mglA* mutants survived significantly longer than wild type-infected flies[44]. They extended this approach by screening for *F. novicida* genes involved in virulence. They performed a directed screen using an *F. novicida* transposon insertion library, and scored the

survival of infected fruit flies[45]. This approach allowed them to identify clusters of genes required for *Francisella* virulence, and established *Drosophila* as a useful *in vivo* system to identify bacterial genes involved in "virulence" or "avirulence" of pathogenic bacteria.

Look and learn: Intravital imaging of Drosophila to monitor the consequences of bacterial effectors during in vivo infection

One of the limits in studying host-pathogen interactions is the visualization of the pathogen as they interact with the host cells *in vivo*, especially during the very early stages of infection. Despite the development of intravital microscopy and luminescence lifetime imaging technology in mice, the resolution and potential for investigation in mammals are limited. Like zebrafish and nematodes, *Drosophila* have proved to be a powerful model for *in vivo* microscopy, and this has been extensively used to study *Drosophila* early embryonic development and wound repair[46,47]. Recently, intravital imaging has been used to follow bacterial infection in real time. Will Wood's group[48] has adapted and developed a powerful imaging system using the *Drosophila* embryo to study in the role of a bacterial effector called Makes Caterpillars Floppy (Mcf1), produced by the insect pathogen *Photorhabdus asymbiotica*. Using this model they show that embryonic hemocytes can sense and phagocytose non-pathogenic *Escherichia coli*. However, when embyros were infected with *P. asymbiotica*, hemocytes bind to the bacteria but become immotile 20 min after infection. Using Drosophila, Mcf1 toxin was identified as the bacterial effector responsible for this striking phenotype, as embryos injected with *E. coli* producing Mcf1 or purified toxin alone, recapitulate the hemocyte immobilization phenotype[48]. This study also used *Drosophila* mutants to show that the immobilization phenotype requires the internalization of the Mcf1 toxin, and that this phenotype is dependent of the GTPase Rac. This work was facilitated by the use of the combination of a genetically tractable host, *Drosophila melanogaster*, and a genetically tractable microbe, *E.coli*, to elucidate the role of the Mcf1 toxin during the early steps of infection *in vivo*[48]. Moreover, these studies demonstrate that it is possible to obtain subcellular resolution in living organisms, and thus highlight the value of *Drosophila* for live cell imaging and intravital microscopy for the study of the immune response *in vivo*.

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