UNIVERSITY OF CALIFORNIA SAN DIEGO

Identification of Effectors of the Toll Pathway in Drosophila Innate Immunity

A dissertation submitted in partial satisfaction of the requirements for
the degree Doctor of Philosophy

in

Biology

by

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The Dissertation of Lianne Beth Cohen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE DISSERTATION

Identification of Effectors of the Toll Pathway in Drosophila Innate Immunity

by

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Fungal infections are a major source of morbidity and mortality throughout the world, especially in agriculturally relevant plants, humans, and insects. To defend against fungal infections, the fruit fly *Drosophila melanogaster* employs a robust innate immune system. Infections induce a large number of immune proteins through a number of signaling pathways including the Toll pathway. Some of these proteins have been studied, such as the antimicrobial peptides (AMPs) and Bomanins (Boms); many, however, remain uncharacterized. In Chapter I, I used a candidate approach to identify putative effectors from

this set of induced uncharacterized genes. I generated CRISPR/Cas9 mutants and assayed them for survival upon infection with various pathogens. I identified an immunodeficiency in a *ΔCG18067* mutant. Additionally, I found a background mutation in the immune induced gene *IM14*. In Chapter II, I examine the role of IM14 and a related peptide, IM4, in innate immunity. By generating a CRISPR/Cas9 knockout of both genes, Δ(IM4,IM14), I find that the IM4 and IM14 peptides are required for defense against a subset of filamentous fungi, including *Fusarium oxysporum*, but not other Toll-inducible pathogens, such as *Enterococcus faecalis* and *Candida. glabrata*. Further genetic analysis revealed that IM4 and IM14 are each required for defense, although they share some functionality. By generating and assaying a genomic epitope-tagged IM14 construct, I detected *in vitro* interaction of IM14 peptide in hemolymph with the hyphae of *F. oxysporum*. These findings identify IM4 and IM14 as a new class of innate immune effectors with humoral activity against a select set of filamentous fungi. Overall, I reveal that several previously uncharacterized genes play a crucial role in the innate immune system of *D. melanogaster* and could aid in the development of new antifungal treatments.

Introduction: Overview of Drosophila Innate Immune Defense Against Fungi

Importance of Host-Pathogen Interactions

For as long as animals have been present on Earth, there have been pathogens that infect them (Poinar, 2014; Poinar and Poinar, 2005). Insects in amber have provided fossil evidence showing various pathogens infecting hosts as early as the Early Cretaceous period. These interactions between humans and other animals with pathogens have undeniably driven evolution (Rausell and Telenti, 2014; Rifkin et al., 2017). For example, host-pathogen competition between humans and the malaria-causing *Plasmodium falciparum* parasite about 100,000 years ago led to the emergence of the causative mutation in sickle cell anemia (Rifkin et al., 2017).

The dynamics between hosts and pathogens are often described in the context of the Red Queen hypothesis. Initially proposed by Leigh Van Valen, it conveys the idea that organisms must constantly evolve and adapt to maintain a level of fitness (Papkou et al., 2019). In the context of host-pathogen interactions, both host and pathogen are obligatorily locked in an arms race, constantly trying to best the other. They must be continually running just to remain in the same position, just as the Red Queen tells Alice, in the classic story *Alice's Adventures in Wonderland*. Through this constant evolutionary pressure, hosts have developed immune systems to combat pathogens.

Studying pathogens over the last 100 years has been a productive ground for research, leading to vast improvements in human health. Almost all of the increase in lifespan in the first half of the 20th century was due to combating infectious diseases (Lederberg, 2000). Public health efforts to improve hygiene and sanitation fought the spread of infectious diseases and saved countless lives. Vaccines have decreased the incidence of viral infections

throughout the world and led to an extraordinary achievement: eliminating small-pox (Bandyopadhyay et al., 2015; Mina, 2017). Antibiotics provided a revolutionary tool for fighting bacterial pathogens.

However, the promises from the early and mid-20th century, of a world where infectious diseases have a minimal impact on human health, have not born out. Emerging antibiotic resistant pathogens and the destructive anti-vaccine social movements threaten to upend the advancements made (Frieri et al., 2016; Pandolfi et al., 2018). Continued research into pathogens and animal immune systems developed to fight off these infections is vital for persistent medical improvements.

Fungi as Pathogens

Fungal pathogens are a key source of morbidity and mortality in the world. Over 1.7 billion people are infected with pathogenic fungi (Delarze and Sanglard, 2015). Furthermore, invasive fungal infections lead to about 1.5 million deaths annually (Kumar et al., 2018). These opportunistic pathogens are able to take advantage of immunocompromised patients, such as organ transplant recipients, leading to rising numbers of fungal infections. The most common invasive fungal pathogens are *Candida* spp, *Aspergillus* spp., and *Cryptococcus* spp. Fungal infections can be particularly insidious to treat because of their complex lifecycles that involve such divergent structures as conidia spores and hyphae.

In addition to infecting humans, fungal pathogens also infect agriculturally important plants. Throughout history, fungal epidemics have devastated crops, leading to significant social and economic changes (Gunther Doehlemann, Bilal Ökmen, Wenjun Zhu, 2017).

Fungal pathogens continue to cause disease today. *Magnaporthe oryzae* infects and destroys rice, the primary source of food for over half the world's population (Dean et al., 2012). *Botrytis cinerea* and *Fusarium* spp. cause disease in a wide range of crops from fruit to cereals.

Insects also experience infections from fungi. Wild-caught *Drosophila* have been found to be infected with numerous pathogens from bacteria to fungi (Cuthbertson and Audsley, 2016; Juneja and Lazzaro, 2009; Sharma and Marques, 2018). Furthermore, the fruit fly *D. melanogaster* has been developed as a model for studying fungal pathogenesis (Brunke et al., 2015; Lamaris et al., 2007).

Drosophila as a model for Innate Immunity

Drosophila melanogaster is an excellent model for studying host-pathogen interactions and immunity. There are two different types of immune systems in animals: innate and adaptive. Innate immunity refers to all parts of the immune system that can combat an infection without an individual being previously exposed to the pathogen. Adaptive immunity requires an organism to be exposed to a particular pathogen before launching a specific immune response, usually through T-cells, B-cells, and antibodies (Hoffmann et al., 1999a). Mammals have both an innate and adaptive immune systems that work together protecting the animal. Insects and other invertebrates have only innate immunity, providing a great model to study this system in isolation (Hoffmann, 2003). These organisms must defense against all invaders using germ-line encoded factors.

Additionally, *D. melanogaster* is particularly resistant to infections, which is vital to its lifecycle spent in great proximity to microbes (Hoffmann et al., 1999a; Kounatidis and Ligoxygakis, 2012). This allows researchers to probe the strong immune system and simply identify immunodeficient mutants. *Drosophila* are readily grown in laboratory conditions and more than a century of study has established many tools and techniques, creating a genetically tractable system that allows for precise investigation into how the immune system is able to fight off infections.

Drosophila **immunity**

The *Drosophila* innate immune system is comprised of two major mechanisms: cellular and humoral immunity (Lemaitre and Hoffmann, 2007). The circulating hemolymph, analogous to mammalian blood, contains thousands of hemocytes that employ various methods to control infections. Plasmatocytes make up the majority of hemocytes and clear pathogens via phagocytosis. Using receptors to bind a wide range of bacteria, they internalize these microbes and destroy them. Plasmatocytes work together with lamellocytes to induce an encapsulation response, usually in response to the laying of eggs by parasitic wasps. Lamellocytes wall off the invading tissue and eventually kill anything inside it. The remaining cell type is crystal cells which together with the activation of prophenoloxidases mediate melanization (Lemaitre and Hoffmann, 2007). This reaction, which occurs at the site of damage to the fly cuticle, aids in wound healing and kills invading pathogens.

Humoral immunity describes the large repertoire of effectors present in the hemolymph of flies. Many of these proteins, which directly interact with pathogens, are

secreted from the fat body, analogous to the mammalian liver, into the hemolymph in response to infection (De Gregorio et al., 2001; Levy et al., 2004). The expression and secretion of the inducible effectors is largely governed by two signaling pathways: Toll and Imd. The Imd pathway is activated by DAP-type peptidoglycan (PGN) found in Gram-negative bacteria (Myllymäki et al., 2014). Once PGN binds the PGRP-LC receptors in the fat body, the signal is transferred into the cytoplasm. Signal transduction leads to the activation of the NF-kappaB transcription factor Relish, which enters the nucleus and initiates expression of a defined set of genes.

In addition to its role in development, Toll signaling activates the immune system in response to the fungi and Gram-positive bacteria with Lys-type PGN (Valanne et al., 2011). For signaling to be triggered in the fat body, the processed ligand Spatzle must bind the Toll receptor in the cell membrane. The precursor form of Spatzle can be cleaved into its mature form by three stimuli. Lys-type PGN binds the PGRP-SA, while β-glucan from fungal cell walls binds GNBP-3, both activating the ModSP protease cascade, which ends with Spatzle cleavage. The third way to induce cleavage of Spatzle is by sensing damage by various pathogens through the processing of the protein Persephone. Intracellular signal transduction leads to the release of the Dif and Dorsal NF-kappaB transcription factors from their sequestration in the cytoplasm. They then move into the nucleus, binding to kappaB sites in the genome and inducing transcription of effector genes.

Among the effectors secreted from the fat body upon activation of Toll and Imd are the classical antimicrobial peptides (AMPs). There are seven classes, represented by: Diptericin, Attacin, Drosocin, Cecropin, Defensin, Drosomycin, and Metchnikowin (Lemaitre and Hoffmann, 2007). Primary studies of AMPs were conducted via isolation or synthesis of

peptides followed determination of antimicrobial activity via *in vitro* killing assays, often including assessment of minimum inhibitory concentrations (MICs). Using these techniques, Diptericin, Attacin and Drosocin were each found to have anti-microbial activity against Gram-negative bacteria (Bulet et al., 1996; Cudic et al., 1999; Hultmark et al., 1983; Kragol et al., 2001). Cecropin, one of the first AMPs identified in animals, has a broad range of activity (Hultmark et al., 1980). It is active against Gram-negative and Gram-positive bacteria as well as filamentous fungi (Ekengren and Hultmark, 1999; Lowenberger et al., 1999; Rabel et al., 2004). Defensins are widely conserved, with homologs in humans, plants, as well as insects (Hoffmann et al., 1999b). MICs indicate that they are exceptionally potent against Gram-positive bacteria, while also possessing anti-fungal effects (Imler and Bulet, 2005). Expression of Drosomycin and Metchnikowin, each of which is antifungal, is controlled by Toll (Levashina et al., 1995).

A key study of the antimicrobial activity of AMPs *in vivo* involved constitutively expressing single peptides in an immunodeficient background (Tzou et al., 2002). This report found Defensin to be protective against Gram-positive bacteria, while Attacin and Drosocin provide some rescue of survival against Gram-negative bacteria. Drosomycin was the most potent AMP in flies challenged with filamentous fungi. However, there are several problems with using this study as a sole account of the natural role of the AMPs. For one, this experimental design tests sufficiency of AMPs to defend against infection, not their necessity for defense. Additionally, in these experiments AMPs are constitutively expressed, not induced upon infection, which could change the dynamics of interactions between AMPs and pathogens as well as interactions AMPs have with other effector peptides.

Recently, genomic editing technology has allowed the evaluation of the requirement for AMPs upon infection with various pathogens via the generation of loss-of-function mutants (Hanson et al., 2019). Deletion of all the major AMPs in *D. melanogaster* except Cecropin revealed that AMPs are required for defense against Gram-negative bacteria. In particular, Diptericin, Attacin and Drosomycin are the major effectors involved in defense against this set of pathogens. Interestingly, analysis of survival after infection with Gram-positive bacteria or fungi demonstrated little to moderate requirement for AMPs. Metchnikowin and Drosomycin both play a role in defense against the yeast *C. albicans*, but for all other Gram-positive bacteria and fungi tested, the effecting of deleting the AMPs was less severe than that of loss-of-function mutants in the Toll pathway. This contrasts markedly with the Bomanin family of effectors, as described next.

One group of Toll-induced effectors required for defense against Gram-positive bacteria and fungi are the Bomanins, a recently discovered family of peptides (Clemmons et al., 2015). Bomanins, or Boms for short, are a group of 12 related peptides induced by Toll. They share a motif that is not found outside of the family and come in three different versions: short, tailed and bicipital. Deletion of 10 out of 12 Bom genes renders flies susceptible to infection with Gram-positive bacteria and fungi. Expression of a single Bom peptide in flies was sufficient to restore resistance to mutant flies (Lindsay et al., 2018). Additionally hemolymph killing activity against the yeast *C. glabrata* is dependent on the presence of Boms.

Study of the *D. melanogaster* immune system provides insight into mammalian innate immune systems. The two systems share significant conservation of key immune factors (Hoffmann et al., 1999a). The Toll pathway in flies has many components in common with

the NF-kappaB pathway in mammals. Both pathways are induced by pattern recognition receptors and use homologous signal transductors to activate expression of effectors. Additionally the AMP Defensin, which is active against Gram-positive bacteria, is conserved between flies and mammals.

What is still unknown about *Drosophila* Immunity?

While the recognition and signaling mechanisms of Drosophila innate immunity are well understood, the discovery of Boms suggests that there could be other undiscovered effectors. Furthermore, there are many infection induced genes that are unknown and so far have not been studied (De Gregorio et al., 2001; Levy et al., 2004). Interestingly, like the Boms, many of these genes are taxonomically restricted to *Drosophila*.

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Chapter I:

Screen for Toll Effectors of the Drosophila Innate Immune System

Introduction

When *Drosophila melanogaster* becomes infected with microbes, a complex immune response works to defend against infections (Lemaitre and Hoffmann, 2007). Much of the immune response is regulated by several signaling pathways that are required for defense, one of which is the Toll pathway (Valanne et al., 2011). Toll is activated by components of microbial cell wall, Lys-type peptidoglycan from Gram-positive bacteria and β-glucan from fungi as well as pathogen originating proteases. The signal is then transmitted into a cell of the fat body, leading to activation of the transcription factor Dif (Lindsay and Wasserman, 2014). Dif binds to kappaB sites in the genome and induces expression of response genes (Busse et al., 2007).

Deletion of components of the Toll signaling pathway leaves flies susceptible to infection by Gram-positive bacteria and fungi (Lemaitre et al., 1996; Tauszig-Delamasure et al., 2002). But how does the Toll pathway protect flies from infections? Among the dozens of genes induced by Toll are proteins involved in defense mechanisms like melanization, as well as effector proteins like antimicrobial peptides (AMPs), Boms and many uncharacterized proteins (De Gregorio et al., 2002, 2001; Levy et al., 2004; Uttenweiler-Joseph et al., 1998).

Initially, the major effectors of the *Drosophila* immune system were thought to be AMPs. There are seven classes of AMPs expressed in *Drosophila* that have *in vitro* activity against various pathogens (Lemaitre and Hoffmann, 2007; Levashina et al., 1995). Regulation of the AMPs is controlled by two signaling pathways, Imd and Toll. The Imd signaling pathway induces a transcriptional response to DAP- type peptidoglycan found in Gram-negative bacteria, while Toll responds mostly to Lys-type peptidoglycan from Gram-positive bacteria and β-glucan from fungi. While AMPs can be regulated by both pathways, Drosocin

and Diptericin are mostly regulated by Imd. Drosomycin and Metchnikowin are controlled by Toll. Attacin, Defensin and Cecropin are regulated by both pathways (Busse et al., 2007; Lindsay and Wasserman, 2014).

In vitro experiments show that Diptericin, Attacin and Drosocin have activity against Gram-negative bacteria (Bulet et al., 1996; Cudic et al., 1999; Hultmark et al., 1983; Kragol et al., 2001). Drosomycin and Metchnikowin have antifungal activity (Levashina et al., 1995). Cecropin is active against Gram-negative and Gram-positive bacteria as well as filamentous fungi (Ekengren and Hultmark, 1999; Lowenberger et al., 1999; Rabel et al., 2004). Defensins are exceptionally deadly to Gram-positive bacteria, while also possessing anti-fungal effects (Imler and Bulet, 2005). A recent *in vivo* study demonstrates that while AMPs, especially Diptericin, Attacin and Drosocin mediate defense against Gram-negative bacteria, the AMPs induced by Toll have been found to not be required for defense against Gram-positive bacteria and fungi (Hanson et al., 2019).

In 2015, our research group described a new class of peptide effectors, the Bomanins (Boms) (Clemmons et al., 2015). Boms are highly expressed after infection and share a sequence motif that is not found outside of the family. The Boms are required for defense against Gram-positive bacteria and fungi (Clemmons et al., 2015; Lindsay et al., 2018). Alongside AMPs and Boms, are many other unstudied peptides that are highly induced upon infection (De Gregorio et al., 2002, 2001). Could these genes of unknown function be effectors as well?

AMPs and Boms share properties that could be instructive for identifying new effectors. They are typically small in size (less than 10 kDa) and are secreted from the fat body into the hemolymph where they could interact directly with pathogens during a systemic

infection. Some AMPs are conserved across flies, mammals and plants like Defensin. Other effectors, like the AMPs Drosomycin and Metchnikowin, as well as the Boms, are specific to the *Drosophila* genus.

Methods and Materials

Generation of mutant flies

The null alleles *ΔIMPPP*, *ΔCG13749*, *ΔCG4269*, and *ΔCG18067* were generated using CRISPR/Cas9 technology, applying methods described previously (Gratz et al., 2014). Pairs of guide RNAs that targeted Cas9 to delete the region 2R: 13,406,843-13,408,003 for *ΔIMPPP)*, 2R: 8,933,195-8,934,564 for *ΔCG13749*, 2R: 22,599,083-22,600,338 for *ΔCG4269*, and 2R: 20,534,264-20,536,153 for *ΔCG18067* were cloned into the pU6-BbsI-chiRNA vector (Addgene plasmid # 45946). Homology arms of approximately 1 kb were cloned into pHD-DsRed (Addgene plasmid # 51434). Constructs were injected into Bloomington Drosophila Stock Center line 51323 (y[1] M{vas-Cas9}ZH-2A w[1118]/FM7c) which provides Cas9. Constructs were based on target sequences in the BL51323 strain.

Microbial cultures

For survival experiments, microbes were cultured as follows. *Enterococcus faecalis* NCTC 775 (ATCC 19433) and *Enterobacter cloacae* were grown overnight at 37°C in LB media and concentrated to an OD_{600} of 10 in 20% glycerol. *Candida glabrata CBS 138* [ATCC 2001] was grown overnight in YPD media at 37°C and concentrated to an OD_{600} of 100 in PBS, 0.1% Tween. Fungal spores were concentrated in 20% glycerol and stored at -

80°C before being used at the following concentrations (in spores/ml): *Fusarium oxysporum* f. sp. *lycopersici* 4287 (FGSC #9935): 3 x 10^8; *Neurospora crassa*: 1 x 10^9.

For the induction of the Toll response, heat-killed *Micrococcus luteus* was prepared as previously described (Lindsay et al., 2018).

Survival assays

Groups of 20-30 adult male flies aged 2-7 days were collected and stabbed with a needle dipped in a suspension of bacteria, yeast, or fungal spores. Flies infected with *E. faecalis* were incubated at 25°C; all other infected flies were incubated at 29°C. Fly deaths were recorded at least twice per day for the duration of each experiment. Any deaths that occurred within the first 6 h were set aside to exclude from the data any deaths due to traumatic injury. Statistical analyses were performed using the Gehan-Breslow-Wilcoxon test.

MALDI-TOF

After Toll induction with heat-killed *M. luteus*, flies were incubated at 29°C for 24 h, after which hemolymph was collected via capillary as previously described (Lindsay et al., 2018). Hemolymph in 0.1% trifluoroacetic acid/50% acetonitrile was mixed 1:1 with Universal Matrix (Sigma-Aldrich). Samples were then dried onto a MALDI plate. Spectra were collected using a Bruker Biflex IV MALDI-TOF mass spectrometer from 1,500 to 10,000 m/z for linear mode with positive polarization. For external mass calibration, equal parts of peptide calibration standard II (Bruker: 8222570) and protein calibration standard I (Bruker: 8206355) were mixed. Representative spectra are shown from three independent

samples. Peaks were identified via corresponding m/z values from previous studies (Levy et al., 2004; Uttenweiler-Joseph et al., 1998). Spectra were visualized using R 3.3.2 and ggplot2 2.2.1 (R Core Team, 2013; Wickham, 2016).

mRNA quantification by qPCR

Adult male flies, 2-7 days old, were stabbed with heat-killed *Micrococcus luteus*. Flies were then incubated at 29°C for 24 hours. Groups of 5-6 flies were collected and frozen in liquid nitrogen. Total RNA was isolated with TRIzol (Ambion) and cDNA was made via SuperScript RT II (Invitrogen). Measurements by qPCR were performed on the iQ5 cycler (BioRad) using iQ SYBR Green Supermix (BioRad) using the primers listed below. Values were normalized to fly mRNA based on expression of the *rp49* gene. Primers: rp49_F1: CAAGGGTATCGACAACAG, rp49_R1: CTTGTTCGATCCGTAACC. IM14_qPCR_F2: AACTGTCTGAAGATCTGCG, IM14_qPCR_R2: AGCATGAATGACTTGGGTG. IM4_qPCR_F1: ACAACCACCAAACCTCAG; IM4_qPCR_R1: GTTGTCGGTCTGGATGAG.

PCR

PCR was performed using Phusion polymerase (New England Biolabs) according to NEB protocol. PCR reaction was then run on a 1% agarose gel with SYBR Safe (Thermo Fisher) and imaged with UV light. Primers used: IM14_seq_F1: GAACTGTCTGAAGATCTGCGGCTT; IM14_seq_R1: TTTCGAACAGCTGATCAGCTGAGA; IM14_seq_F2: TCTTCGCTCGTCAATCAGTG;

IM14_seq_R2: CAGTGTGACCCTTTCCCACTA; IM14_seq_F3: CGAACATGGCCAAGAGAAG; IM14_seq_R3: CAAAGCCGCAATCAGAGC.

Results

In order to identify putative effectors of the *Drosophila* innate immune system, I focused on uncharacterized proteins that are small, induced by the Toll pathway, secreted out of cells and conserved, either within the *Drosophila* genus or across a wider phylogeny. A previous RNA-seq experiment in the lab, performed by Scott Lindsay, identified genes induced by infection with the fungus, *Beauveria bassiana*, in four species: *D. melanogaster*, *D. virilis, D. ananassae,* and *D. mojavensis*. Comparative transcriptomics revealed 17 core genes that are induced by all four species. Among this group were eight genes identified as members of the Bomanin family, a group of effectors required for Toll-mediated defense (Clemmons et al., 2015). Of the remaining nine genes were three that stood out for their predicted secretion by SignalP (Almagro Armenteros et al., 2019) into the hemolymph and lack of identifiable domains: IMPPP, CG18067, and CG4269.

IMPPP. IMPPP (Immune induced molecule prepropeptide) is a gene the codes for the precursor of seven distinct peptides found in the hemolymph of Toll induced flies (Levy et al., 2004; Uttenweiler-Joseph et al., 1998). These seven peptides – IM5, 6, 8, 10, 12, 13, and 24 – are cleaved from the precursor via furin-like sites and range in size from 17 to 96 amino acids. Preliminary RNAi experiments showed no effect on survival, but complete knockdown of the transcripts was not shown (Levy et al., 2004). In addition to the four *Drosophila* species

studied in the RNA-seq experiment, IMPPP is found in many other *Drosophila* species, but not outside the genus. BLAST searches against the *Drosophila* genome identified an IMPPP paralog, CG13749 (Johnson et al., 2008). CG13749 shares 62% protein sequence identity with IMPPP. While this gene has no signal peptide, it was found to be induced two-fold by fungal infection (Scott Lindsay, unpublished data). In a genome-wide screen for heat avoidance, RNAi against CG13749 was found to be lethal (Neely et al., 2010).

CG18067. CG18067 is induced five-fold in D. melanogaster by fungal infection (Scott Lindsay, unpublished data). It is conserved within the *Drosophila* genus, but no homologues have been found in other insects (Johnson et al., 2008). At 222 aa, it is on the larger side of proteins considered and has a recognizable coil-coiled domain, but no other distinguishing features.

CG4269. The 79 aa peptide CG4269 was found via RNA-seq to be induced seven-fold by fungal infection. This gene was also identified previously by microarray for its strong induction upon septic injury and fungal infection (De Gregorio et al., 2001). In wild-type flies it has a sustained production that is regulated by both the Imd and Toll pathways (De Gregorio et al., 2002). Unlike the most of the other genes found in the core 17 Toll responsive genes, CG4269 is conserved outside of the genus. In addition to being widespread in Drosophila, homologs are found in many insects, including the silkworm *Bombyx mori*, the mosquitoes *Aedes* spp., *Anopheles* spp., and *Culex quinquefasciatus*, as well as the flies, *Musca domestica* and *Stomoxys calcitrans* (Johnson et al., 2008). Furthermore, gene expression levels have been studied in some of these species and immune induction was observed. For the pollen beetle, *Meligethes aeneus*, a homolog for CG4269 is upregulated

162-fold upon introduction of an immunogenic mixture of LPS and microbes (Vogel et al., 2014).

CG16978. While CG16978 was not identified as part of the core 17 genes, it is strongly upregulated specifically by the Toll pathway upon infection (De Gregorio et al., 2002, 2001). It also exhibits many of the hallmarks of possible effectors with its small size (96 aa) and signal peptide as predicted by SignalP. Similar to IMPPP and CG18067, CG16978 is only conserved within *Drosophila*.

CG14567. In addition to considering the transcriptomics data from *Drosophila*, I also surveyed studies of immune challenge in other insects. Among the immune induced genes in the pollen beetle was a homolog of CG14567 which was expressed 21x greater in the immune challenged adults compared to controls (Vogel et al., 2014). In *D. melanogaster*, CG14567 is predicted to be secreted and is upregulated two-fold in hemocytes after immune challenge (Johansson et al., 2005). Homologs of CG14567 are found a number of different insects including silkworms and honey bees (Johnson et al., 2008). Within *D. melanogaster*, a paralog of CG14567, CG33290, is also present, with 47% sequence identity shared.

Having chosen the seven genes listed in Table 1.1 as putative effectors, I set out to make CRISPR/Cas9 knockouts. I designed guide RNAs, and homology repair templates for each gene and injected into a stock that expressed Cas9 in the germline, BL51323. I was able to isolate mutants for IMPPP, CG4269, CG13749 and CG18067. Mutants for IMPPP, CG4269 (two independent isolates), and CG18067 were homozygous viable and fertile. However, two independent isolates of CG13749 homozygous mutants were lethal, imitating the phenotype seen in the RNAi stock (Neely et al., 2010).

In order to investigate the role these genes play in Drosophila immunity, I tested these mutants in survival assays against pathogens controlled by the Toll-pathway. Surprisingly, as I will describe below, multiple deletions appeared to have similar effects on immunity. However, the course of the investigations suggests that a background mutation was present in the parent BL51323 stock and underlies the shared phenotype initially identified. At the same time, the screen did identify an additional immune phenotype linked to deletions of a specific gene.

Using w^{1118} as an immunocompetent control and $Bom^{\Delta 55C}$ flies as a positive control for immunodeficiency, I tested the *ΔIMPPP*, *ΔCG4269*, and *ΔCG18067* mutant lines for survival against a variety of pathogens. In initial experiments these mutant lines showed little defect in immunity. For example, I found that *ΔIMPPP* and *ΔCG4269* mutant lines were not susceptible to systemic infection with the Gram-positive bacteria *E. faecalis*, whereas $Bom^{\Delta 55C}$ flies succumbed quickly to infection (Figure 1.1 A). Additionally upon infection with the yeast *C. glabrata*, both *ΔIMPPP* and *ΔCG4269* mutant lines were resistant to infection (Figure 1.1 B), while $Bom^{\Delta 55C}$ flies are not. Lastly, in experiments with the filamentous fungi *N. crassa*, the *ΔIMPPP* mutant line phenocopies the resistance of the wild type, whereas $Bom^{\Delta 55C}$ flies were susceptible (Figure 1.1 C).

The problem of the shared phenotype and background first appeared in experiments with the filamentous fungi *F. oxysporum*. In testing *ΔIMPPP*, *ΔCG4269*, and *ΔCG18067* mutant lines with this pathogen, I found that they were all significantly more susceptible to infection than w^{1118}. *ΔIMPPP* and *ΔCG4269* mutant lines had an intermediate survival curve between w^{1118} and $Bom^{\Delta 55C}$, while *ΔCG18067* flies died at a similar rate to $Bom^{\Delta 55C}$ flies after infection (Figure 1.1 D).

I was surprised to see all three mutants have disrupted survival against *F. oxysporum*, but not any of the other pathogens tested. I decided to test the parent stock, Bloomington stock 51323, from which these mutants were derived, to determine if there was a preexisting immunodeficiency. We found that BL51323 is also susceptible to *F. oxysporum*, dying at a similar rate to *ΔIMPPP* and *ΔCG4269* mutants, between w^{1118} and $Bom^{\Delta 55C}$, but not as severe as *ΔCG18067* flies (Figure 1.1 E).

In parallel experiments, I set out to determine if the deletion stocks were altered solely for production of the targeted genes or if there might be pleiotropic effects on other immune induced peptides, due to the deletion or any background mutation. For this purpose, Samuel Lin, a fellow graduate student, and I used MALDI-TOF mass spectrometry to examine the Toll-induced peptides (Figure 1.2). In w^{1118} induced hemolymph, we observe members of the Bomanin family, immune induced peptides and antimicrobial peptides, like Drosomycin. When examining *ΔIMPPP* hemolymph, we found that the peptides derived from the prepropeptide, IM10, IM12 and IM13, are absent as expected. Curiously, we also found that the peak at 2694 m/z for IM14 was missing in *ΔIMPPP* hemolymph, as well as in *ΔCG4269* hemolymph.

Having found that IM14 peptide is not present in hemolymph in both *ΔIMPPP* and *ΔCG4269* mutants, I asked whether *IM14* transcripts were expressed. I measured *IM14* transcripts upon Toll-induction by qRT-PCR in *ΔIMPPP* flies and w^{1118} flies (Figure 1.3 A). I found that *IM14* was upregulated upon Toll-induction in w^{1118} flies, but that *IM14* transcripts were completely absent from ΔIMPPP flies. On the other hand, another immune induced gene *IM4*, adjacent to *IM14*, is Toll-induced in both w^{1118} and *ΔIMPPP* flies, but it was expressed at a higher level in *ΔIMPPP* than in the wild-type (Figure 1.3 B). From these experiments, I

conclude that IM14 is absent from hemolymph due to a defect at the transcription level and that there is potentially a chromosomal lesion affecting expression of both *IM4* and *IM14*.

To test whether a change in genomic sequence is responsible for the lack of *IM14* expression, I investigated the genomic structure of the *IM14* locus via PCR (Figure 1.4). Amplification of the IM14 coding sequence and a portion of the adjacent gene *Ipk1* using the F1/R1 primers yielded the expected product of 403 base pairs in both wild-type controls and the *ΔIMPPP* mutant (Figure 1.4 B). However, attempts to amplify genomic sequence between *IM4* and *IM14* with either the F2/R2 or F3/R3 primer pairs yielded no product in the *ΔIMPPP* mutant, while expected products of 1.4 kb and 1.3 kb were observed in both wild-type controls (Figure 1.4 C). These data suggest that there is a chromosomal aberration the region in between *IM4* and IM14 that blocks expression of *IM14*, while increasing expression of *IM4*.

Discussion

I have identified an immunodeficiency observed after *F. oxysporum* infection for *ΔIMPPP*, *ΔCG4269,* and *ΔCG18067* mutant lines, but also reported evidence that this may is due a background chromosomal aberration affecting *IM14*. We see a defect in survival observed in the parent stock BL51323 similar to that of *ΔIMPPP* and *ΔCG4269* mutants (Figure 1.1 E). Further experiments described in Chapter II of this dissertation reveal that *IM4* and *IM14* are required in defense against a subset of filamentous fungi. Taken together, the susceptibility of *ΔIMPPP* and *ΔCG4269* mutants to *F. oxysporum* is most likely due to the lack of *IM14* expression. Whether there could also be a role for IMPPP and CG4269 for

defense against *F. oxysporum* infection is unsettled until the targeted CRISPR/Cas9 mediated mutations are isolated from the background chromosomal aberration at the *IM14* locus.

Infection of *ΔIMPPP* and *ΔCG4269* mutants with the Gram-positive bacteria *E. faecalis* and the yeast *C. glabrata* demonstrates no defect in their survival compared to wild-type flies, suggesting that these genes play no role in defense against these pathogens. In addition, *ΔIMPPP* flies are resistant against infection with the filamentous fungus *N. crassa*. From these studies we are unable to identify any essential, nonredundant role of IMPPP or CG4269 in the immune system. However, the experiments conducted were not exhaustive and invite more investigations. In particular, exploration of their role in defense against numerous and diverse pathogens is important understand the function of these genes.

I do see an effect of the *ΔCG18067* mutation that is greater than the BL51323 background after infection with *F. oxysporum*. *ΔCG18067* mutant flies have a survival curve that more closely resembles that of the $Bom^{\Delta 55C}$ mutant. Further examination by Samuel Lin of the role of CG18067 in the immune system found a striking function for CG18067 in mediating defense through Bomanins. CG18067 has been renamed Bombardier and work on that locus constitutes a major chapter of Samuel Lin's PhD dissertation.

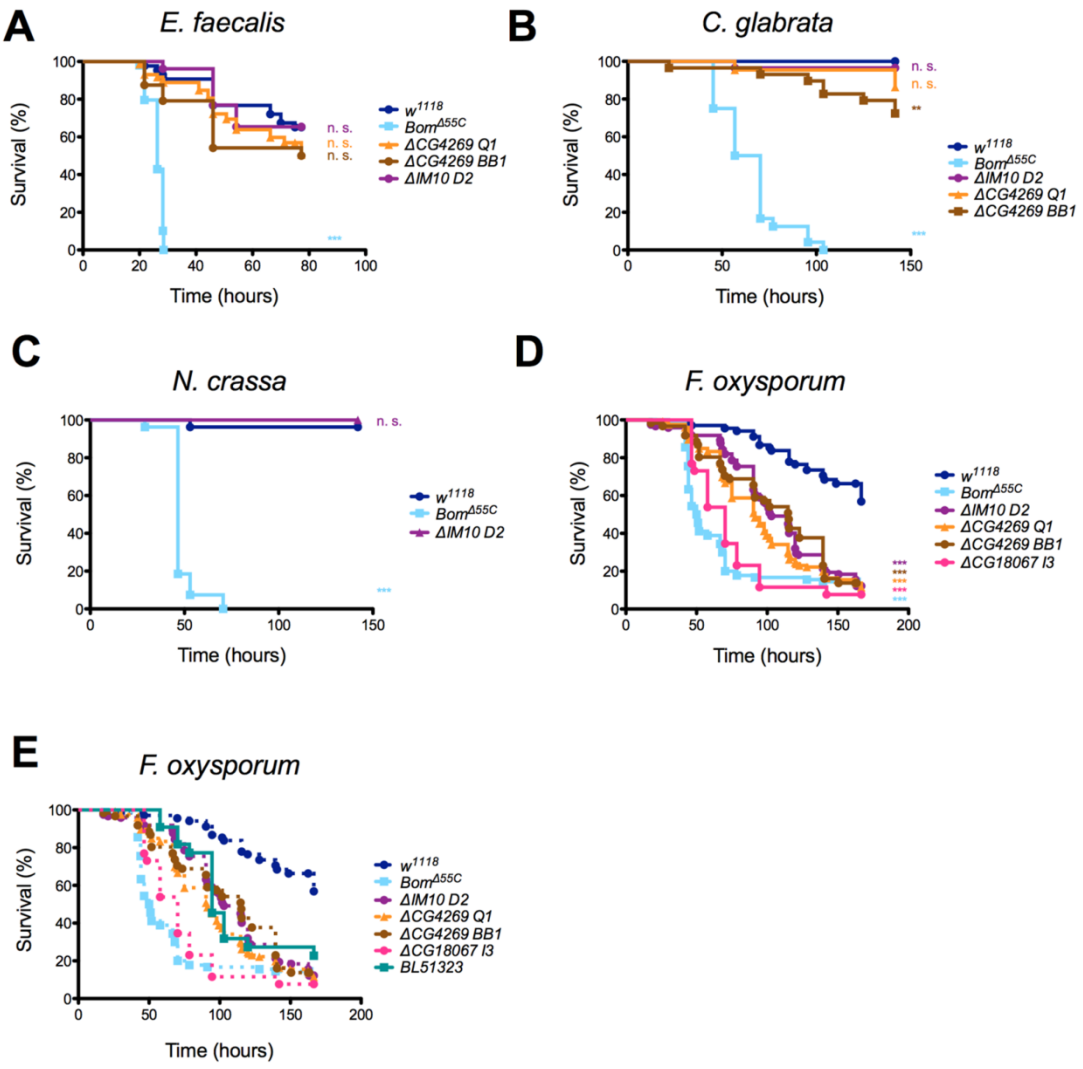

Figure 1.1: Survival of *ΔIMPPP*, *ΔCG4269*, *ΔCG18067* mutant lines against *E. faecalis*, *C. glabrata*, *N. crassa*, and *F. oxysporum*. Panel A shows a combination of two independent experiments. Panels B and C show data from one experiment each. Panel D shows the combination of three independent experiments, for all lines expect for ΔCG18067 which represents one experiment. Dotted lines in panel E represent data shown in panel D; data for Bl52323 is from one experiment. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to w^{1118} (***, p<0.0001; n.s. = not significant, p> 0.05)

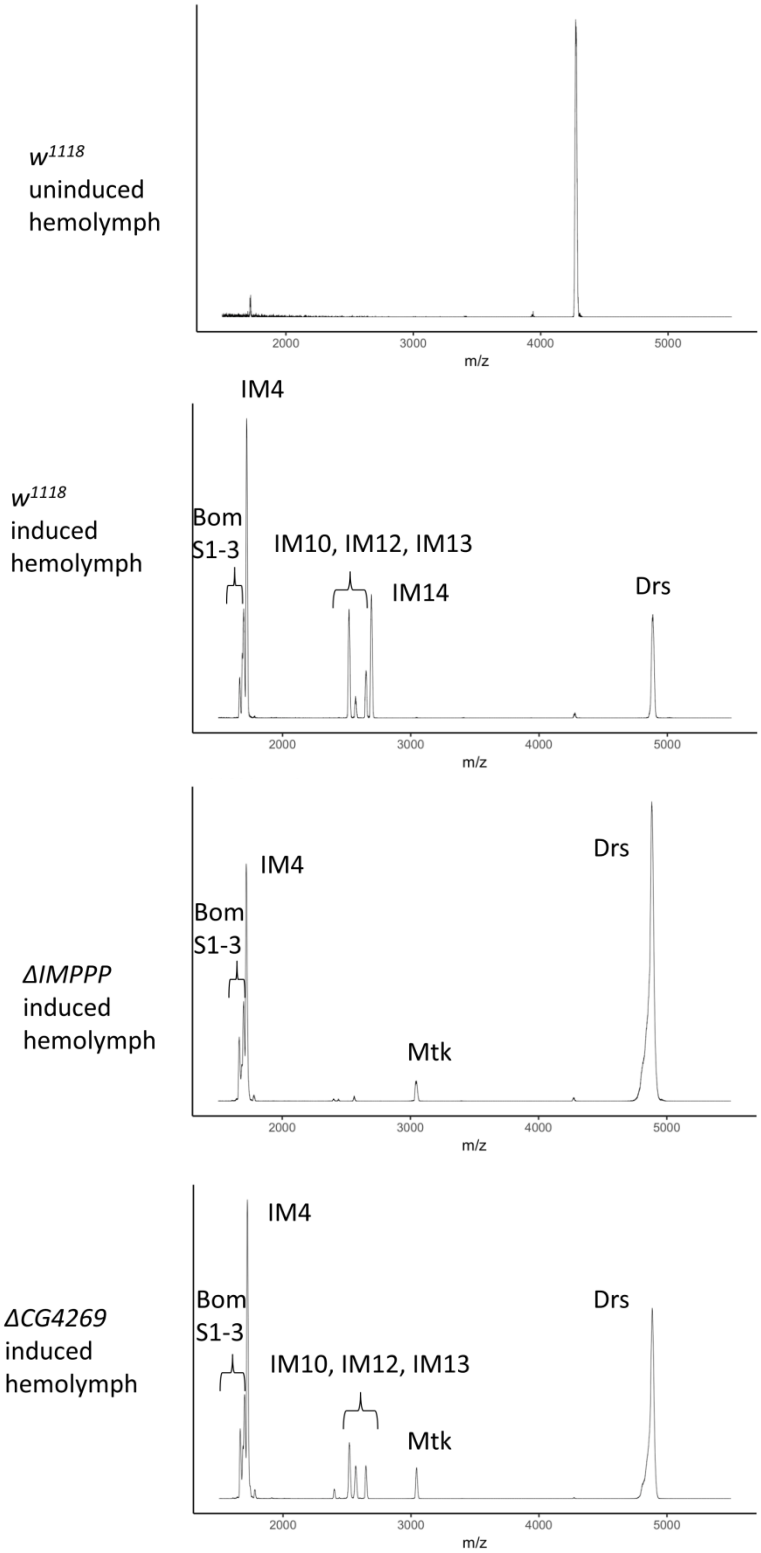

Figure 1.2: MALDI-TOF spectra for w^{1118}, *ΔIMPPP* and *ΔCG4269* hemolymph. Mass spectrometry analysis of Toll-induced hemolymph in linear mode, highlighting loss of IM14 in all deletion mutant lines. Mtk, Metchnikowin. Drs, Drosomycin.

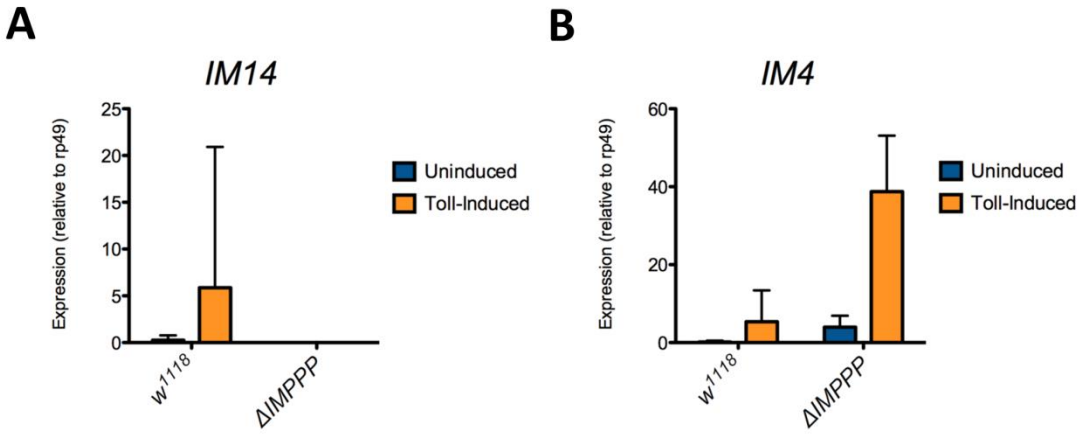

Figure 1.3: mRNA expression levels of *IM14* and *IM4* in w^{1118} and *ΔIMPPP*. Flies were collected 24h after Toll induction with heat-killed *M. luteus*. RNA was extracted from groups of 5-6 flies and *IM14* and *IM4* transcripts were measured relative to *rp49* transcripts. Shown is an average of three independent experiments. Error bars represent standard error of the mean.

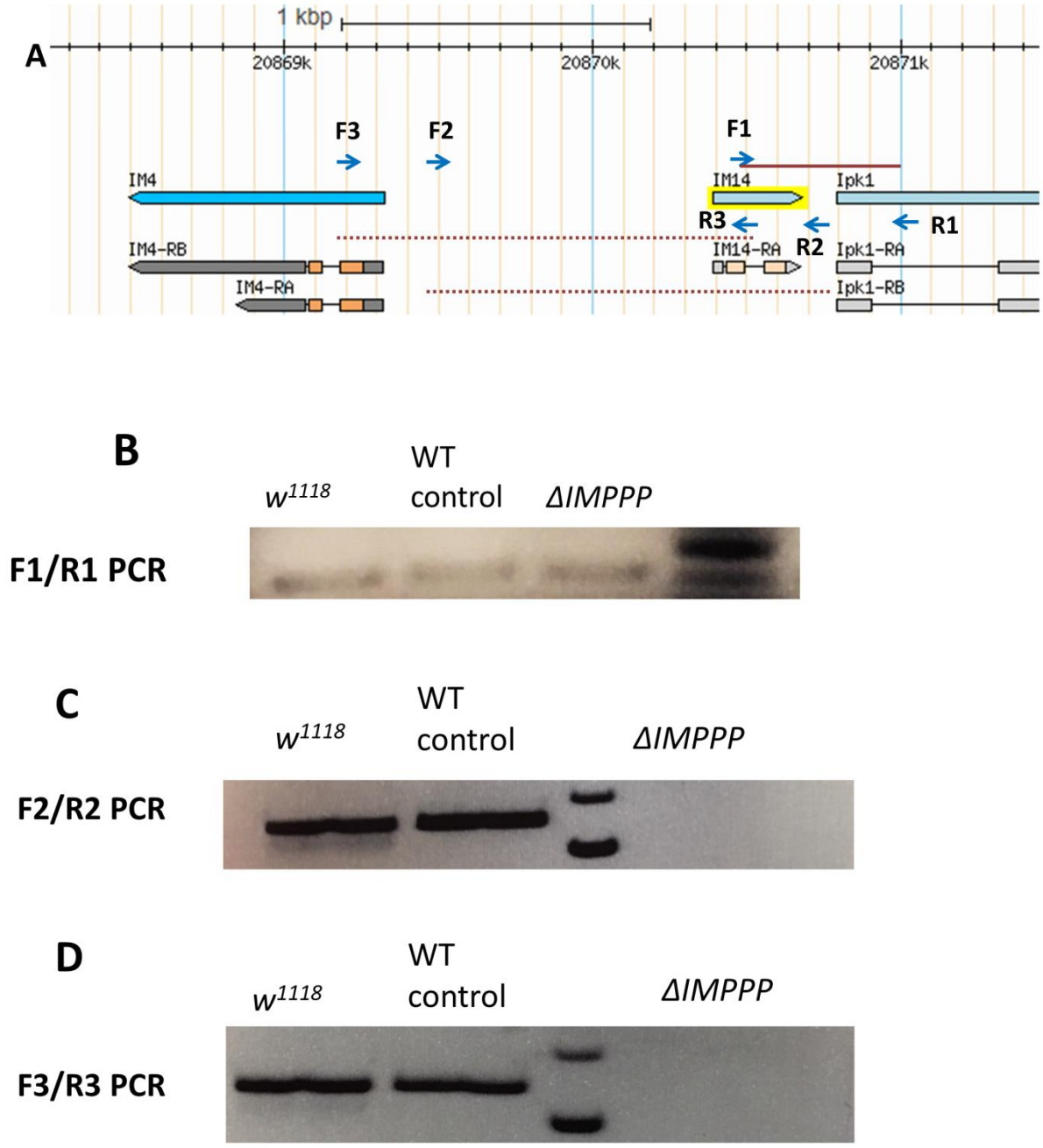

Figure 1.4: Genomic aberration at IM14 locus in *ΔIMPPP* mutant line. (A) Screenshot from FlyBase showing the IM4 and IM14 genomic locus. PCR primer binding sites are shown with blue arrows. Predicted PCR products are shown in red lines. (B, C, D) DNA agarose gels of PCR reactions stained with SYBRsafe. Ladder bands for (B) are 500 bp and 400 bp. Ladder bands for (C and D) are 1500 bp and 1000 bp.

Table 1.1 Putative Toll Effectors.

Gene Name	Length (aa)	Induction after fungal infection (RNA-seq)	Conservation	Secretion predicted by SignalP
IMPPP (CG18279)	238	9x	limited to Drosophila	yes
CG13749	183	2x	limited to Drosophila; IMPPP homolog	no
CG18067	222	5x	limited to Drosophila	yes
CG4269	79	7x	Drosophila and other insects: flies, mosquitoes, silkworm.	yes
CG16978	96	6x	limited to Drosophila	yes
CG14567	190	4x	Drosophila and other flies, silkworms, honey bees	yes
CG33290	171	0.66 x	Drosophila and other flies, silkworms, honey bees; homolog to CG14567	yes

Acknowledgments

I thank Scott Lindsay for his RNA-seq data of genes induced by *Beauveria bassiana*, in four species: *D. melanogaster*, *D. virilis, D. ananassae,* and *D. mojavensis*. I thank Anita Hermann and the McGinnis lab for the generous gift of the BL51323 stock.

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Chapter II: IM4 and IM14 mediate *Drosophila* defense against a subset of filamentous fungi

Abstract

Fungal infections, widespread throughout the world, affect a broad range of life forms, including agriculturally relevant plants, humans, and insects. In defending against fungal infections, the fruit fly *Drosophila melanogaster* employs the Toll pathway to induce a large number of immune peptides. Some have been investigated, such as the antimicrobial peptides (AMPs) and Bomanins (Boms); many, however, remain uncharacterized. Here, we examine the role in innate immunity of two related peptides, IM4 and IM14, found in hemolymph following Toll pathway activation. By generating a CRISPR/Cas9 knockout of both genes, Δ(IM4,IM14), we find that the IM4 and IM14 peptides are required for defense against a subset of filamentous fungi, including *Fusarium oxysporum*, but not other Toll-inducible pathogens, such as *Enterococcus faecalis* and *Candida. glabrata*. Analysis of null alleles and transgenes revealed that IM4 and IM14 are each required for defense, although their functions partially overlap. Generating and assaying a genomic epitope-tagged IM14 construct, we detected interaction *in vitro* of IM14 peptide in hemolymph with the hyphae of *F. oxysporum*. Together, these results identify IM4 and IM14 as a new class of innate immune effectors with humoral activity against a select set of filamentous fungi.

Introduction

Fungal infections have a devastating impact on a wide range of organisms. They are destructive to agricultural plants around the world, including rice, wheat, and tomatoes (Dean et al., 2012). Additionally, fungi infect more than one million humans annually (Janbon et al., 2019). Existing antifungal treatments are limited, with only one new class of drugs, echinocandins, developed in the past 15 years. Furthermore, extensive usage of limited classes

of related antifungals has led to the increasingly frequent appearance of drug-resistant fungi (Janbon et al., 2019). An enhanced understanding of naturally occurring antifungal defenses is thus of tremendous potential benefit.

The fruit fly *Drosophila melanogaster* is a robust model for fungal infections, replicating many features of murine fungal infections (Brunke et al., 2015; Dionne and Schneider, 2008). In the wild, flies have been found to be infected with a number of filamentous fungi, including *Beauveria*, *Metarhizium*, and *Fusarium* species (Cuthbertson and Audsley, 2016; Sharma and Marques, 2018). In combatting these infections, flies rely on the Toll innate immune pathway (Imler, 2014; Valanne et al., 2011). Toll provides defense against not only filamentous fungi, but also yeasts and those Gram-positive bacteria that produce a cell wall containing Lys-type peptidoglycan (Lemaitre and Hoffmann, 2007; Lindsay and Wasserman, 2014; Valanne et al., 2011). A second innate immune pathway, defined by the Imd receptor, provides defense against Gram-negative bacteria and the limited number of Gram-positive bacteria that produce a cell wall containing DAP-type peptidoglycan (Buchon et al., 2014; Georgel et al., 2001).

Systemic activation of Toll signaling induces a broad set of genes first identified by microarray analysis and mass spectroscopy (De Gregorio et al., 2002, 2001; Levy et al., 2004; Uttenweiler-Joseph et al., 1998). Many of the induced innate immune genes are transcribed in the fly fat body, with the protein products secreted into the hemolymph. These include antimicrobial peptides (AMPs), the Bomanin peptides, and a number of uncharacterized peptides.

Although AMPs such as the antifungal peptide Drosomycin (Drs) directly kill pathogens *in vitro* (Fehlbaum et al., 1994; Levashina et al., 1995) and are immunoprotective

when ectopically expressed *in vivo* (Tzou et al., 2002), recent loss-of-function studies reveal little or no requirement for AMPs in defense against fungi and Gram-positive bacteria (Hanson et al., 2019). In contrast, the Bomanin family of peptides (Boms) is required for defense against both classes of pathogens (Clemmons et al., 2015). Boms, which are *Drosophila*-specific, are readily detected in hemolymph following Toll activation. Here we describe the functional characterization of additional immune effectors, IM4 and IM14, which appear in hemolymph following systemic infection and are required for defense against a subset of filamentous fungi.

Materials and Methods

Fly husbandry and strain generation

Flies were raised on standard cornmeal agar media at 25°C. The w^{1118} strain was used as the wild type. *MyD88*$^{-}$ flies were $MyD88^{kra1}$, and *imd*$^{-}$ flies were imd^{shadok}

The null alleles *Δ(IM4,IM14)*, *ΔIM4*, and *ΔIM14* were generated using CRISPR/Cas9 technology, applying methods described previously (Gratz et al., 2014). Pairs of guide RNAs that targeted Cas9 to delete the region 2R: 20,868,460-20,870,480 for *Δ(IM4,IM14)*, 2R: 20,868,783-20,869,392 for *ΔIM4*, and 2R: 20,870,332-20,870,728 for *ΔIM14* were cloned into the pU6-BbsI-chiRNA vector (Addgene plasmid # 45946). Homology arms of approximately 1 kb were cloned into pHD-DsRed (Addgene plasmid # 51434). Cas9 was provided by plasmid pBS-Hsp70-Cas9 (Addgene plasmid #46294). Constructs were based on target sequences in the w^{1118} strain and injected into w^{1118}. See Table 2.2 for primer sequences.

The FLAG epitope tag was cloned between the signal sequence and mature peptide of IM14 in the context of the pHD-DsRed homologous repair template. This FLAG-IM14 construct was introduced at the IM14 genomic locus using the *ΔIM14* guide RNAs. Plasmids expressing IM4 or IM14 from the *pBomS3* promoter were made using methods previously described (Lindsay et al., 2018). Briefly, the *BomS3* gene promoter was placed 5' to the ORF of either *IM4* or *IM14*. These constructs were then each integrated via ΦC31-mediated transgenesis at an *attP* landing site located at 86Fb on the *D. melanogaster* 3rd chromosome (BDSC stock #24749). The transgenes were crossed into the *ΔIM4* and *ΔIM14* backgrounds and homozygous stocks were derived. An empty vector control was also introduced at the 86Fb *attP* landing site.

Microbial cultures

For survival experiments, microbes were cultured as follows. *Enterococcus faecalis* NCTC 775 (ATCC 19433) and *Enterobacter cloacae* were grown overnight at 37°C in LB media and concentrated to an OD_{600} of 10 in 20% glycerol. *Candida glabrata CBS 138* [ATCC 2001] was grown overnight in YPD media at 37°C and concentrated to an OD_{600} of 100 in PBS, 0.1% Tween. All filamentous fungi were grown on malt extract agar plates at 29°C until sporulation was observed (10-15 days). Fungal material was then strained through glass wool with sterile water to collect spores, which were concentrated in 20% glycerol and stored at -80°C before being used at the following concentrations (in spores/ml): *Aspergillus flavus* (sequenced strain): 5×10^9; *A. fumigatus* AF293 (FGSC# A1100): 6×10^9; *A. parasiticus* Nor-1 mutant (NRRL #6111): 3×10^9; *Botrytis cinerea* (B05.10): 3×10^9; *Fusarium graminearum* (NRRL #5883): 8×10^8; *F. oxysporum* f. sp. *lycopersici* 4287 (FGSC #9935): 3×10^8; *F. verticillioides* (FGSC #7415): 3×10^9; *Neurospora crassa*: 1×10^9.

For the induction of the Toll response, heat-killed *Micrococcus luteus* was prepared as previously described (Lindsay et al., 2018).

Survival assays

Groups of 20-25 adult male flies aged 2-7 days were collected and stabbed with a needle dipped in a suspension of bacteria, yeast, or fungal spores. Flies infected with *E. faecalis* were incubated at 25°C; all other infected flies were incubated at 29°C. Fly deaths were recorded at least twice per day for the duration of each experiment. Any deaths that occurred within the first 6 h were set aside to exclude from the data any deaths due to traumatic injury. The experiment was repeated three times and results combined. Statistical analyses were performed using the Gehan-Breslow-Wilcoxon test.

MALDI-TOF

After Toll induction with heat-killed *M. luteus*, flies were incubated at 29°C for 24 h, after which hemolymph was collected via capillary as previously described (Lindsay et al., 2018). Hemolymph in 0.1% trifluoroacetic acid/50% acetonitrile was mixed 1:1 with Universal Matrix (Sigma-Aldrich). Samples were then dried onto a Bruker MSP 96 ground steel plate. Spectra were collected from 1,500 to 10,000 m/z for linear mode, and 1,000 to 5,000 m/z for reflectron mode, both with positive polarization. Peptide calibration standard II (Bruker) was used as an external calibration standard. For each genotype, at least five independent samples were collected. Representative spectra are shown. Peaks were identified via corresponding m/z values from previous studies (Levy et al., 2004; Uttenweiler-Joseph et al., 1998). Spectra were visualized using R 3.3.2 and ggplot2 2.2.1 (R Core Team, 2013; Wickham, 2016).

Peptide gel electrophoresis and immunoblotting

Hemolymph samples were collected via Zymo-Spin IC column method (Lindsay et al., 2018) from 30 male flies aged 2-7 days that had been induced with heat-killed *M. luteus* and incubated for 24 h at 29°C. Samples were run on a SDS-tricine, 18% separating /10% spacer /4% stacking, acrylamide gel (Heuer and Szostak, 1998). Protein samples were then transferred to a PDVF membrane, blocked with 5% milk in TBST and stained with primary α-FLAG M2 (Sigma) (1:500) and secondary secondary sheep α-mouse HRP (Amersham Biosciences) (1:1000). The immunoblot was then treated with West Pico PLUS substrate (Thermo Scientific) and exposed to film.

Peptide hyphal binding and immunofluorescence

The immunostaining protocol was adapted from Luo et al 2019 (Luo et al., 2019). *F. oxysporum* was grown in 5 ml malt extract broth from a starting concentration of 2.9 x 10^5 spores/ml. After overnight shaking at room temperature, fungal hyphae were collected by centrifugation at 1000 g for 10 min and resuspended in PBS. Hemolymph was collected via the Zymo-Spin IC column method (Lindsay et al., 2018) from 420 male flies that had been induced with heat-killed *M. luteus* 24 h prior and incubated at 29°C, yielding ~35 µl cell-free hemolymph. Next, aliquots of 200 µl hyphae and 35 µl hemolymph were shaken at room temperature for 30 min. The samples were washed three times with PBS before fixation with 4% formaldehyde for 1 h . After washing another three times with PBS, samples were blocked for 1 h with 5% BSA. Samples were then incubated with α-FLAG antibody (1:200) overnight at 4°C. After washing with PBS, samples were stained for 2 h with donkey α-mouse Alexa555 (1:400) and DAPI (1:200) and then washed and mounted on slides. Samples were imaged

with aTi2 Widefield microscope (Nikon) and analyzed with the NIS- elements software and OMERO.

Results

Generation of flies null for the gene pair *IM4* and *IM14*

Pioneering mass spectrometry experiments by Bulet and colleagues identified two dozen peptide IMs (immune-induced molecules) that accumulate in *Drosophila* hemolymph upon induction of the innate immune response, principally the Toll pathway (Levy et al., 2004; Uttenweiler-Joseph et al., 1998). Among these, the Bomanins have been found to play an essential role against a broad range of pathogens (Clemmons et al., 2015; Lindsay et al., 2018) while several, including IM4 and IM14, have unknown functions.

IM4 and IM14 are closely related to one another and occupy adjacent positions in the genome, where they are divergently transcribed (Figure 2.1). As shown in Figure 2.2 A, the 15 amino acid sequence of amidated mature IM4 has 67% identity with the corresponding region of the slightly longer (24 aa) mature IM14 peptide. Like the Bomanins, IM4 and IM14 have been found to be widespread among the *Drosophila* genus, but not identified elsewhere. To investigate the potential role of IM4 and IM14 in innate immunity, we used CRISPR/Cas9 technology to delete both genes. The 2.0 kb deleted region includes the entire *IM4* gene, the upstream region for both genes, and the first exon of *IM14* (including the start codon). Flies homozygous for the *Δ(IM4, IM14)* deletion, hereafter *Δ4-14*, were viable and fertile.

With the *Δ4-14* stock in hand, we carried out MALDI-TOF studies of hemolymph (Figure 2.2 B-E). As described above, following Toll activation wild-type hemolymph displays robust expression of immune peptides, including IM4, IM14, Bomanins, and AMPs.

The signals from IM4 and IM14 were ablated in *Δ4-14*, as evidenced by the loss of signal at 1722 mass/charge (m/z) (IM4) and 2694 m/z (IM14). Furthermore, the spectra of induced *Δ4-14* hemolymph was wild-type for all previously identified peaks other than IM4 and IM14, including the Bomanins and AMPs, Metchnikowin (Mtk) and Drosomycin (Drs). The absence of IM4 and IM14 thus did not detectably alter the accumulation or modification of other Toll-induced peptides in the hemolymph.

In addition to previously identified peaks, *Δ4-14* hemolymph contained one previously unseen signal. The 1724 m/z signal of this peak, readily apparent in reflectron mode, is identical to that predicted for the BomS5 amidated peptide, previously known as CG15065 (Figure 2.2 E). This signal had not been detected previously because in the wild type it lies in the shoulder of the robust IM4 peak. Its existence in Toll-induced hemolymph was expected, however, on the basis of microarray and RNA-seq data demonstrating strong Toll-activated induction of the *BomS5* locus (De Gregorio et al., 2002; Valanne et al., 2019).

IM4 and *IM14* are specifically required for defense against *F. oxysporum*

We next turned to a functional assay to determine whether the absence of IM4 and IM14 impaired survival following systemic infection. Because the Toll pathway responds to and protects against infection by many Gram-positive bacteria and fungi, we focused on these classes of pathogens. We stabbed adult flies with a needle dipped in a suspension of bacteria, yeast, or fungal spores and then monitored survival. We used w^{1118} flies as our wild-type, i.e. immunocompetent, control and $Bom^{\Delta 55C}$ flies, which lack the 10-gene *Bom* cluster, as an immunodeficient control (Clemmons et al., 2015).

For a number of the pathogens tested, *Δ4-14* flies behaved identically to the wild type. Roughly 50% of both wild-type and *Δ4-14* flies survived six or more days following infection with the Gram-positive bacteria *Enterococcus faecalis*, whereas 100% of $Bom^{\Delta 55C}$ flies died within two days (Figure 2.3A). Likewise, wild-type and *Δ4-14* flies survived a week or longer after infection with the yeast *Candida glabrata*, whereas $Bom^{\Delta 55C}$ flies died in four days or fewer (Figure 2.3 B). We also found no effect of *Δ4-14* on immune defenses mediated by the Imd pathway: Wild-type, *Δ4-14*, and $Bom^{\Delta 55C}$ flies all survived infection with the Gram-negative bacteria *Enterobacter cloacae*, whereas control *imd*- flies died within one day (Figure 2.3 C).

For one pathogen in the initial test set, the filamentous fungus *Fusarium oxysporum*, deletion of *IM4* and *IM14* had a marked effect on survival (Figure 2.3 D): Fifty percent of flies homozygous for *Δ4-14* died within four days of infection. In contrast, greater than 70% of wild-type flies survived seven or more days post-infection. Thus, loss of the IM4 and IM14 peptides disrupts defense against *F. oxysporum*, but not other tested pathogens. Interestingly, loss of IM4 and IM14 did not impact survival as severely as loss of the Boms, which lead to 50% death after two days, very similar to complete loss of Toll signaling (Clemmons et al., 2015).

Δ4-14 is susceptible to some but not all filamentous fungi

We next investigated whether the susceptibility of *Δ4-14* flies to *F. oxysporum* reflected a general susceptibility to filamentous fungi. For these studies, we focused on filamentous fungi for which flies deficient for Toll signaling, and thus for induction of IM4, IM14, and other Toll effectors, exhibit a significantly decreased survival relative to wild type

(Figure 2.4). The control fly strains in each case were w^{1118} (wild type) and *kra-1* (*MyD88*$^{-}$), a loss-of-function allele for an essential mediator of Toll signaling (Tauszig-Delamasure et al., 2002).

As shown in Figure 2.4, susceptibility of *Δ4-14* flies to the filamentous fungi species varied. Survival was significantly less than wild-type for *F. verticillioides* and *F. graminearum* (panels A, B), two *Fusarium* species closely related to *F. oxysporum.* In the case of *F. graminearum*, survival of *Δ4-14* flies was intermediate between that of wild-type and *MyD88*$^{-}$ flies, a pattern very similar to that observed with *F. oxysporum*, where *Δ4-14* survival falls between wild type and $Bom^{\Delta 55C}$, which behaves similarly to *MyD88*$^{-}$ (Clemmons et al., 2015). In contrast, *Δ4-14 flies* displayed a much greater immune impairment upon infection with *F. verticillioides* than with *F. oxysporum*, dying to a comparable extent and at a similar rate as the *MyD88*$^{-}$ control (compare Figures 2.4 A and 2.3 D).

Variation in survival was also observed among *Aspergillus* species. The survival curves of *Δ4-14* infected with either *A. parasiticus* or *A. flavus* largely tracked with *MyD88*$^{-}$ (panels C, D). Upon *A. fumigatus* infection, however, *Δ4-14* flies survived at least twice as long *as MyD88*$^{-}$ flies (Figure 2.4 E).

For some filamentous fungi, loss of IM4 and IM14 did not affect survival. For example, 80% of wild-type and *Δ4-14* flies survived for at least seven days after infection with *Botrytis cinerea*, whereas >50% of *MyD88*$^{-}$ flies died after two days (Figure 2.4 F). Likewise, wild-type and *Δ4-14* flies survived *Neurospora crassa* infection for six days or more, but 50% of *MyD88*$^{-}$ flies died after three days (Figure 2.4 G). Overall, we find that the IM4 and IM14 peptides play a vital role in survival after infection with certain species of filamentous fungi, but are not important for infections with others.

IM4 and *IM14* are each required for defense

IM4 and IM14 are highly similar in sequence and expression pattern. Are they functionally redundant? To address this question, we explored the function of each individual locus. We again used CRISPR/Cas9, generating deletions that removed the entire coding sequence for either IM4 or IM14. The 5' endpoints of each deletion were chosen to lie within 100 bp of the transcriptional start site, minimizing potential disruption of elements in the regulatory region separating the two genes (Figure 2.1). For both deletions, MALDI-TOF analysis of induced hemolymph confirmed loss of the deleted gene product but no other peptides, indicating that either IM4 or IM14 can be stably expressed in the absence of the other (Figure 2.5).

To test the effect on defense of deleting *IM4* or *IM14*, we stabbed adults with *F. verticillioides* spores, for which *Δ4-14* flies have a reduced survival. Deleting either the *IM4* or *IM14* gene resulted in susceptibility to *F. verticillioides* markedly different from wild-type and comparable to that of the double deletion (Figure 2.6). Thus, IM4 and IM14 each act in defense against *F. verticillioides* infection.

Since deletion of either *IM4* or *IM14* had as severe an effect on survival as the double mutant, it was possible that each gene has a specific and distinct function in antifungal defense. Alternatively, survival might depend only on total dosage for the two genes, with loss of either dropping expression below the threshold required. To distinguish between these models, we generated transgenes placing each ORF under control of *pBomS3*, shown previously to be strongly Toll-responsive promoter (Lindsay et al., 2018), and then assayed the transgenes for rescue of *ΔIM4* or *ΔIM14*. As shown in Table 2.1, *pBomS3*-driven *IM4* rescued *ΔIM4*, improving the median survival from 46 h to 93 h ($p<0.0001$). The same was

true of *pBomS3*-driven *IM14* in the *ΔIM14* background ($p<0.0001$) (see Figure 2.7 for full survival curves). Flies expressing the empty vector construct at the same chromosomal location did not show any increase in survival (Figure 2.8).

Having confirmed the activity of the two constructs, we expressed each in a background deficient for the other. *IM14* expression significantly improved survival of *ΔIM4* flies, increasing median survival from 46 to 78 h ($p<0.0001$). Similarly, *IM4* expressed in a *ΔIM14* background improved median survival from 46 to 55 h ($p=0.0005$). Nevertheless, rescue was incomplete. The median survival of *IM14* expressed in *ΔIM4* background (78 h) did not reach median survival of Δ*IM4* rescued with *IM4* (93 h) (n.s., $p=0.09$). Furthermore, *IM4* did not rescue survival of *ΔIM14* (55 h) to the same level as *IM14* (93 h) ($p<0.0001$). The data thus indicate that the two loci encode functions that are neither fully distinct nor fully redundant.

FLAG-IM14 binds to *F. oxysporum* hyphae

To explore whether IM14 interacts directly with fungal pathogens against which it provides defense, we tagged IM14 with the FLAG epitope, using CRISPR/Cas9. The tag was introduced at the amino-terminus of the endogenously expressed mature peptide. Immunoblot analysis of induced hemolymph from FLAG-IM14 flies revealed a single band detectable with α-FLAG antibody (Figure 2.9 A). MALDI-TOF analysis of hemolymph confirmed the loss of the IM14 peak at 2694 m/z and the appearance of a peak with an m/z ratio of 3689, the value expected for FLAG-IM14 (Figure 2.9 B).

Having confirmed that FLAG-IM14 peptide is stably expressed, we next assayed its activity in providing antifungal defense. Specifically, flies homozygous for *FLAG-IM14* at the *IM14* locus were infected with *F. verticillioides* and their survival was compared to both wild-type flies and *ΔIM14* flies. Survival of FLAG-IM14 flies was not wild-type, but was significantly better than that of flies lacking the *IM14* gene (Figure 2.10). We conclude that the FLAG-IM14 peptide is active in providing defense against *F. verticillioides* infection.

Next, we assayed FLAG-tagged IM14 peptide in hemolymph for its ability to bind fungus. We collected hemolymph from Toll induced flies, incubated it with hyphae from *F. oxysporum*, and fixed samples. Staining with α-FLAG, we detected labeling along the surface of *F. oxysporum* hyphae (Figure 2.11). Staining was absent in parallel experiments with untagged wild-type hemolymph. We conclude that IM14 binds to *F. oxysporum* hyphae.

In summary, our results demonstrate that the pair of immune-induced peptides, IM4 and IM14, mediate Toll-induced defense against specific filamentous fungi, most likely via a humoral effect on fungal hyphae.

Discussion

Role of IM4 and IM14 in antifungal defense

In this study we found that the related peptides IM4 and IM14 are required in *D. melanogaster* for defense against a subset of filamentous fungi. We have also demonstrated that the two peptides have partially overlapping functions. Survival data reveal a dependence on the overall level of IM4 and IM14, with each peptide able to partially compensate for the absence of the other. Furthermore, each peptide accumulates in the absence of the other.

IM4 and IM14 lack known motifs of defined function. As noted previously (Clemmons et al., 2015), there is a similarity in size and sequence between IM4 and IM14 and the Bomanin peptides. There are, however noteworthy differences, including the presence of a CxxC motif in all of the Bomanins and the broader requirement for the Bomanins in Toll-mediated defense.

Among those fungi for which deleting *IM4* and *IM14* decreases survival, *Δ4-14* flies nevertheless often exhibit significantly greater survival than do *MyD88*$^{-}$ or Δ*Bom*^{55C} flies (see, e.g., *F. oxysporum* and *F. graminearum*). Thus, in contrast to the Bom effectors, which are strictly required for Toll defenses against a broad range of pathogens, IM4 and IM14 appear to be required for some, but not all Toll functions and to be active against only a select group of pathogens against which Toll mounts defense.

Like the Bomanins, IM4 and IM14 are found only within the *Drosophila* genus. Taxonomically-restricted genes (TRGs), while often studied only sparingly, represent 10-20 % of most genomes and frequently have essential functions (Khalturin et al., 2009). TRGs have been identified in the immune pathways of many invertebrates, including flies, mosquitoes, and cnidarians. Within immune systems they are abundant among effectors, but rare among signal transduction factors (Sackton et al., 2007; Waterhouse et al., 2007).

Specificity of IM4 and IM14 in antifungal defense

In tracking survival following systemic infection, we find considerable variability with regard to which pathogens exhibit increased virulence toward *D. melanogaster* in the absence of IM4 and IM14. Categorizing the fungi against which IM4 and IM14 provide defense, we

detect no simple relationship to fungal phylogeny. For example, IM4 and IM14 are required to defend against all the *Fusarium* species tested and some of the *Aspergillus* species, but not *Neurospora crassa*. Yet *Fusarium* and *Neurospora* are both members of the class Sordariomycetes, whereas *Aspergillus* is part of the less closely related Eurotiomycetes class (Schoch et al., 2009; Zhang et al., 2006). Furthermore, *Δ4-14* flies exhibit differential susceptibility to fungi within a single genus: the *Δ4-14* deletion substantially decreases survival against *A. flavus* and *A. parasiticus*, but has a much smaller effect on survival following *A. fumigatus* infection.

Although susceptibility of *Δ4-14* flies does not track simply with fungal phylogeny, susceptibility does appear to be closely related to fungal pathogenicity. Consider four filamentous fungi that are particularly pathogenic for wild-type flies: *F. verticillioides*, *F. graminearum*, *A. flavus*, and *A. parasiticus*. Infection with any of these four pathogens kills greater than 50% of wild-type flies within seven days. For each of these four, *Δ4-14* greatly decreases survival. By comparison, consider filamentous fungi with low pathogenicity, e.g., *A. fumigatus*, *N. crassa*, and *B. cinerea*. For each, greater than 80% of wild-type and *Δ4-14* flies survive for seven or more days post infection. Note that we observe this association of susceptibility with pathogenicity only among filamentous fungi: For the strongly pathogenic Gram-positive bacterium *E. faecalis*, the *Δ4-14* deletion had no effect on survival.

Although the Bomanins are strictly required for Toll humoral defenses, we have found a correlation between pathogenicity and the level of Bomanin function required to confer resistance (Clemmons et al., 2015). It thus appears that for both Bomanins and the IM4 and IM14 gene pair, pathogenicity tracks with the strength of effector function required for defense.

Activity of IM4 and IM14

How do IM4 and IM14 provide defense against filamentous fungi? One mechanism could be directly binding and killing the pathogens. Supporting this model is our immunofluorescence data, demonstrating that IM14 interacts *in vitro* with at least one filamentous fungus that it targets. Antifungal peptides, such as mammalian LL-37 and plant defensin NaD1, also been bind hyphae of fungal pathogens against which they are active (Luo et al., 2019; Van Der Weerden et al., 2008). IM14's ability to bind fungal hyphae could indicate an antimicobial function. Given that our assay was carried out with crude hemolymph, we cannot state whether the observed interaction of IM14 with hyphae is direct or is mediated by one or more unidentified hemolymph components.

IM4 and IM14 might themselves interfere with pathogen growth, survival, or proliferation, or they might enable the fungicidal activity of other factors. The same is true of the Bomanins, which are required for hemolymph mediated killing of *C. glabrata*, but for which fungicidal activity of synthetic peptides has not been observed (Lindsay et al., 2018). Given that IM4 and IM14 are required for defense against only a subset of Toll and Bomanin targets, the function of IM4 and IM14 may be to meet a specific challenge posed by certain fungi to the entry or activity of antimicrobial factors. Further investigation of IM4 and IM14, as well as other hemolymph immune effectors, is likely to be informative in this regard.

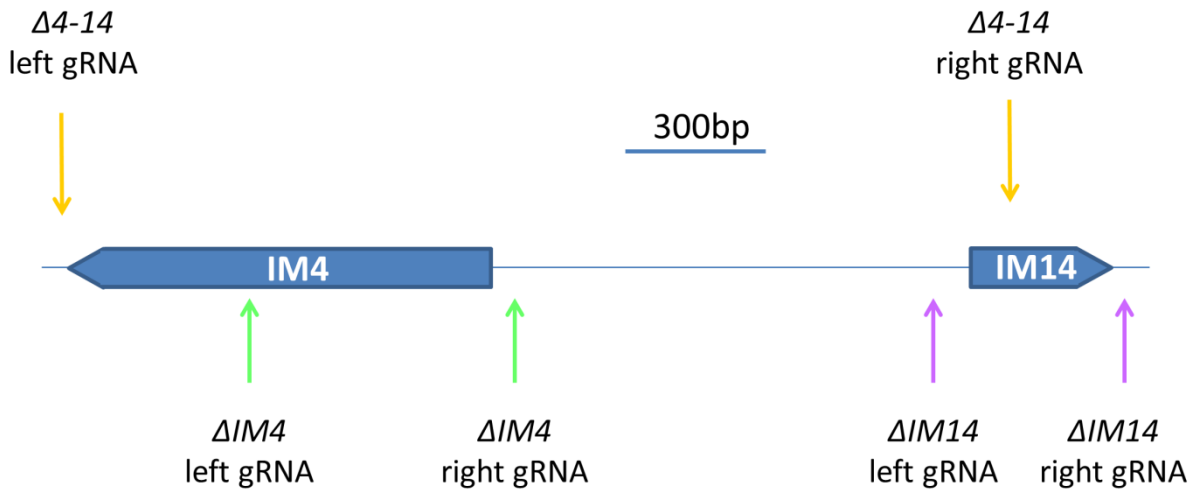

Figure 2.1: Generation of deletions in *IM4* and *IM14* genomic region. Blue shaded regions indicate transcription units. Arrows represent guide RNA target sites for mutants generated with CRISPR/Cas9 in this study

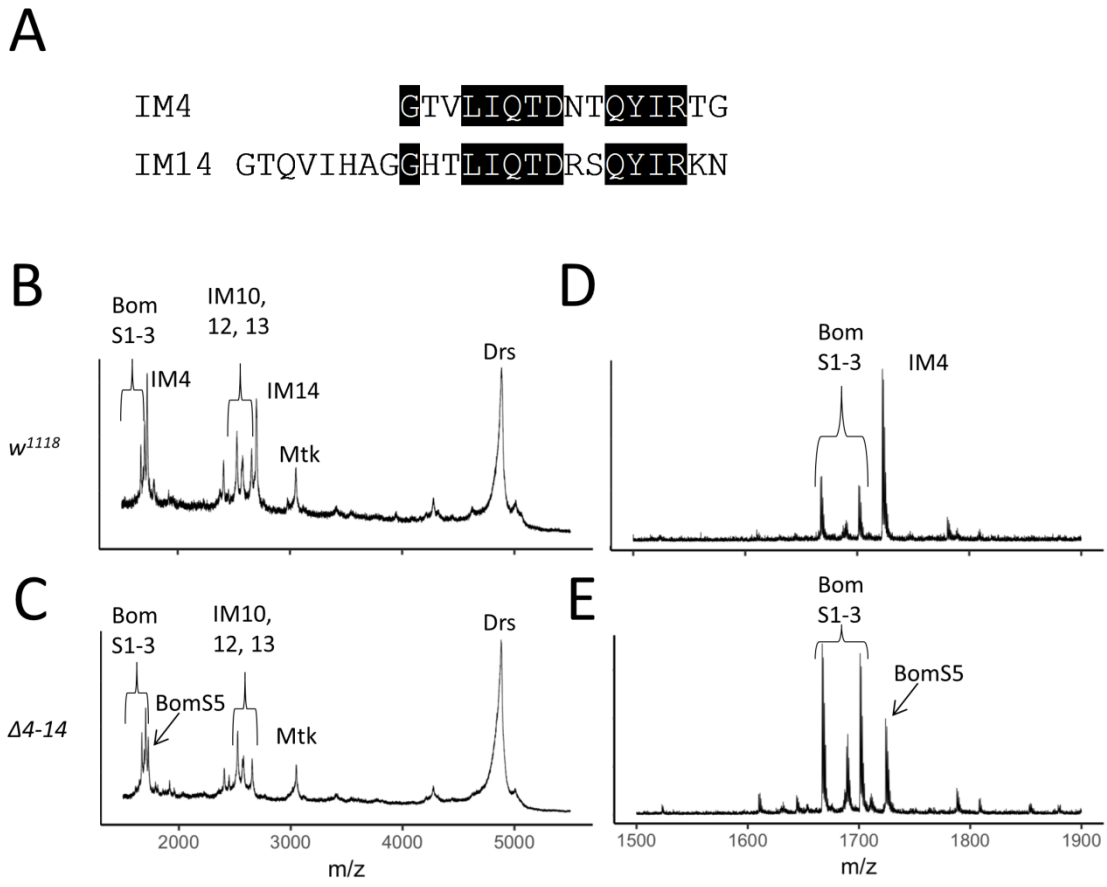

Figure 2.2: Deletion of *Drosophila IM4* and *IM14* gene pair. **(A)** Alignment of mature IM4 and IM14 peptide sequences. Identical residues are highlighted. **(B-E)** Mass spectrometry analysis of Toll-induced hemolymph in linear **(B, C)** and reflectron **(D, E)** mode, illustrating loss of IM4 and IM14 signal in *Δ4-14* deletion mutant. IM4 signal overlaps with the BomS5 signal, which is readily apparent in the *Δ4-14* mutant analyzed in reflectron mode. Mtk, Metchnikowin. Drs, Drosomycin.

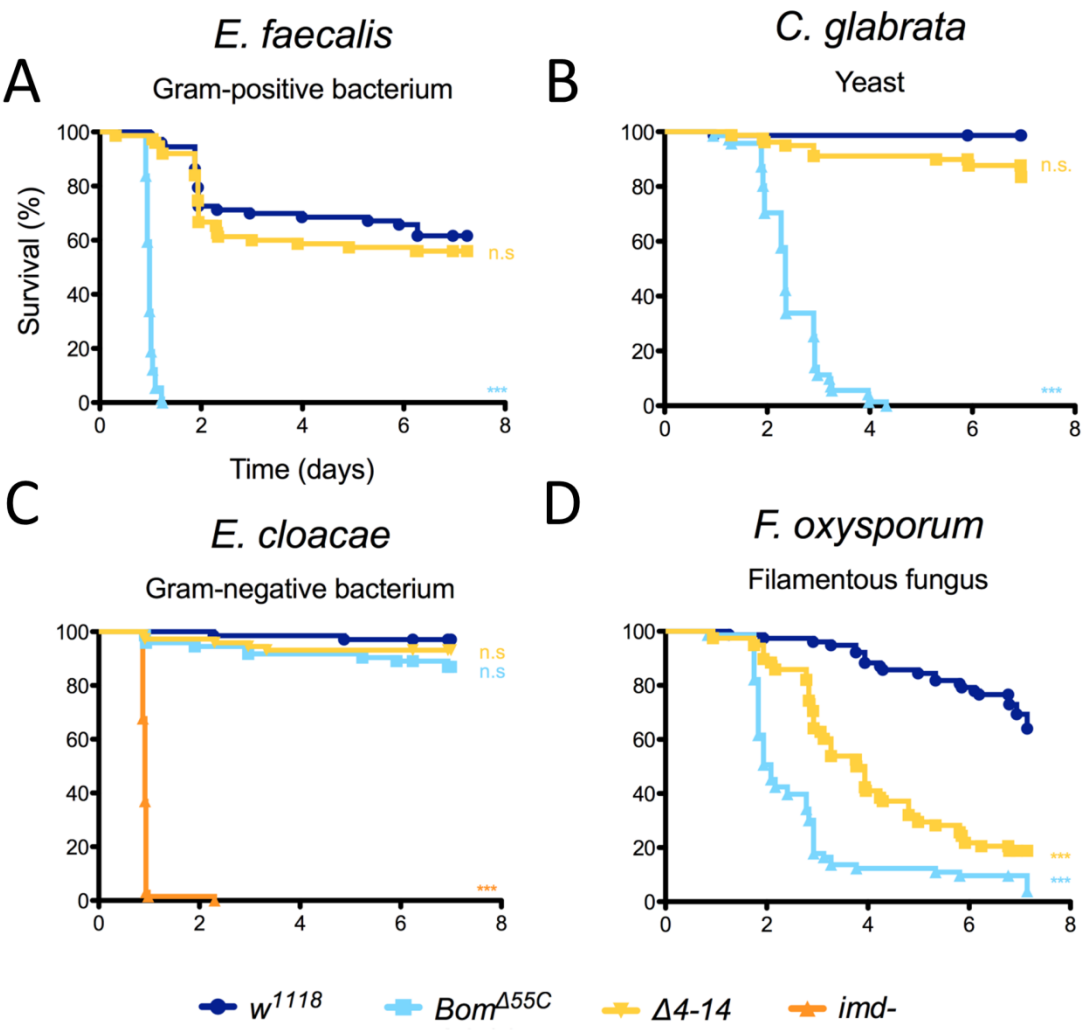

Figure 2.3: Survival of *Δ4-14* against *E. faecalis*, *C. glabrata*, *E. cloacae*, and *F. oxysporum* infection. Shown is the combination of three independent experiments for each pathogen with 20-25 flies per genotype per experiment. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to w^{1118} (***, $p<0.0001$; n.s. = not significant, $p> 0.01$)

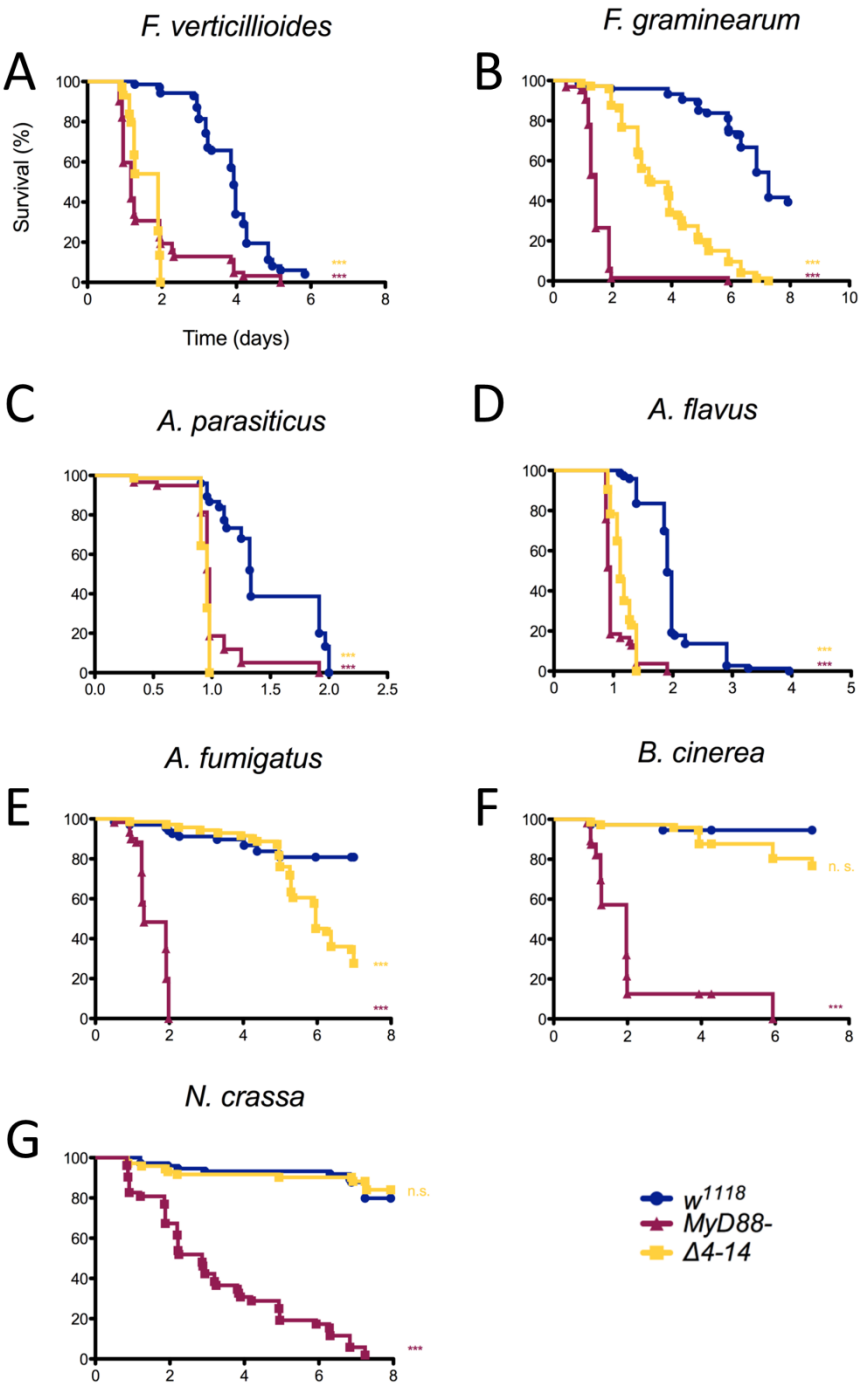

Figure 2.4: Survival of *Δ4-14* against *F. verticillioides, F. graminearum, A. parasiticus, A. flavus, A. fumigatus, Botrytis cinerea, and N. crassa.* The combination of three independent experiments for each pathogen with 20-25 flies per genotype per experiment is shown. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to w^{1118} (***, p<0.0001; n.s. = not significant, p> 0.01)

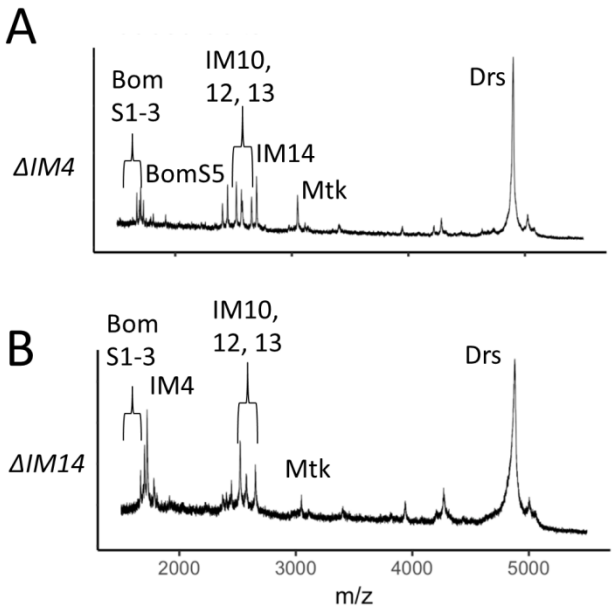

Figure 2.5: MALDI-TOF spectra for *ΔIM4* and *ΔIM14* hemolymph. **(A, B)** Mass spectrometry analysis of Toll-induced hemolymph in linear mode, highlighting loss of IM4 **(A**) and IM14 **(B)** in deletion mutants.

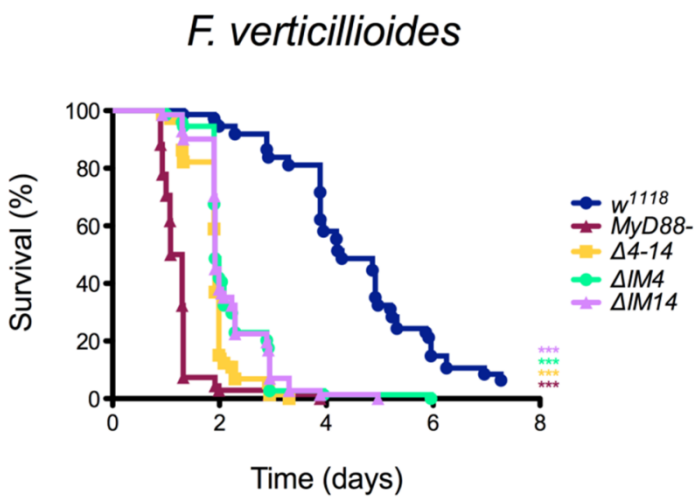

Figure 2.6: Survival of *ΔIM4* and *ΔIM14* against *F. verticillioides.* Shown is the combination of three independent experiments with 20-25 flies per genotype per experiment. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to w^{1118} (***, p<0.0001).

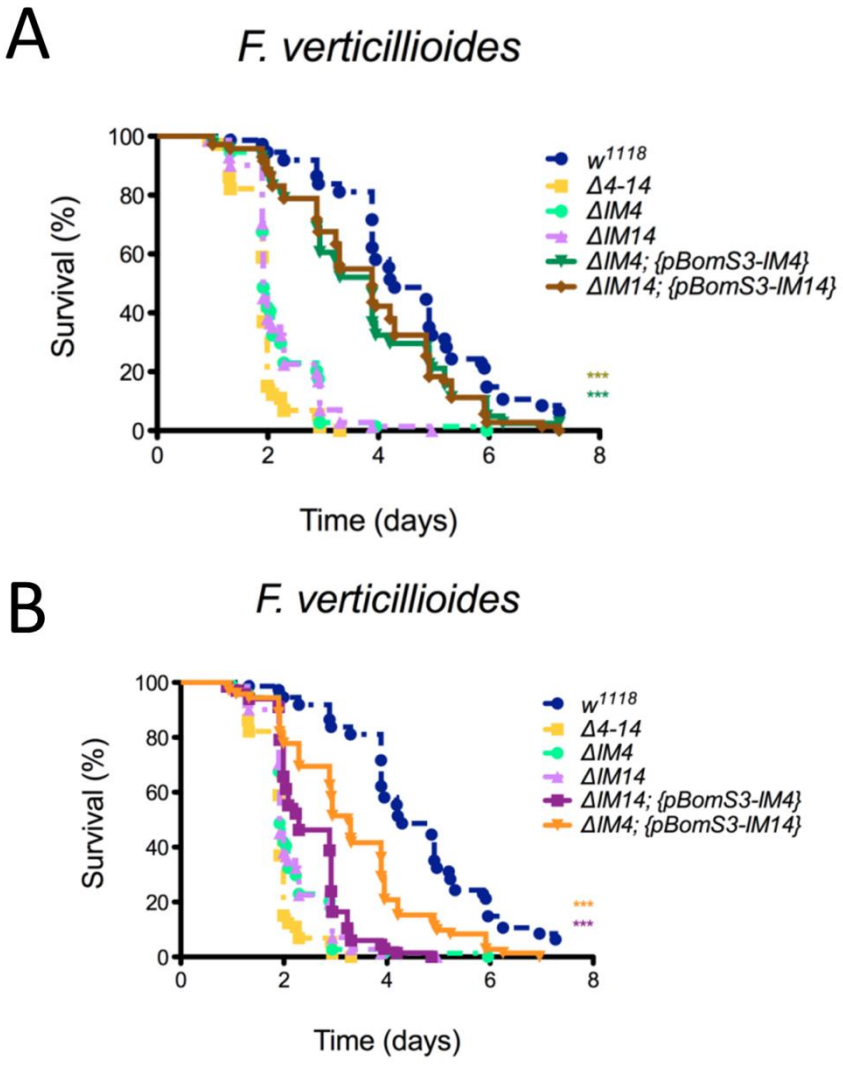

Figure 2.7: Survival against *F. verticillioides* of *ΔIM4* and *ΔIM14* rescued with IM4 or IM14. Shown is the combination of three independent experiments with 20-25 flies per genotype per experiment. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Dotted lines represent data shown in Figure 5. (A) Cis-rescue. Peptides rescue deletion of the corresponding gene. Significance is shown relative to *ΔIM4* for *ΔIM4; pBomS3>IM4* and relative to *ΔIM14* for *ΔIM14; pBomS3>IM14* (***, p<0.0001) (B) Trans-rescue. Peptides rescue deletion of other member of gene pair. Significance is shown relative to *ΔIM4* for *ΔIM4; pBomS3>IM14* and relative to *ΔIM14* for *ΔIM14; pBomS3>IM4* (***, p<0.0001)

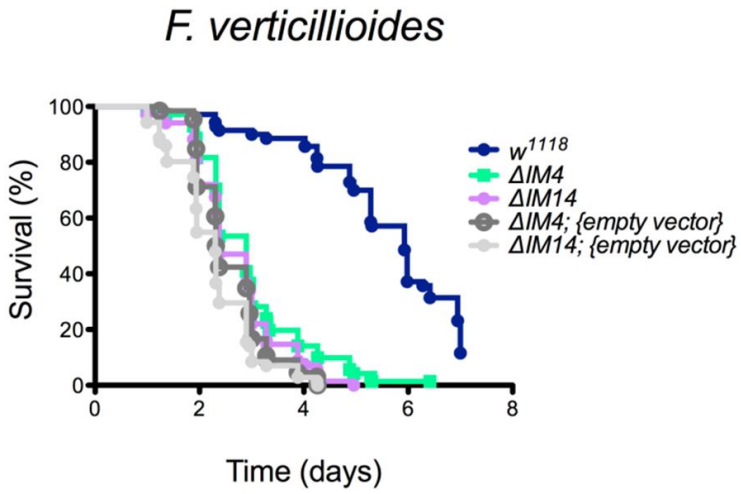

Figure 2.8: Survival against *F. verticillioides* of empty vector controls. Shown is the combination of three independent experiments with 20-25 flies per genotype per experiment.

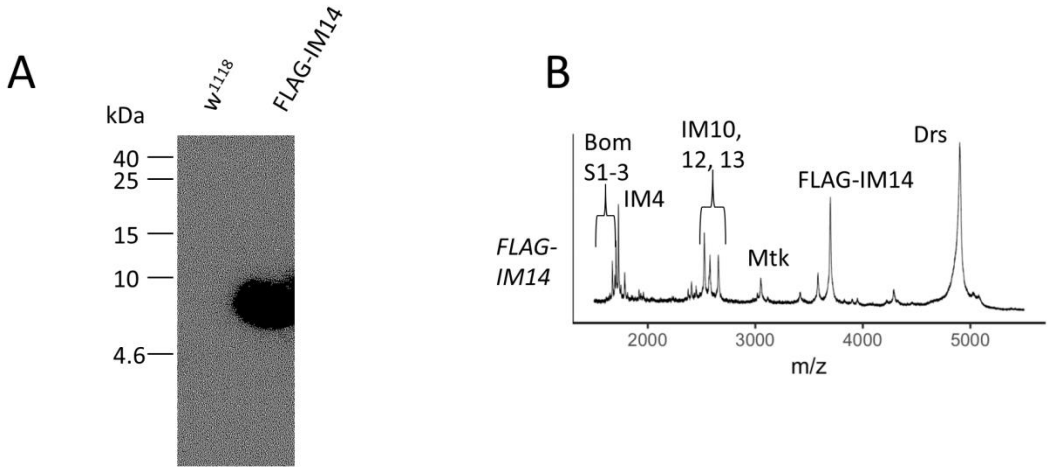

Figure 2.9: Characterization of FLAG-IM14 gene product. (A) Immunoblot stained with mouse α-FLAG M2 (1:500) and sheep α-mouse HRP (1:1,000). Two µl of Toll-induced hemolymph was loaded per lane. (B) MALDI-TOF analysis of FLAG-IM14 Toll-induced hemolymph in linear mode.

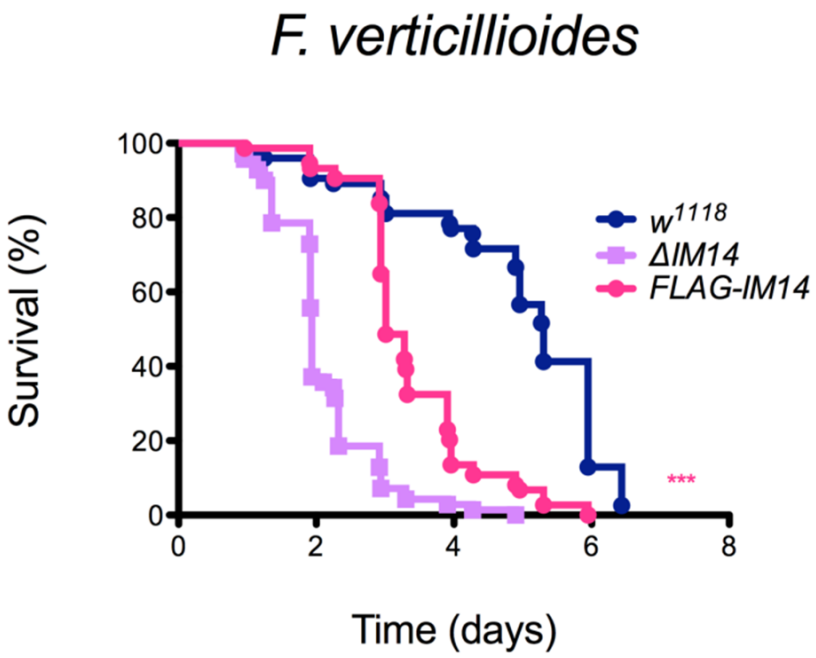

Figure 2.10: Survival against *F. verticillioides* of *FLAG-IM14*. Shown is the combination of three independent experiments with 20-25 flies per genotype per experiment. Survival curves were compared using the Gehan-Breslow-Wilcoxon test. Significance is shown relative to *ΔIM14* (***, $p<0.0001$)

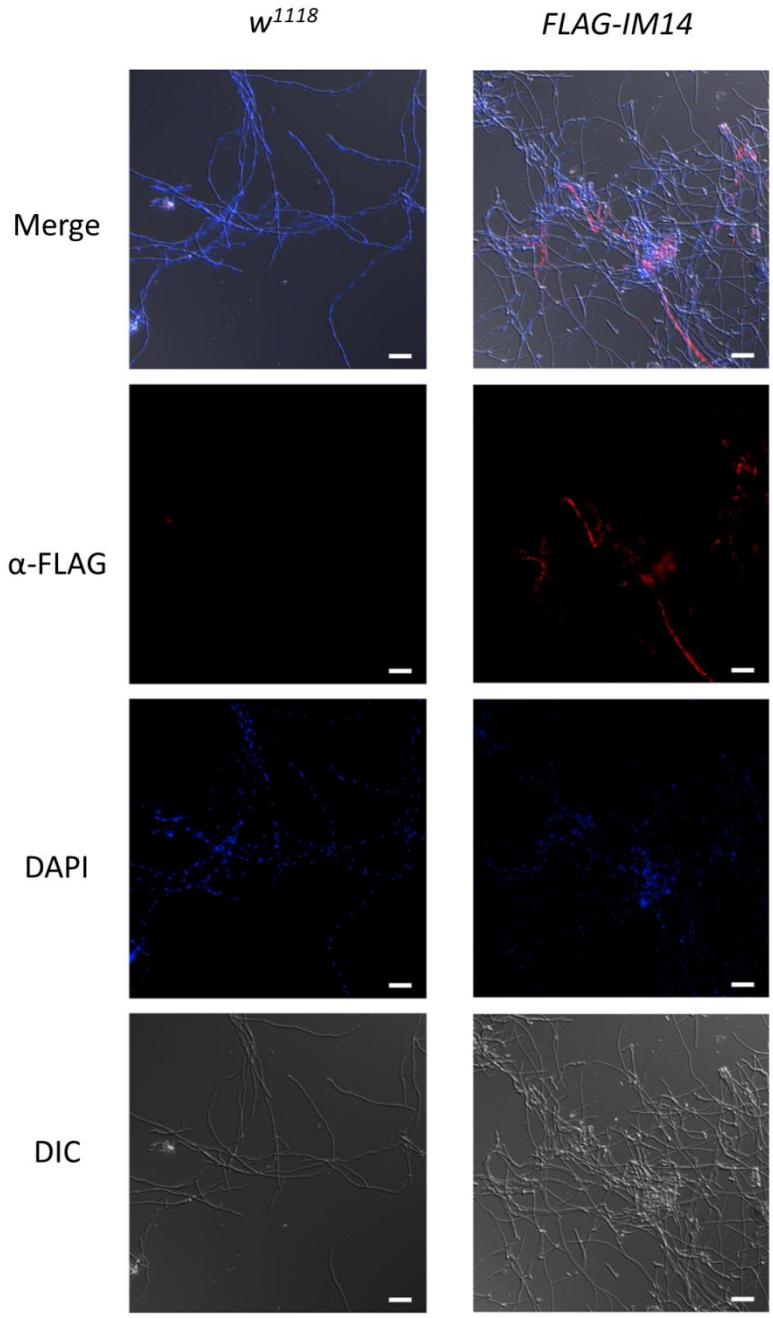

Figure 2.11: Immunofluorescence of *F. oxysporum* hyphae. Hyphae were incubated with w^{1118} or FLAG-IM14 hemolymph and then stained with mouse α-FLAG M2 (1:200) and donkey α-mouse Alexa 555 (1:400). DAPI marks fungal DNA. Scale bar is 30 μm. Images were generated as compressed Z-stacks.

Table 2.1: Median survival in hours of *ΔIM4* and *ΔIM14* mutants rescued by homotypic and heterotypic transgenes. Data derived from Figure 2.7. n.a. = not applicable

	No transgene	***pBomS3-IM4***	***pBomS3-IM14***
MyD88^{-}	29	n.a.	n.a.
Δ4-14	46	n.a.	n.a.
ΔIM4	46	93	78
ΔIM14	46	55	93
w^{1118}	103	n.a.	n.a.

Table 2.2: Primers used generating constructs for CRISPR/Cas9 mutants *Δ4-14, ΔIM4, ΔIM14* and *FLAG-IM14*.

	Left sense oligo	Left antisense oligo	Right sense oligo	Right antisense oligo	homology arm 1 forward primer	homology arm 1 reverse primer	homology arm 2 forward primer	homology arm 2 reverse primer
Δ4-14	CTTCGGATGTCAATCAAACTC AAC	AAACGTTGAGTTTGATTGACATCC	CTTCGATTGCGGCTTTGGCGACGG	AAACCCGTCGCCAAAGCCGCAATC	GCAGCCTGAAACTGAACGAT	GAGTTTGATTGACATCGAATCT	CGGCGGAGGCTGGTGAGTGCAT	TCGCCAGGCTCTGATAAAAT
ΔIM4	CTTCGATTACTACTAGTATTGCA	AAACTGCAATACTTAGTAGTAATC	CTTCGGGCGATTCCAAGACAACCT	AAACAGGTTGTCTTGGAATCGCCC	TAGTTGGCTACTTCAAAAGC	AATACTTAGTAGTAATCAAAG	CCTGGGTGATTACACAGGTT	TGGCGGCTTTAAAAGTAGG
ΔIM14	CTTCGATGCAGTGAGCTCGCCAGC	AAACGCTGGCGAGCTCACTGCATC	CTTCGAGCGCGCACATAGTGGGAA	AAACTTCCCACTATGTGCGCGCTC	CTGGGCGATTCCAAGACAAC	AGCTGGCGGCTTTAAAAGTAGG	GAAAGTGTCACACTGACTAAATATAC	GTTGTCGTTGTCAAAATG
FLAG-IM14								
	sequence cloned into pHD-DsRed							
	GGAACATCCGCGGGGCGAGCTCACTGCATCGGATGGGTGAGCTATATAAGCGCGCCCTGTCCGGATCGCTAATCAAAGTAGAATTTGAATTCAAACTGTAAACATGAACTGTCTGAAGATCTGCGGCTTTTTCTTCGCTCTGATTGCGGCTTTGGCGACGGCGGAGGCTGACTACAAAGACGATGACGACAAGGGTGAGTGCATAAAAAAGCAATCTTAAAGATCGTTTTTTGCTTATCAGCATTTTATTATTGATAGGCACCCAAGTCATTCATGCTGGCGGACACACGTTGATTCAAACTGATCGCTCGCAGTATATACGCAAAAACTAAAAAAAAAACCTCAAATAAATATTTAAAGAATAAAAATGTTTTGAAACAGCATATGATGTTCC							

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

LC and SW conceived the project. LC, SAL, YX, and SJHL performed and analyzed experiments. LC and SW wrote the paper.

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Chapter III:

Discussion and Future Directions

Discussion and Future Directions

In the course of my dissertation work, I identified three putative effectors that play a crucial role in the *Drosophila* innate immune system - IM4, IM14 and CG18067. This report reveals that although the immune system has been studied extensively, there are still rich avenues for research. One key technological development that allowed these genes to be studied is genome editing and CRISPR/Cas9 in particular (Cong et al., 2013; Gratz et al., 2014). Using this technology, I was able to make specific direct deletions of genes that appeared to play a role in immunity. Many of the genes involved in the immune system were initially found through classical forward genetic screens (Hashimoto et al., 1988). By using a different reverse genetics approach, I was able to find a distinctive set of genes that forward genetic screens did not identify. *IM4* and *IM14* would have been particularly difficult to identify via forward genetic screens because their small size would have a low hit rate by random mutagenesis or P-element insertion. Also, the development of tools and protocols around CRISPR/Cas9 has made process of generating mutants fast and straightforward, especially in *D. melanogaster*.

Previous antimicrobial peptides were found by assaying biochemical activity *in vitro* (Imler, 2014; Levashina et al., 1995). CG18067 has not shown antimicrobial activity when isolated protein was tested (Samuel Lin). Purified IM4 and IM14 peptides have not been tested for antifungal activity. Since IM 4 and IM14 were not found previously via screens for in vitro antimicrobial activity, they may not have direct antifungal activity on their own. By asking for genes that are required for defense in vivo, I was able to distinguish a new set of immune effectors.

Curiously, all three of these genes are taxonomically restricted genes (TRGs), meaning they do not have significant sequence homology found outside of their own taxa (Khalturin et al., 2009). Since much of the established *Drosophila* immune system has homology to the mammalian system, especially signaling components, this observation represents somewhat of a departure. However, immune genes that act as effectors or recognition factors have been found more likely to be TRGs (Sackton et al., 2013; Waterhouse et al., 2007). Even classic antimicrobial peptides, like Drosomycin and Metchnikowin are not found outside of *Drosophila*.

Is it meaningful that these effectors are TRGs? First of all, the finding of required TRGs for defense highlights the importance of looking beyond conservation, which has traditionally been a marker of value when selecting genes for further research. Secondarily, it prompts the question: Why are these genes that are required for immunity not present in other species? There are several possible explanations their limited scope. TRGs could be providing novel functions that are not needed in other species. At first, unique functions for TRGs seems unlikely because all animals use immune systems to combat pathogens. However, perhaps these peptides are providing a novel function that we are currently unable to detect because of a limited understanding of the Drosophila immune system. For example, the specific anatomy of *Drosophila* might require antimicrobial peptides that access certain compartments or function in a specific biochemical environment. Another explanation for the frequency of TRG among effectors is the continuous external evolutionary pressure from pathogens forces effectors to diverge between taxa. The Red Queen hypothesis has been extensively used to describe this constant evolutionary arms race between host and pathogens (Papkou et al., 2019). Perhaps effectors direct interaction with pathogens could cause them to quickly

diverge, which masks any conservation with genes in other species that share a last common ancestor.

IM4 and IM14 function

In Chapter II of this dissertation, I showed that *IM4* and *IM14* are both required for defense against a subset of filamentous fungi. Additionally, *IM4* and *IM14* each have unique functionality, while still retaining the ability to partially rescue for the absence of the other. Furthermore, FLAG- tagged IM14 displays the capacity to bind hyphae of the fungal pathogen *F. oxysporum*. However, the molecular mechanism of either of these peptides in defense is still unknown.

IM4 and IM14 are small peptides that are secreted into the hemolymph, where they could come in direct contact with fungal pathogens during a systemic infection. One simple explanation for their role in immunity is they bind actively growing fungal hyphae and kill them. Measuring their ability to control fungal load *in vivo* provided some hints that they directly combat fungal growth (Appendix). Furthermore, preliminary experiments examining the filamentous fungi *F. oxysporum* exposed to wild-type hemolymph yielded no antimicrobial activity. IM4 and IM14 could still be directly antifungal but technical problems with these assays have allowed this activity to evade detection.

Further investigation into the exact nature of the binding of IM14 to *F. oxysporum* hyphae could elucidate the function of IM14. In particular, determining what components of the fungal cell wall IM14 interacts with and whether this interaction is direct or there are other *Drosophila* factors needed would provide insights into IM14's mechanism of action.

Additionally, whether IM4 shares IM14's ability to bind hyphae is unknown. Further research on this question would not only clarify the role of the individual peptides, it could also explain the relationship between the peptides and their somewhat overlapping function.

Another explanation for the requirement of IM4 and IM14 for defense against fungal pathogens could be that they support the antifungal activity of other effectors. Antimicrobial peptides, like Drosomycin and Metchnikowin, proteins known to have direct *in vitro* activity against filamentous fungi, do not appear to be required for defense *in vivo* in *Drosophila* (Hanson et al., 2019). Could IM4 and IM14 aid Bomanin-mediated defense against fungal pathogens? Boms are required for defense against Gram-positive bacteria and fungi, while IM4 and IM14 are only required for a subset of these pathogens (Clemmons et al., 2015). Clearly during infections with Gram-positive bacteria and certain fungi, Boms are able to protect flies against these pathogens without IM4 and IM14. There is some evidence that IM4 and IM14 are required for defense against particularly pathogenic fungi (Chapter II). Conceivably IM4 and IM14 could be working to provide extra support to the Boms in these cases.

Preliminary immunoprecipitation experiments to detect *Drosophila* proteins bound to IM14 did not find any interactions. This could be a true reflection of the lack of any protein-protein interactions involving IM14 or it could represent the technical limitations of the assay. Any interaction between IM14 and Boms or other effectors could be too transient to be detected by immunoprecipitation. Furthermore, these interactions might only occur in the presence of pathogen, which was not included in these experiments.

Development of New Antifungal Treatments

There is an increasing need for new antifungal therapies. The incidence of more immunocompromised patients, including those with AIDS, receiving chemotherapy or organ transplant recipients, has led to an increase in fatal invasive fungal infection (Delarze and Sanglard, 2015). Moreover, fungal pathogens are increasingly found to be resistant to the current array of antifungal drugs. Resistance is both due to the existing properties of fungi and extensive use of these drugs (Janbon et al., 2019). Traditional drugs like, Amphotericin B and triazoles are prescribed for prolonged treatment. There is also a deficit in the generation of new drugs. Echinochandins are the only new class of antifungal developed in the last fifteen years. Investigations of naturally occurring antifungal strategies, like those presented in this dissertation, could provide insights into the improvement of antifungal treatments, either by finding new antifungal factors or by indicating fungal targets that could be studied further for the development of antagonists.

Could IM4 and IM14 be used as antifungal therapies? If further research into their mechanisms of function reveals direct antimicrobial activity, IM4 and IM14 could be candidate antifungals. Their small size and ability to function in the hemolymph of flies suggest that they could be developed for medical use.

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Appendix: Analysis of the role of IM4 and IM14 in pathogen load upon infection with fungal infections

Introduction

As shown in Chapter II, flies homozygous for *Δ(IM4, IM14),* also known as *Δ4-14,* have decreased survival compared to wild-type flies after infection with certain filamentous fungi. Survival upon infection can occur by two mechanisms: resistance and tolerance. Resistance refers to the ability to fight the pathogen directly by reducing the number or growth of pathogen, while tolerance refers to mitigating the impact of the pathogen on the health of the organism (Ayres and Schneider, 2012). A mutant that is deficient in resistance allows pathogen growth to exceed that in the wild-type organism. If a mutant is deficient in tolerance, the health of the organism will be more severely impacted by infection than wild type at the same pathogen load (Troha et al., 2018).

To evaluate the role of resistance during an infection, two metrics need to be measured and compared: health and pathogen level. Health can be evaluated based on a number of factors, including reproductive fitness, body mass, or survival. Experiments described in Chapter II used survival, as it is a clear indicator of whole body health and encompasses many elements that affect health. *Δ4-14* flies have decreased health, as determined by survival compared to wild-type flies after infection with particular filamentous fungi. Pathogen level can be defined in several ways (Ayres and Schneider, 2012). For infections in which pathogen number rises and falls in waves, it is useful to measure the total amount of pathogen throughout the infections. In contrast, when an infection leads directly to death of the organism the pathogen load compared across a set time point is most suitable.

There is now a consensus that tolerance can best be assayed by considering at the pathogen load upon death. Previous studies have shown that wild-type *Drosophila* die with

the same pathogen load for a given microbe, regardless of when death occurs (Duneau et al., 2017). Mutations that affect only resistance are expected to change the median survival time, but not the pathogen load at death. In contrast mutations that lower tolerance should result in death at a lower pathogen load (Troha et al., 2018).

In measuring pathogen load, one must evaluate the best method for the pathogen at hand. When measuring bacterial load in *Drosophila*, the most common method is counting colony forming units (CFU's) for a whole fly (Clemmons et al., 2015; Neyen et al., 2014; Troha and Buchon, 2019). Since each bacterial cell typically grows independently, this is an effective technique for acquiring a viable cell count. On the other hand, filamentous fungi grow as hyphae, which are structures that contain many cells growing in chains (Leslie and Summerell, 2007). Therefore, to accurately measure the number of fungal cells, a different approach is needed. Quantitative PCR (qPCR) of fungal DNA or RNA allows for assessment of filamentous fungal load (Dostálová et al., 2017). In the case of measuring fungal RNA, a reference gene needs to be chosen whose expression level does not change throughout the infection or in different growth conditions. Analysis of *Fusarium* genes found *EF1A* to be an appropriate reference gene to use as a proxy for fungal load (Kim and Yun, 2011; Lin et al., 2019).

Methods and Materials

Flies were raised on standard cornmeal agar media at 25°C. The w^{1118} strain was used as the wild type. $MyD88^{-}$ flies were $MyD88^{kra1}$ and *Δ4-14* flies were *Δ(IM4, IM14)*.

Adult male flies, 2-7 days old, were stabbed with a needle dipped in *F. verticillioides* at 3 x 10^9 spores/ml. Flies were then incubated at 29°C. Groups of 5-6 flies were collected at the stated times and frozen in liquid nitrogen. For fungal load upon death (FLUD), newly dead flies were collected within 30 mins of death. Total RNA was isolated with TRIzol (Ambion) and cDNA was made via SuperScript RT II (Invitrogen). EF1A was selected as a proxy gene for fungal load based on its stable expression (Kim and Yun, 2011). Measurements by qPCR were performed on the iQ5 cycler (BioRad) using iQ SYBR Green Supermix (BioRad) using the primers listed below. Values were normalized to fly mRNA based on expression of the rp49 gene.

Primers: Fv_EF1A_F1: GGCTTTCACTGACTACCCTCCTCT, Fv_EF1A_R1: ACTTCTCGACGGCCTTGATGACAC, rp49_F1: CAAGGGTATCGACAACAG, rp49_R1: CTTGTTCGATCCGTAACC.

Results

To determine if a decrease in resistance or tolerance was responsible for the decrease in survival of *Δ4-14* flies, I measured pathogen load after infection in *Δ4-14* flies, in Toll pathway deficient mutants (*MyD88*$^-$), and in wild-type flies. After stabbing adult males with *F. verticillioides*, groups of 5-6 infected flies were collected and RNA was extracted. Fungal *EF1A* transcript levels were measured and normalized to the fly reference gene *rp49*. At two hours and eight hours post infection, there was no significant difference between *Δ4-14*, *MyD88*$^-$ and wild-type flies by two-way ANOVA (Figure 3.1). However, *MyD88*$^-$ flies had

increased pathogen load at 24 hours post infection ($p<0.001$). *Δ4-14* flies also have a statistically insignificant increase in pathogen load ($p>0.05$).

To ask if IM4 and IM14 were promoting survival after infection by providing increasing tolerance to fungi, I measured the fungal load upon death (FLUD) in *Δ4-14* flies and MyD88- flies and compared to wild-type flies. After stabbing adult flies with F. verticilliodes, individual flies were collected within 30 minutes of death and RNA was extracted. Fungal RNA was measured by EF1A transcripts and normalized to a fly housekeeping gene rp49. I found that the fungal load upon death of *Δ4-14* flies was not lower than wild-type flies (Figure 3.2). Similarly, *MyD88*$^{-}$ flies had a higher FLUD than wild-type flies ($p<0.05$ by one-way ANOVA, Dunn's multiple comparisons). FLUD was in fact slightly higher for *Δ4-14* flies than for *MyD88*$^{-}$ flies, although the difference was statistically insignificant ($p>0.05$). From this experiment I conclude that *Δ4-14* flies did not have a defect in tolerance; if there is any difference, they appear to have a slightly increased tolerance of *F. verticilliodes* compared to wild-type flies.

Discussion

Analysis of fungal load by qPCR at set time points indicates that *Δ4-14* flies have a statistically insignificant increase in fungal load compared to wild-type flies (Figure 3.1). When using FLUD to investigate IM4 and IM14's role in tolerance of fungal pathogen, I found that *Δ4-14* flies did not have a defect in tolerance compared to wild-type flies (Figure 3.2). This finding suggests that IM4 and IM14 do not play a role in mediating files tolerance to fungal infections.

By asking how *MyD88*$^{-}$ flies perform in these experiments, I see a significant increase in fungal load at 24 h. This finding mimics previous studies that indicate that Toll deficient flies have a higher pathogen load than wild type, suggesting the Toll pathway acts in flies mainly by establishing resistance to pathogen (Clemmons et al., 2015). However, our FLUD analysis via qPCR of *MyD88*$^{-}$ flies does not agree with the pattern previously observed for bacterial load upon death (Duneau et al., 2017). Immunodeficient flies, like *Imd*$^{-}$ and *MyD88*$^{-}$ mutants, show no change in bacterial load upon death compared to wild type, whereas I report a significant increase in FLUD in *MyD88*$^{-}$ flies.

Since IM4 and IM14 are regulated by the Toll pathway (Levy et al., 2004; Uttenweiler-Joseph et al., 1998), I expected to find *Δ14-14* flies to show an increase in fungal load as seen in *MyD88*$^{-}$ flies, but to a lesser degree. Additionally, our FLUD measurement of *MyD88*- flies differs with previous data showing pathogen load upon death does not change in Toll-deficient flies.

Overall, a comparison of these results with previous studies indicates that this assay for fungal load could be improved. Since I am unable to directly count viable cells, as is done for bacterial load assays, I rely on the chosen gene, EF1A, to be an effective proxy for fungal load (Kim and Yun, 2011; Lin et al., 2019). However, the reference gene I selected - EF1A - was identified based on expression data from fungal growth in plants and in various *in vitro* conditions, not growth in *Drosophila*. A better assay might be developed with transcriptomic data specific to *Fusarium* infections in *Drosophila*. Even so, developing a qPCR assay based on DNA rather than RNA could circumvent these gene expression issues. This method might allow for more direct counting of nuclei.

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The appendix, in part, has been submitted for publication as it may appear in Frontiers in Immunology, 2019, Cohen LB, Lindsay SA, Xu Y, Lin SJH, Wasserman SA. IM4 and IM14 mediate *Drosophila* defense against a subset of filamentous fungi. The dissertation author was the primary investigator and author of this paper.

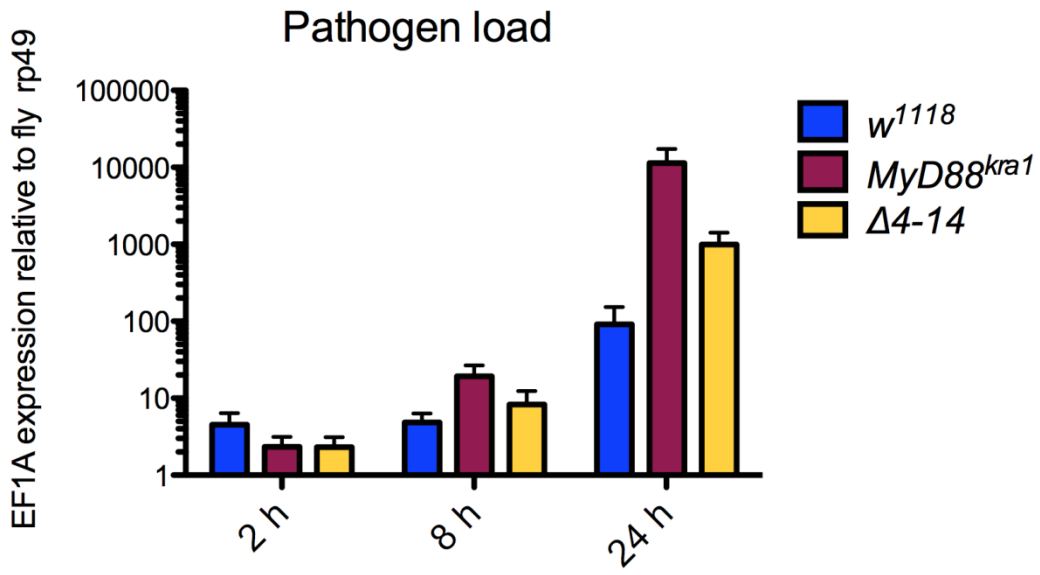

Figure 3.1: Pathogen load for *w*^{1118} and *Δ4-14*. Flies were collected at two, eight and twenty-four hours after infection with *F. verticillioides*. RNA was extracted from groups of 5-6 flies and fungal *EF1A* transcripts were measured relative to fly *rp49* transcripts. The *rp49* level was set to 10,000. Data are displayed on a log scale. Shown is an average of five independent experiments. Error bars represent standard error of the mean.

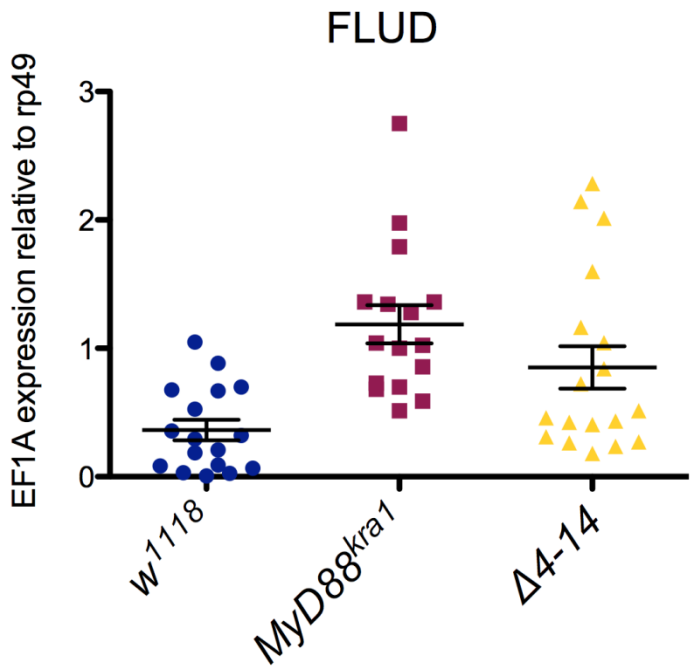

Figure 3.2: Fungal load upon death (FLUD) for w^{1118} and *Δ4-14*. Flies were collected individually within 30 minutes of death. RNA was extracted and fungal *EF1A* transcripts were measured relative to fly *rp49* transcripts. Points represent individual flies collect from three independent experiments.

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