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Conserved signaling mechanisms in the *D. melanogaster* cellular immune response

By

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Conserved signaling mechanisms in the *D. melanogaster* cellular immune response

By

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A.A., Oxford College of Emory University, 2011

Advisor: Todd A. Schlenke, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
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ABSTRACT

Conserved signaling mechanisms in the *D. melanogaster* cellular immune response
By Susanna E. Brantley

The model organism *Drosophila melanogaster* provides a system and excellent genetic tools for studying highly conserved innate immune responses. Numerous screens and mutant fly strains have implicated conserved signaling pathways, including Toll, JAK/Stat, and JNK, in the fly cellular immune response against parasitic wasps (Zettervall, 2004; Avet-Rochex, 2010; Irving, 2005; Braun, 1997). This study was conducted to better understand the tissue specific activity of Toll, JAK/Stat, and JNK signaling pathways in the differentiation of a specialized blood cell type, the lamellocyte, which is actively involved in encapsulation of wasp pathogens. The pro-immune activity of Toll, JAK/Stat, and JNK signaling in *Drosophila* indicates that conserved signaling pathways could be the targets of wasp virulence strategies. Many wasps are successful pathogens of *D. melanogaster*, but the mechanisms of wasp virulence, such as the action of wasp venom components, are not well studied. It is not known if or how wasps use virulence mechanisms specifically targeting conserved signaling pathways implicated in lamellocyte differentiation. This study will investigate the effect of parasitism by diverse wasp species on JAK/Stat activity in *D. melanogaster*. By better understanding the precise mechanisms of conserved signaling and the ways in which parasites affect the pathways involved in immune responses, we may gain insight into how signaling pathways implicated in mammalian development, immunity, and disease can be controlled and modulated.

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CHAPTER 1

Introduction

Innate immune mechanisms are highly conserved, evolutionarily ancient defenses against pathogens encoded by the germ line. All organisms have innate defenses to identify and fight off diverse pathogens, from foreign DNA to large parasites. In mammals, the innate immune response is the fast acting, first response to infection that is thought to initiate the slower but more specific adaptive immune response (Fearon and Locksley, 1996). Adaptive immunity is defined by highly specific, somatically recombined receptors and long lived memory cells (Brennan, 2004). In the past few decades, mechanisms of innate immunity have increasingly been shown to play equally important roles as classical adaptive immunity in mammalian responses to invading pathogens. Innate immunity has historically been studied in mammalian systems, but the inability to separate innate from adaptive immune responses in these models makes the study of innate immune mechanisms difficult. Many immune signaling pathways, defense strategies, and mechanisms of pathogen recognition are conserved across insect and vertebrate lineages. The genetic model organism *Drosophila melanogaster* provides excellent genetic tools for studying highly conserved innate immune responses. For example, the mammalian system for innate pathogen recognition via Toll-like receptors (TLRs) that activate Nfk-B signaling cascades was identified by homology to the *Drosophila* Toll signaling pathway responsible for fly humoral immunity (Valanne, 2011).

Drosophila utilizes both humoral and cellular innate immune responses to combat fungal, bacterial, and parasitic pathogens. The humoral response depends mainly on the production of antimicrobial peptides (AMPs) after bacterial or fungal infection (Lemaitre, 2007). AMPs are produced in the *Drosophila* "liver," the fat body, following infection

and are actively involved in eliminating invading pathogens. The fat body is the site of most signaling in the humoral immune response and involves the cooperation of the two Nfκ-B signaling pathways, Toll and Imd. Toll signaling is activated by Gram-positive bacteria and fungal pathogens via the ligand Spatzle, leading to production of the AMP, drosomycin. Gram-negative bacteria activate Imd signaling and diptericin production via peptidoglycan recognition proteins (PGRPs). Bacterial challenge also initiates activity of JAK/Stat and JNK signaling pathways, which play important roles in fly cellular immunity (Brennan, 2004; Lemaitre, 2007).

A healthy fly larva constitutively produces two hemocyte (blood cell) types – plasmatocytes and crystal cells – which circulate in the hemolymph (Figure 1.1). A population of plasmatocytes is specified in the embryo and is involved in phagocytosis of apoptotic cells during development. In the larva, preexisting embryonic plasmatocytes and newly differentiated plasmatocytes from the hematopoietic organ, the lymph gland, are responsible for phagocytosis of bacteria and also play a role in encapsulation of larger pathogens. Plasmatocytes are the most abundant cell type in the hemolymph, making up almost 95% of the hemocytes in circulation. Crystal cells, which make up approximately 5% of the circulating hemocytes in a *Drosophila* larva, contain prophenoloxidase crystals. These crystals are the cleavage substrate of a melanization cascade, producing melanin during wound healing (Crozatier, 2007; Meister, 2004; Meister, 2003).

Melanization also plays an important role in the specialized *Drosophila* immune response against parasitoid wasps. Diverse species of parasitoid wasps are natural pathogens of

Drosophila. These wasps inject their eggs along with venom into the hemocoel of fly larvae and pupae. If these eggs are not killed, they will hatch into wasp larvae, which begin to eat the flies from the inside out, eventually eclosing as adult wasps. However, upon attack, some *Drosophila* species can mount an immune response involving cellular encapsulation and melanization of the egg. In this response, plasmatocytes are the first hemocytes to bind to the wasp egg. This is followed by the binding of a specialized hemocyte called a lamellocyte and blackening of the capsule due to melanization (Carton, 1986; Carton, 2008; Schlenke, 2007).

Lamellocytes are not present in circulating hemolymph of a healthy fly larva, but are produced within the first 24 hours after parasitic wasp oviposition (Markus, 2009). Lamellocytes are large, flat cells that express integrin cell adhesion molecules as well as precursors required for melanization, characteristics that are necessary for melanotic encapsulation (Irving, 2005) (Figure I.1D). Lamellocytes differentiate in the lymph gland, as shown by expression of the lamellocyte-specific marker *misshapen* in the lymph gland in response to wasp attack (Evans, 2003). Lineage-tracing studies using plasmatocyte and lamellocyte specific markers, as well as studies showing that lamellocytes appear in circulation prior to observation of lamellocyte-specific cell markers in the lymph gland following wasp attack, confirm that lamellocytes also differentiate from preexisting plasmatocytes via an intermediate activated plasmatocyte type (a podocyte) (Markus, 2009; Stofanko, 2010). However, the mechanisms of lamellocyte differentiation are not well understood.

Numerous screens and mutant fly strains have implicated conserved signaling pathways, such as Toll, JAK/Stat, and JNK, in lamellocyte differentiation (Zettervall, 2004; Avet-Rochex, 2010; Irving, 2005; Braun, 1997). However, it is unclear how these pathways work synergistically to produce a lamellocyte and whether their actions differ in regard to lamellocyte origin. Specifically, it is not known if Toll, JAK/Stat, or JNK signaling is specific to particular tissues in the context of lamellocyte differentiation. This study will investigate the ability of Toll, JAK/Stat, and JNK signaling pathways to induce lamellocyte differentiation specifically via activity in the fat body, hemocytes, or the lymph gland. Additionally, this study will show in which tissues these signaling pathways may be required for encapsulation. Finally, this study will investigate the interactions between Toll, JAK/Stat, and JNK signaling in an effort to shed light on the precise order and mechanism of signaling in the context of lamellocyte differentiation.

Furthermore, the pro-immune activity of Toll, JAK/Stat, and JNK signaling in *Drosophila* indicates that conserved signaling pathways could be the targets of wasp virulence strategies. Many wasps are successful pathogens of *D. melanogaster*, but the mechanisms of wasp virulence, such as the action of wasp venom components, are not well studied. It is not known if or how wasps may use defense mechanisms specifically targeting conserved signaling pathways implicated in lamellocyte differentiation. This study will investigate the effect of parasitism by diverse wasp species on JAK/Stat activity in *D. melanogaster*. By better understanding the precise mechanisms of conserved signaling and the ways in which parasites affect the pathways involved in

immune responses, we may gain insight into how signaling pathways implicated in mammalian development, immunity, and disease can be controlled and modulated.

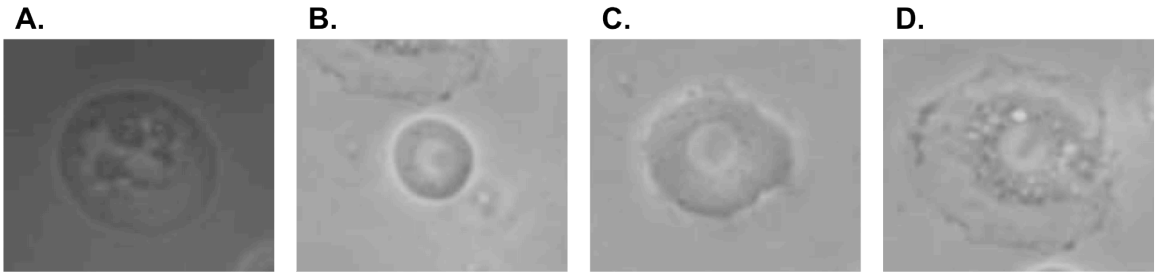

Figure 1.1. ***D. melanogaster*** **hemocytes.** (A) Crystal cell; (B) plasmatocyte; (C) podocyte; (D) lamellocyte

CHAPTER 2

The role of conserved signaling pathways in the *D. melanogaster* cellular immune response to parasitic wasps

Abstract

The *Drosophila* model system provides excellent tools for studying the mechanisms of conserved signaling pathways in the context of innate immunity. Activation of Toll, JAK/Stat, and JNK signaling in the fly leads to differentiation of a specialized blood cell type, the lamellocyte, which is actively involved in the fly's cellular immune response to parasitic wasp infection. Using the binary *Gal4-UAS* system and the tools of *Drosophila* genetics, we investigated the tissue specificity of Toll, JAK/Stat, and JNK signaling in lamellocyte differentiation. We found that Toll signaling in the lymph gland was necessary for successful encapsulation and induced lamellocyte differentiation. In contrast, JAK/Stat and JNK signaling play important roles in activation of preexisting hemocytes. Exploitation of the fly-wasp interaction combined with the tools of *Drosophila* genetics can provide valuable new information regarding conserved signaling pathways implicated in mammalian immunity, development, and disease.

Introduction

The innate immune response in *Drosophila* involves both humoral and cellular components. Using the fly as a model genetic system, much has been discovered about the conserved aspects of innate immune responses. For example, we now understand the contribution of NFk-B signaling via Toll-like receptors (TLRs) to mammalian innate immunity because of work on Toll signaling in *Drosophila* (Valanne, 2011). Toll signaling is generally associated with the humoral response in the fly. Toll activation by Gram-positive bacterial or fungal pathogens leads to the production of antimicrobial peptides (AMPs) that can actively kill invading pathogens (Lemaitre, 2007).

In addition to its role in humoral immunity, Toll signaling may also play an important role in the cellular immune response. Gain of function mutations in Toll result in overproliferation of blood cells and constitutively high numbers of the specialized lamellocyte blood cell type. $Toll^{10B}$, a specific gain of function mutant, displays a temperature sensitive melanotic tumor phenotype due to the aggregation of excess lamellocytes in circulation (Lemaitre, 1995). This phenotype has been observed in other mutants and used in various screens to identify other genes that, when upregulated, may be involved in lamellocyte differentiation. Specifically, components of Toll, JAK/Stat, and JNK signaling have been implicated in blood cell proliferation and melanotic tumor formation (Luo, 1995; Zetterval, 2007). However, the details of how these signaling pathways are involved in the mechanism of lamellocyte differentiation are poorly understood. Using the tools of *Drosophila* genetics, this study will investigate the tissue

specificity of Toll, JAK/Stat, and JNK signaling pathways in the cellular immune response to parasitic wasps.

Materials and Methods

Insect Species

Flies were maintained on standard medium of cornmeal, yeast, and molasses. The Gal4-UAS binary system was used to overexpress candidate genes using the following genotypes obtained from Bloomington Stock Center (Bloomington, IN): *UAS-Dif, UAS-dl, UAS-lwr, UAS-hop, UAS-Socs36e*^{EP}*, UAS-Rac1, UAS-Src64b, UAS-Pak,* and *UAS-hep*. The following genotypes were used for knockdown experiments: *UAS-Dif*^{TRiP}, *UAS-dl*^{TRiP}*, UAS-lwr*^{DN}*/TM3Ser-actGFP, UAS-Rac1*^{TRiP}*, UAS-Src64b*^{TRiP}*, UAS-dPIAS*^{TRiP}*, UAS-Pak*^{TRiP}*, UAS-puc*^{TRiP} and *UAS-Bsk*^{DN}. Tissue specific drivers used were *He-Gal4* (blood cells), *C833-Gal4* (fat body), and *Dot-Gal4* (lymph gland). The Gal4 driver outcrossed to w^{1118} was used as a control for all experiments.

Leptopilina clavipes (LcNet) wasps were maintained on *D. virilis* hosts as described in Kacsoh and Schlenke (PLoS One, 2012). *L. clavipes* is generally avirulent (i.e. is encapsulated) in wild-type *D. melanogaster* strains.

Hemocyte Counts

Crosses were carried out at 18°C, as many of the overexpression constructs were lethal at 25°C. This temperature may have resulted in less pronounced phenotypes, as the Gal4-

UAS system works best at a higher temperature. However, for consistency, all crosses were done at the same temperature, including controls. Five to ten female virgin Gal4 drivers were mated with 3-5 UAS construct or wild type males in vials. Crosses were performed in triplicate. From each cross, 15 relatively similar sized third instar larvae were washed in *Drosophila* Ringer's solution and dried on a kimwipe. Three sets of 5 larvae were bled into 20μL of 1X Fly PBS with 0.01% PTU (to prevent blood cell lysis and melanization). Hemocyte dilutions were transferred to a disposable hemocytometer (Incyto C-Chip DHC-N01). Hemocytes from sixteen 0.25 x 0.25 x 0.1 mm hemocytometer squares were counted for each replicate. Three hemocyte types were identified: plasmatocytes (small, dark cells), podocytes (larger, rounder cells with cytoplasmic projections), and lamellocytes (large, flat cells with cytoplasmic ring).

Encapsulation Rate

All experiments were carried out at 25°C. Approximately 30 virgin female Gal4 flies were mated overnight with 10 UAS or wild type males. These crosses were transferred to egg lay chambers containing molasses plates supplemented with yeast paste (water, baker's yeast, and melon extract) and allowed to lay for 24 hours. Egg lay plates were then incubated for 48 hours. Thirty early third instar larvae were picked from egg lay plates and transferred to regular food in 35mm diameter Petri dishes. Three replicate plates were set up for both control and UAS crosses. Three female LcNet wasps were allowed to attack larvae for 3 days. Following wasp attack, wasps were removed and the attacked and control (unattacked) larvae were dissected for observation of melanotically

encapsulated wasp eggs. A successful encapsulation was scored as a dark, black capsule with a clear outer layer of cells (Figure 2.3).

Results

While many studies have screened for the ability of Toll, JAK/Stat, and JNK pathway genes to affect blood cell numbers and lamellocyte differentiation, this is the first study to investigate the tissue specificity of pathway activity. We chose three classes of genes from each signaling pathway to investigate: upstream components, signaling components, such as kinases and transcription factors, and downstream negative regulators that participate in feedback loops (Table 2.1).

Table 2.1. Candidate genes

Candidate Gene	Pathway	Details	Overexpression Line	Knockdown Line
Dorsal	Toll	GOF causes melanotic tumor formation [Evans, 2003].	UAS-dl	dl-RNAi
Dif	Toll	Dimerizes with dorsal in Toll signaling pathway. Possibly redundant with dorsal or relish [Lemaitre,2007; Qiu, 1998].	UAS-Dif	Dif-RNAi
Lwr	Toll	Mutation causes hyperproliferation of hemocytes. Interacts with Toll transcription factors [Huang, 2005].	UAS-lwr	Lwr-DN (dominant neg)
Hopscotch	JAK/Stat	GOF induces lamellocyte differentiation and melanotic tumor formation [Luo, 1995].	UAS-hop hopTum-1 (GOF mutant)	Hop-RNAi
dPIAS	JAK/Stat	Downstream negative regulator of JAK/Stat activity [Betz, 2001].	UAS-dPIAS	dPIAS-RNAi
Socs36e	JAK/Stat	Regulated by JAK/Stat activity [Agaisse, 2004].	UAS-Socs36e	
Src64B	JAK/Stat JNK	Downstream effector of JAK/Stat activity. GOF induces lamellocyte differentiation. Regulates JNK signaling in development [Williams, 2009].	UAS-Src64B	Src64B-RNAi
Rac1	JNK	Overexpression induces lamellocyte differentiation. Upstream of JNK signaling [Zettervall, 2004; Williams, 2006].	UAS-Rac1	Rac1-RNAi
Pak	JNK	Upstream of JNK signaling in mammalian system [Rudel, 1998]	UAS-Pak	Pak-RANi
Hemipterous	JNK	Overexpression induces lamellocyte differentiation. Upstream of JNK signaling [2].	UAS-hep	Hep-RNAi
Basket	JNK	Overexpression of JNK signaling induces lamellocyte proliferation [Zettervall, 2004].		Bsk-DN
Puckered	JNK	Negative regulator of JNK signaling [Martin-Blanco, 1998].	UAS-puc	puc-RNAi

We first looked at overactivation of Toll, JAK/Stat, and JNK signaling in hemocytes, lymph gland, and fat body by overexpressing genes that promote signaling or knocking down genes that contribute to negative feedback loops (Table 2.2). We first ectopically expressed Toll, JAK/Stat, and JNK pathway genes using hemocyte-specific *He-Gal4*. We found that hemocyte-specific activity of JAK/Stat and JNK pathway genes led to increased lamellocyte and podocyte populations at the expense of plasmatocytes (Table 2.2A; Figure 2.1), while hemocyte-specific expression of Toll pathway genes had no effect. Next, we ectopically expressed pathway genes using the lymph gland-specific *Dot-Gal4*. Using this driver, Toll pathway genes *Dif* and *dl* induced higher lamellocyte numbers and decreased circulating plasmatocytes (Table 2.2B). However, this trend was not observed for JAK/Stat or JNK pathway genes. Ectopic expression of *Dif*, JAK/Stat, and JNK signaling in the fat body using *C833-Gal4* led to increases in lamellocyte numbers, but had no effect on other hemocyte populations (Table 2.2C). While expression of *Src64b* and *hep* was lethal, a few second instar larvae survived *He-Gal4>UAS-hep* and their blood cells imaged. High levels of lamellocytes were observed in circulation, but we could not recover enough larvae to quantify hemocyte numbers (Figure 2.1).

Table 2.2. Overexpression of Toll, JAK/Stat, and JNK signaling pathway components alters hemocyte composition in a tissue specific manner. The binary Gal4-UAS system was used to activate Toll, JAK/Stat, and JNK signaling in (A) hemocytes, (B) lymph gland, or (C) fat body using tissues specific drivers (*He-Gal4, Dot-Gal4,* and *C833-Gal4*, respectively). Note that overexpression of *Dot-Gal4>UAS-Src64b, C833-Gal4>UAS-Src64b, He-Gal4>UAS-hep, Dot-Gal4>UAS-hep,* and *C833-Gal4>UAS-hep* crosses were lethal. Red expression profile indicates a decrease in blood cell numbers/percent in comparison to average control by student's t-test; green indicates an increase in comparison to control ($p<0.05$). Gray indicates no significant change compared to control. Each data point represents the average of three replicates.

A.

	Plasmatocytes (Cell count)	Podocytes (Cell count)	Lamellocytes (Cell count)	Total Cells (Cell count)	Plasmatocytes (Percent)	Podocytes (Percent)	Lamellocytes (Percent)
UAS-Dif							
UAS-dl							
UAS-lwr^{RNAi}							
UAS-hop							
UAS-dPIAS^{RNAi}							
UAS-Socs36e							
UAS-Src64B							
UAS-Rac1							
UAS-Pak							
UAS-hep	lethal	lethal	lethal	lethal	lethal	lethal	lethal
UAS-puc^{RNAi}							

B.

	Plasmatocytes (Cell count)	Podocytes (Cell count)	Lamellocytes (Cell count)	Total Cells (Cell count)	Plasmatocytes (Percent)	Podocytes (Percent)	Lamellocytes (Percent)
UAS-Dif							
UAS-dl							
UAS-lwr^{RNAi}							
UAS-hop							
UAS-dPIAS^{RNAi}							
UAS-Socs36e							
UAS-Src64B	lethal	lethal	lethal	lethal	lethal	lethal	lethal
UAS-Rac1							
UAS-Pak							
UAS-hep	lethal	lethal	lethal	lethal	lethal	lethal	lethal
UAS-puc^{RNAi}							

C.

	Plasmatocytes (Cell count)	Podocytes (Cell count)	Lamellocytes (Cell count)	Total Cells (Cell count)	Plasmatocytes (Percent)	Podocytes (Percent)	Lamellocytes (Percent)
UAS-Dif							
UAS-dl							
UAS-lwr^{RNAi}							
UAS-hop							
UAS-dPIAS^{RNAi}							
UAS-Socs36e							
UAS-Src64B	lethal	lethal	lethal	lethal	lethal	lethal	lethal
UAS-Rac1							
UAS-Pak							
UAS-hep	lethal	lethal	lethal	lethal	lethal	lethal	lethal
UAS-puc^{RNAi}							

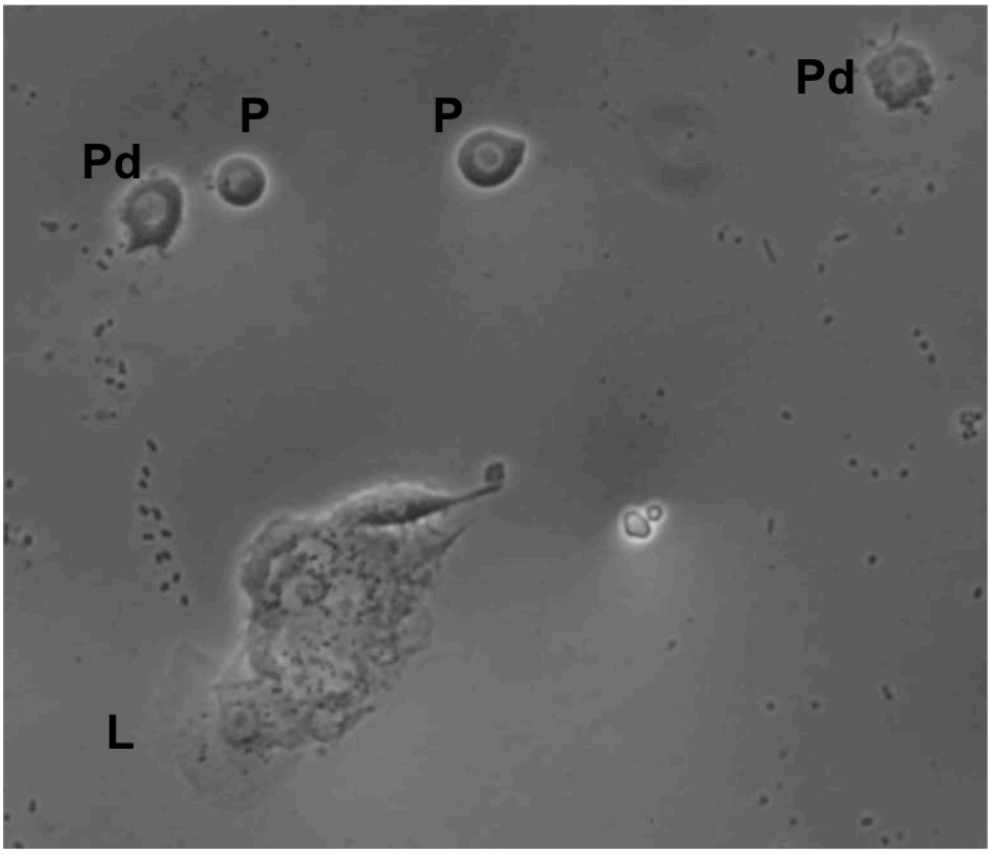

Figure 2.1. *He-Gal4>UAS-hep* leads to lamellocyte differentiation. Three hemocyte types were found in circulation when *hep* is overexpressed in blood cells: plasmatocytes (P), podocytes (Pd), and lamellocytes (L).

Overactivation of Toll, JAK/Stat, and JNK signaling pathways in a tissue specific manner shows that pathway activity is sufficient for lamellocyte differentiation. Based on these data, we wanted to investigate whether tissue-specific pathway activation was also necessary for a successful wasp encapsulation response. To test this, we decreased activity of Toll, JAK/Stat, or JNK signaling using RNAi, dominant negative mutants, or overexpression of negative regulators in blood cells or hemocytes. While these experiments are still ongoing, the current data set is shown in Figure 2.2. Of those genes tested to date, none were shown to be necessary in blood cells, as all flies exhibited wild type encapsulation of the avirulent wasp *L. clavipes* (LcNet), that is usually encapsulated by *D. melanogaster* (Figure 2.2A). However, we also knocked down the Toll pathway transcription factors *Dif* and *dl* in the lymph gland and found that they were necessary for proper melanotic encapsulation LcNet (Figure 2.2B). A wild type melanotic encapsulation (Figure 2.3A) is very dark and has an outer layer of lamellocyte cells. When *Dif* and *dl* were knocked down in lymph glands, we observed more live wasps, many broken capsules, and most capsules were defective with lighter melanization and no cells bound (Figure 2.3B).

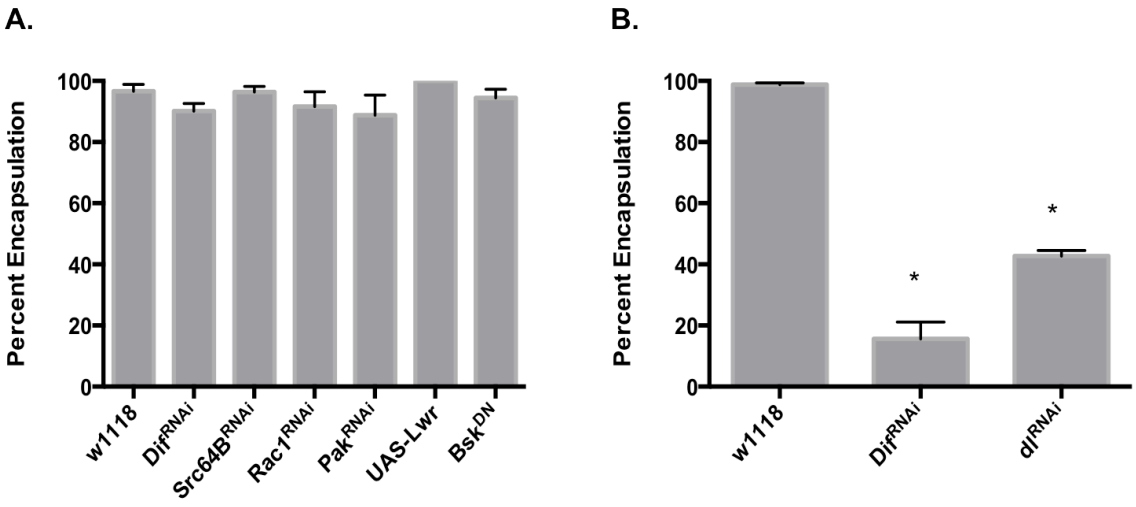

Figure 2.2. Effect of knockdown on encapsulation rate of LcNet. Genes that showed a significant impact on lamellocyte differentiation were investigated for their effect on melanotic encapsulation. Pathway activity was knocked down in hemocytes (A) or lymph gland (B) using tissue specific drivers (*He-Gal4* and *Dot-Gal4*, respectively). Each data point represents the average of three replicates. (*) indicates significance under the Holm-Sidak method for multiple comparisons, $p<0.0002$.

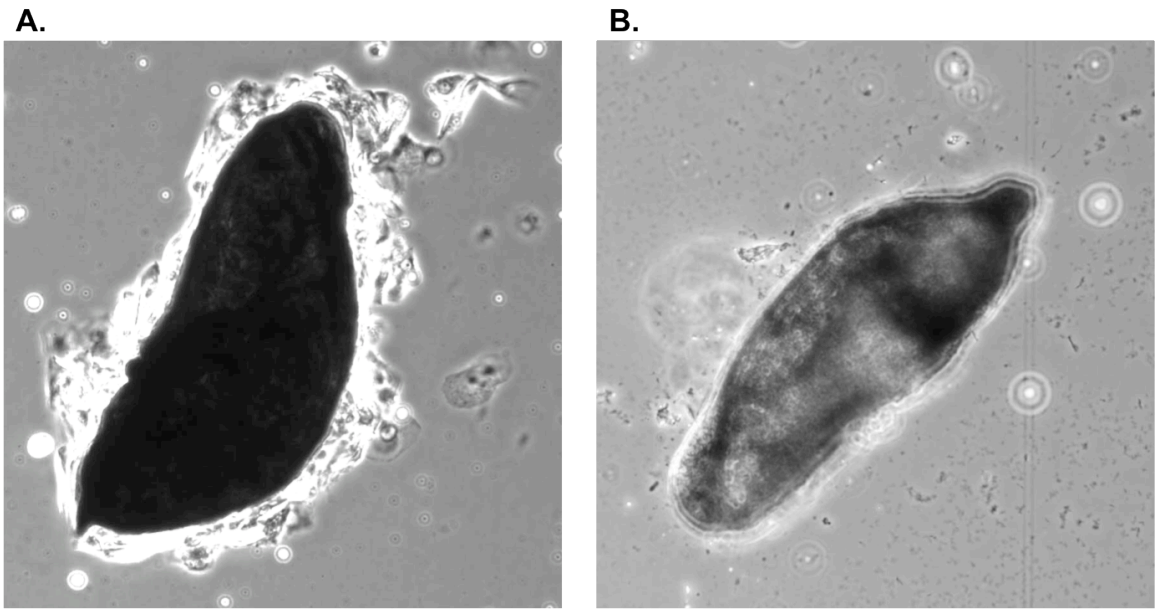

Figure 2.3. Defective capsule. Wild type melanotic encapsulation (A) produces a dark capsule with an outer layer of blood cells. Defective, lighter, patchy capsules with no blood cells bound (B) were recovered from *Dot-Gal4>Dif*^{RNAi} and *Dot-Gal4>dl*^{RNAi}.

Discussion

This study investigated the tissue specificity of conserved signaling pathways previously implicated in lamellocyte differentiation. Our data suggest that Toll signaling in the lymph gland is necessary and sufficient for lamellocyte differentiation. One report (Qiu, 1998) has shown that *cactus*, a transcription factor in the Toll signaling pathway, is expressed in the lymph gland of flies with Toll pathway gain of function mutations. However, this is the first study to show that Toll signaling in the lymph gland is necessary for melanotic encapsulation of parasitic wasps. Hemocyte counts of infected *Dif* and *dl* knockdown larvae should be conducted to determine whether blood cells are not being made or because the blood cells that are produced are ineffective.

Additionally, our data show that JAK/Stat and JNK signaling in hemocytes are each sufficient to induce lamellocyte differentiation. However, these pathways may be redundant, as knocking down activity of either pathway independently had no significant effect on melanotic encapsulation. Perhaps if one pathway is inhibited, the other can still become activated to produce enough lamellocytes for a successful encapsulation response. The result may also be due to the incomplete power of RNAi to inhibit gene expression, especially because the blood cell driver used in this study, *He-Gal4* (Zettervall, 2004), only expresses in approximately 80% of circulating hemocytes. Finally, inhibition of signaling in preexisting blood cells may in fact decrease the number of lamellocytes that differentiate from plasmatocytes, but production of new lamellocytes in the lymph gland after wasp attack may be sufficient for encapsulation. Perhaps the

lymph gland population is more important for the encapsulation of the wasp than the "first responders" that originate in the hemolymph (Markus, 2009; Stofanko, 2010). Assaying encapsulation at earlier time points would show whether wild type flies have cells binding around wasp eggs more quickly than flies with lower JAK/Stat and JNK signaling in preexisting hemocytes.

In conclusion, the system of lamellocyte differentiation in response to wasp attack provides an excellent system for understanding how signaling pathways act synergistically across multiple tissues. Exploitation of this system could provide novel insights regarding conserved signaling pathways implicated in mammalian immunity, development, and disease.

CHAPTER 3

The interaction of parasitic wasps with JAK/Stat pathway activity in the *D. melanogaster* cellular immune response

Abstract

The JAK/Stat signaling pathway is a conserved pathway implicated in mammalian leukemias and lymphomas (Chen, 2012). A model of hyperproliferation of blood cells exists in the model system *Drosophila*. Constitutively active JAK/Stat signaling results in overproduction of a specialized blood cell, the lamellocyte, which is involved in encapsulation of parasitic wasp eggs. Here we show that Stat activity is required for successful wasp encapsulation. Diverse wasp species induce Stat activity in hemocytes (blood cells) and the fat body in varying degrees and patterns. However, using a gain of function mutant for the *Drosophila* JAK, *hopscotch*, we show that two wasp species (*Leptopilina guineaensis* and *Ganaspis xanthropoda*) can effectively reduce a tumor phenotype caused by the presence of excess lamellocytes. These data suggest that wasps can target JAK/Stat activity as part of their virulence strategies. Exploitation of the fly-wasp system and further investigation of wasp venom and virulence strategies may provide novel information regarding ways in which a conserved pathway implicated in human cancers can be modulated or suppressed.

Introduction

Since its discovery just over twenty years ago, the JAK (Janus kinase)/Stat (signal transducers and activators of transcription) pathway has been shown to play an essential role in mammalian hematopoiesis (Stark, 2012). There are four JAKs and seven Stats in the human system. *Drosophila* has one JAK, (*hopscotch*) and one Stat (*Stat92E*), a much simpler system with similar functionality and sequence conservation to the human JAK/Stat pathway (Agaisse, 2004). Dominant, gain of function *hopscotch* alleles have been associated with hemocyte (blood cell) hyperproliferation and premature differentiation. For example, the mutant fly strain $hop^{Tum\text{-}l}$ constitutively produces more hemocytes than wild type flies, and at higher temperatures, $hop^{Tum\text{-}l}$ hemocytes differentiate into a specialized blood cell type called a lamellocyte (Luo, 1995).

In wild type flies, lamellocyte production is induced to encapsulate pathogens too large to be phagocytosed by the constitutive plasmatocyte blood cell population. For example, oviposition by parasitoid wasps stimulates differentiation of the large, adhesive lamellocytes to form a capsule around the invading wasp egg. Melanin and reactive oxygen species (ROS) are released within the capsule to suffocate and kill the pathogen (Carton, 2008). However, some wasp species employ poorly understood virulence strategies to overcome the fly immune response. Two studies (Govind, 2002; Schlenke, 2007) have investigated the ability of one wasp species, *Leptopilina heterotoma* (Lh14), to suppress the *D. melanogaster* cellular immune response. Lh14 venom contains some factor with the ability to lyse $hop^{Tum\text{-}l}$ derived lamellocytes *in vitro*. Furthermore, Lh14

wasps survive to eclosion even in $hop^{Tum\text{-}l}$ larvae. These data suggest that some wasp virulence strategies may depend on inhibiting the production or action of lamellocytes.

Because activity of the JAK/Stat pathway is sufficient for lamellocyte differentiation, we hypothesized that regulators, signaling components, and downstream targets of JAK/Stat activity may be targets of wasp virulence strategies. Here I show that JAK/Stat activity is necessary and sufficient in blood cells for successful melanotic capsule formation. I also characterize the role of JAK/Stat signaling in the cellular immune response against diverse species of parasitic wasps in an effort to better understand how wasp virulence strategies may affect a conserved signaling pathway.

Materials and Methods

Insect Species

Flies were maintained on standard medium of cornmeal, yeast, and molasses. The {PZ}$Stat92E^{06346}$, Hemese-Gal4, C833-Gal4, Dot-Gal4, and $hop^{Tum\text{-}l}$ strains were obtained from Bloomington Stock Center (Bloomington, IN). The Gal4-UAS binary system was used to overexpress activated Stat92E using a transgenic line constructed in Erika Bach's lab (New York University, New York). The JAK/Stat reporter strain (10xSTAT-GFP) was constructed by Erika Bach and can be requested from Bloomington Stock Center (Bach, 2007).

Wasp strains were obtained and maintained as described in Kacsoh and Schlenke (PLoS

One, 2012). Briefly, all wasps were maintained on the *D. melanogaster* strain Canton S, with the exception of *L. clavipes* (strains LcNet and LcAtl), which was maintained on *D. virilis*. Notably, strain LbG486 was provided by D. Hultmark, LcNet was provided by J. van Alphen, strain GxUg was provided by J. Pool. All other wasp strains were collected by the Schlenke lab.

Hemocyte Counts

All crosses and experiments were carried out at 25° C with the exception of experiments carried out with $hop^{Tum\text{-}l}$ temperature sensitive mutants, which were incubated at 28° C for up to 72 hours after a 24-hour wasp attack at 25° C. For $hop^{Tum\text{-}l}$ experiments, flies were allowed to lay eggs on molasses plates supplemented with yeast paste (water, baker's yeast, and melon extract) for 24 hours at 25° C. Egg lay plates were then placed at 28° C and incubated for 48 hours. Thirty early third instar larvae were picked from egg lay plates and transferred to regular food in 35mm diameter Petri dishes. This was done in triplicate for two groups: attacked and unattacked larvae. For attacked plates, three female wasps were allowed to attack for 24 hours at 25° C. Following wasp attack, wasps were removed and the attacked and control (unattacked) larvae were allowed to incubate at 28° C for up to 72 hours. Blood cells were counted at 24 and 48 hours after being moved to 28° C. For Stat overexpression blood cell counts, crosses between Gal4 virgin females and UAS-Stat92E or w^{1118} (wild type control) males were done at 25° C. Crosses were performed in triplicate. Wandering third instar larvae were selected for hemocyte counts.

From each cross, 15 size-matched third instar larvae were washed in *Drosophila* Ringer's solution and dried on a kimwipe. Three sets of 5 larvae were bled into 20 μL of 1X Fly PBS with 0.01% PTU (to prevent blood cell lysis and melanization). Hemocyte dilution was transferred to a disposable hemocytometer (Incyto C-Chip DHC-N01). Sixteen 0.25 x 0.25 x 0.1 mm squares were counted for each replicate. Three hemocyte types were identified: plasmatocytes (small, dark cells), podocytes (larger, rounder cells with cytoplasmic projections), and lamellocytes (large, flat cells with cytoplasmic ring).

Imaging

Wasp attacks of Stat-GFP reporter flies were carried out as described above with the exception that the entirety of the experiment was done at 25° C.

Stat-GFP imaging was done at 24 and 48 hours after wasp attack. Five larvae were dissected under immersion oil from each of three replicate plates. For quantification of Stat-GFP corrected total cell fluorescence, 20 blood cells from each of the 5 larvae were analyzed for fluorescence in comparison to background using ImageJ software.

$Hop^{Tum\text{-}l}$ larvae were imaged as pupae after 72 hours of incubation at 28° C after wasp attack to allow adequate time at higher temperature for development of the tumor phenotype. Penetrance was determined by grouping larvae into two groups: tumors or no tumors. ImageJ software was used to quantify tumor area (data not shown).

Statistics

Student's t-tests and linear regressions comparing penetrance and hemocyte data were

conducted using GraphPad Prism 6.0 software.

Results

To show that JAK/Stat activity is necessary for successful melanotic capsule formation, we used a genetic mutant, $Stat^{06346}$, containing a P-element insertion in the coding region of *Stat92E*. Because $Stat^{06346}$ is a lethal allele, we compared a heterozygous outcrossed strain to wild type background flies. Mutant and wild type flies were parasitized by an avirulent wasp species, *L. clavipes* (LcNet). LcNet is generally encapsulated by wild type *D. melanogaster*, and genetic background controls flies encapsulated close to 100% of wasp eggs. However, *Stat92E* mutant flies showed a significantly lower ability to encapsulate LcNet (Figure 3.1A).

We wanted to investigate the tissue specificity of JAK/Stat activity in the encapsulation response. Overexpression of activated Stat92E ($Stat^{CA}$) by a hemocyte- specific Gal4 driver, Hemese-Gal4, induced differentiation of lamellocytes from plasmatocytes. However, overexpression of activated Stat92E by fat body specific driver C833-Gal4 promoted lamellocyte differentiation without significant changes to other hemocyte populations. Finally, the lymph gland specific driver Dot-Gal4 did not induce any constitutive lamellocyte differentiation (Figure 3.1B).

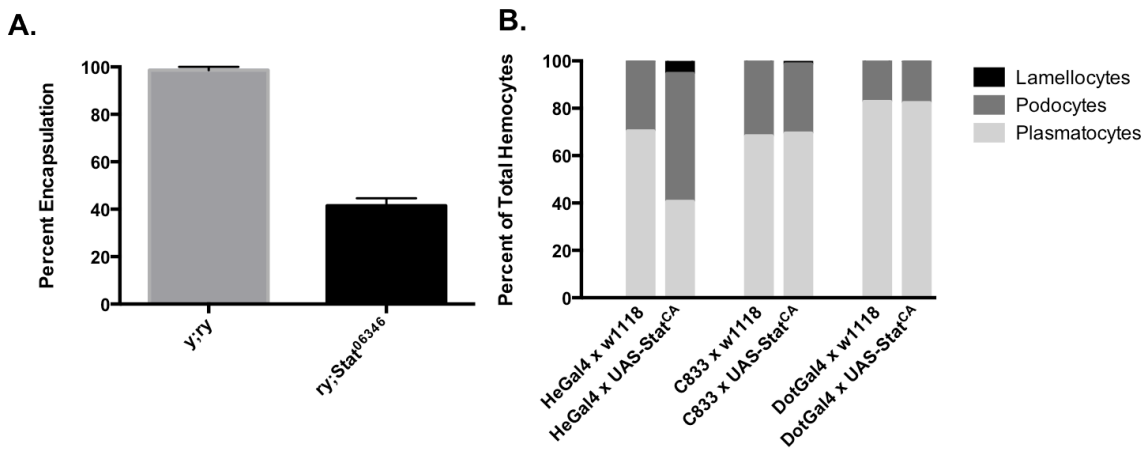

Figure 3.1. JAK/Stat activity is required for melanotic encapsulation. Heterozygous $Stat^{06346}$ loss of function mutant decreases encapsulation rate of avirulent wasp, LcNet, in comparison to control (y;ry) (A). Plasmatocyte, podocyte, and lamellocyte populations changed significantly in $Stat^{CA}$ expression by HeGal4 (blood cell driver) ($p<0.05$); lamellocyte population changed significantly in StatA expression driven by C833 (fat body driver) ($p<0.002$) (B).

To characterize JAK/Stat activity in the context of a wasp attack, we challenged Stat92E GFP reporter flies (Bach, 2007) with a panel of diverse wasp species. In unattacked reporter flies, no expression is observed in immune tissues (hemocytes, fat body, and lymph gland) (Figure 3.2A). However, challenge with the avirulent wasp, LcNet, a wasp that induces a healthy immune response and is usually encapsulated by *D. melanogaster,* altered this expression pattern. At 48 hours following wasp attack, the Stat activity was detected in hemocytes and fat body (Figure 3.2B). Immune challenge by a diverse panel of wasp species induced wasp-specific Stat expression patterns (Figure 3.2; Table 3.1). Quantification of GFP fluorescence in hemocytes 24h (Figure 3.2C) and 48h (Figure 3.2D) after wasp attack showed that virulent wasps (those that normally eclose as adult wasps in *D. melanogaster* hosts) inhibit JAK/Stat activity in comparison to *L. clavipes* (LcNet and LcAtl), an avirulent wasp species that is generally encapsulated by *D. melanogaster*. Fat body expression was qualitatively assessed at 24 and 48 hours following wasp attack. Avirulent wasps LcNet and LcAtl induced high levels of Stat activity in comparison to other wasp species. The virulent wasp LgCam also induced high levels of Stat activity in the fat body, but the closely related strain, LgSA, induced no activity (Table 3.1).

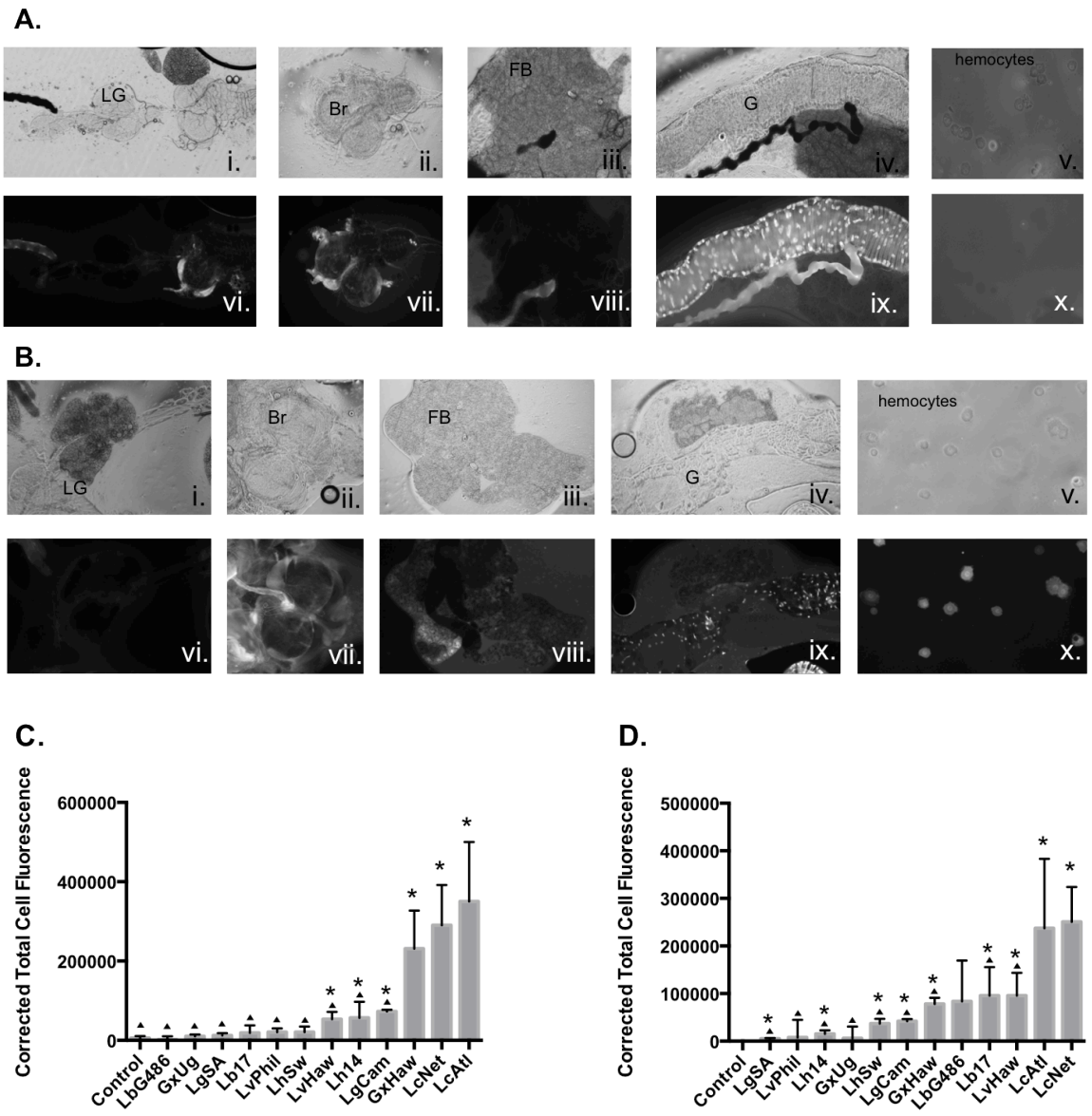

Figure 3.2. Stat activity following wasp attack. Unattacked control (A) tissues (lymph gland (LG), brain (Br), fat body (FB), gut (G), and hemocytes) and LcNet attack (B) 48 hours following wasp attack. Top panels brightfield; bottom panels FITC channel. 10XStatGFP activity in hemocytes at 24 (C) and 48 (D) hours following wasp attack. Each data point represents average corrected total cell fluorescence for 180 blood cells ($p<0.03$ for C; $p<0.05$ for D). Stat activity in hemocytes quantified and corrected for

background in ImageJ. Significance versus control (*) and versus LcNet (▲) determined by student's t-test.

Table 3.1. 10XStatGFP activity in fat body following wasp attack.

(+) indicates estimated observed fluorescence intensity.

	24h	48h
Unattacked	None	None
Lb17	+	+
LbG486	++	++
Lh14	None	None
LhSw	None	None
LgCam	++++	++++
LgSA	None	None
LvPhil	None	+
LvHaw	None	+
LcNet	+++	+++
LcAtl	+++	+++
GxUg	None	None
GxHaw	None	None

Our results show that JAK/Stat signaling is required for the melanotic encapsulation response and may be modulated by wasp virulence strategies. Next, we wanted to test whether wasps can target lamellocyte production downstream of JAK/Stat activation. To do this, we used the *hopscotch* gain of function mutant fly strain, $hop^{Tum\text{-}l}$. At high temperatures, these flies produce constitutively high numbers of lamellocytes. These adhesive cells have no target for encapsulation and often bind to each other, resulting in melanized cell nodules that are often referred to as "melanotic tumors." We attacked $hop^{Tum\text{-}l}$ flies with a panel of wasps and observed the effect of wasp attack on penetrance of the tumor phenotype. Two wasp species, *L. guineaensis* (LgCam) and *Ganaspis xanthropoda* (GxHaw), were able to significantly reduce the penetrance of the tumor phenotype (Figure 3.3).

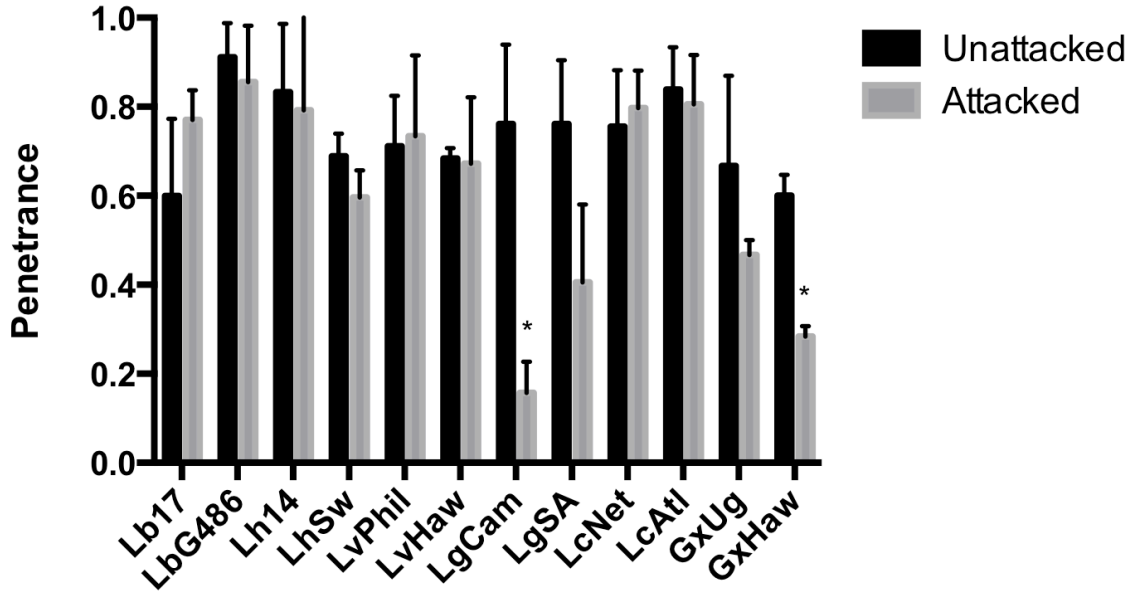

Figure 3.3. Wasp virulence strategies target lamellocytes downstream of JAK activation. Penetrance of $hop^{Tum\text{-}l}$ tumor phenotype observed following wasp attack. Each data point represents the average penetrance of three replicates of thirty observed pupae. Significance determine by student's t-test versus daily control (black bars) (* indicates $p<0.02$).

We measured hemocyte numbers at 24 and 48 hours following wasp attack to predict how wasp virulence strategies affect circulating hemocytes in hosts. At 24 hours following attack, lamellocytes have been produced and plasmatocytes surround the wasp egg, beginning the process of encapsulation. By 48 hours, lamellocytes are incorporated into the capsule, and melanization begins (Mortimer, 2012). At 24 hours following wasp attack, the avirulent wasp LcAtl induces higher numbers of circulating lamellocytes in host larvae (Table 3.2A). A higher percent of circulating lamellocytes is also observed in host larvae at 48 hours after LcNet attack, indicative of a successful encapsulation response (Table 3.2B). In contrast, Lh14 decreases lamellocyte levels at 24 hours following wasp attack (Table 3.2A), and by 48 hours Lh14-infected hosts show no significant differences in hemocyte numbers in comparison to unattacked flies (Table 3.2B). This is consistent with literature (Schlenke, 2007) that describes Lh14 as an immune-suppressive wasp, or one that fights the immune system, in this case by killing blood cells. In contrast, Lb17 is an immune-evasive wasp that can hide from the fly's immune response, presumably by attaching to host tissue (Schlenke, 2007). At 48 hours after wasp attack, Lb17-infected larvae mount an immune response and increase the number of lamellocytes in circulation, much like LcNet (Table 3.2B).

To look more closely at the possible mechanisms of wasp virulence, we focused on the hemocyte data for wasp strains that significantly reduced tumor phenotype penetrance. At 24 hours after wasp attack, LgCam-infected flies produced a significantly higher percentage of circulating plasmatocytes and lower percentage of podocytes than controls (Table 3.2A). A closely related wasp, LgSA, also decreased the percentage of circulation

podocytes at 24 hours after wasp attack (Table 3.2A). While GxHaw did not seem to have any significant effect on hemocyte numbers, closely related GxUg significantly reduced the percent of circulating lamellocytes 48 hours after attack (Table 3.2B).

Table 3.2. Wasp attack induces changes in circulating host hemocytes. Hemocytes were counted at (A) 24 and (B) 48 hours after wasp attack. Red indicates a decrease in blood cell numbers/percent in comparison to average control by student's t-test; green indicates an increase in comparison to control ($p<0.05$). Gray indicates no significant change compared to control. Each data point represents the average of three replicates.

A.

	Plasmatocytes (Cell count)	Podocytes (Cell count)	Lamellocytes (Cell count)	Total Cells (Cell count)	Plasmatocytes (Percent)	Podocytes (Percent)	Lamellocytes (Percent)
Lb17							
LbG486							
Lh14							
LhSw							
LvPhil							
LvHaw							
LgCam							
LgSA							
LcNet							
LcAtl							
GxUg							
GxHaw							

B.

	Plasmatocytes (Cell count)	Podocytes (Cell count)	Lamellocytes (Cell count)	Total Cells (Cell count)	Plasmatocytes (Percent)	Podocytes (Percent)	Lamellocytes (Percent)
Lb17							
LbG486							
Lh14							
LhSw							
LvPhil							
LvHaw							
LgCam							
LgSA							
LcNet							
LcAtl							
GxUg							
GxHaw							

Melanotic tumor formation and hemocyte numbers were highly variable phenotypes, decreasing power to detect significant differences. However, if we look at data correlations, we find a significant trend relating hemocyte composition to tumor phenotype penetrance. At 48 hours after attack, high tumor penetrance is associated with a higher percentage of circulating lamellocytes (Figure 3.4).

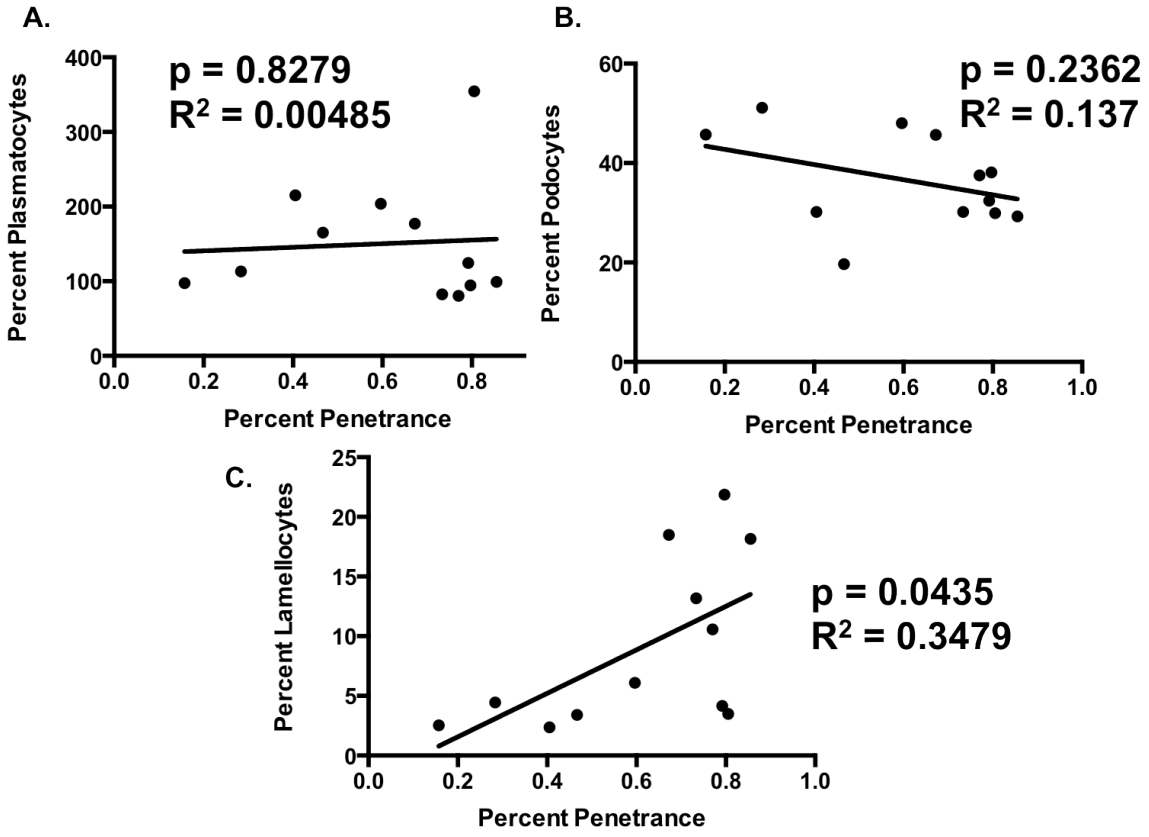

Figure 3.4. Hemocyte composition altered by wasps is associated with changes in Hop$^{Tum\text{-}l}$ tumor phenotype penetrance. Percent penetrance is not significantly associated with the percent plasmatocytes (A) or podocytes (B) at 48 hours after wasp attack. In contrast, percent penetrance is positively associated with percent lamellocytes (C) at 48 hours after wasp attack. Correlations were determined by GraphPad Prism 6.0 software.

Discussion

Here we have shown that Stat activity is necessary for the *D. melanogaster* encapsulation response against the avirulent wasp, LcNet. JAK/Stat signaling does not seem to be acting in the lymph gland of the fly to produce the lamellocytes involved in the encapsulation response, as overexpression of activated Stat in the lymph gland did not lead to a higher percent of circulating lamellocytes. Stat activity in the hemocytes and the fat body does induce lamellocyte differentiation, although in different ways. In the hemocytes, Stat overexpression leads to more lamellocytes at the expense of circulating plasmatocytes, suggesting that preexisting plasmatocytes were the progenitors of the newly synthesized lamellocytes. This is consistent with studies that have shown through lineage tracing that plasmatocytes differentiate into lamellocytes in the context of the immune response against parasitic wasps (Markus, 2009; Stofanko, 2010). Our data suggest that this differentiation may be stimulated by JAK/Stat activity in blood cells.

In contrast, overexpression of activated Stat in the fat body induced higher levels of circulating lamellocytes without affecting other hemocyte populations. This suggests that JAK/Stat activity in the fat body may act through an unknown, non-autonomous signaling mechanism to induce production of new hemocytes that then differentiate into lamellocytes. The origin of this lamellocyte population is unclear from these data, but two hypotheses can be suggested. First, lamellocytes can be produced in the *Drosophila* lymph gland from prohemocytes (Evans, 2003). Perhaps a cytokine signal downstream of Stat activation is secreted by the fat body to stimulate lamellocyte differentiation and

release of new hemocytes into circulation from the lymph gland. A second hypothesis could be that a cytokine produced by JAK/Stat signaling in the fat body is sent to a population of hemocytes known as sessile hemocytes. These blood cells remain attached to the interior of the larval cuticle and remain out of circulation in wild type, unattacked flies. When marked with GFP, these cells can be observed as a distinct banding pattern on the fly cuticle. However, upon wasp attack, this banding pattern disappears, suggesting that the sessile hemocytes have entered circulation (Markus, 2009; Zettervall, 2004). Differentiation of these cells into lamellocytes would also change total lamellocyte numbers without affecting other hemocyte populations in circulation.

Consistent with the induction of lamellocyte differentiation by Stat overexpression, here we show that wasp attack leads to Stat activity in the hemocytes and the fat body. Interestingly, more successful or virulent wasps seem to induce lower levels of Stat activity in their fly hosts than less virulent wasps (LcNet and LcAtl). This suggests that successful wasp parasites evade or suppress upstream *D. melanogaster* immune mechanisms, direct components of JAK/Stat signaling, or downstream feedback loops as a virulence strategy. The necessity of Stat activity in the encapsulation response (Figure 3.1) supports the idea that the JAK/Stat pathway would be a good target for wasp virulence.

Two virulent wasps, LgCam and GxHaw, not only blocked Stat activity in blood cells, but also decreased the penetrance of the tumor phenotype in $hop^{Tum\text{-}l}$ flies. This suggests that LgCam and GxHaw virulence strategies either target JAK/Stat activity directly at the

level of Stat activity or act both upstream and downstream of JAK/Stat activation. While this study did not look at the details of wasp virulence mechanisms, we did investigate the relationship of tumor phenotype to hemocyte composition of attacked larvae. At 24 hours after wasp attack, high plasmatocyte numbers significantly correlate to low penetrance of the tumor phenotype. In contrast, low lamellocyte numbers at 48 hours after attack were significantly associated with wasps that lower tumor phenotype penetrance (Figure 3.4). These trends are consistent with findings by Luo et al. (1995) where changes to Stat92E in the $hop^{Tum\text{-}l}$ background did not have any effect on the overproliferation phenotype, only on the formation of melanotic tumors. These data further support the hypothesis that wasps that inhibit tumor phenotype penetrance do so by blocking lamellocyte differentiation directly at the level of Stat activity.

To look more closely at the possible mechanisms of wasp virulence, we focused on the wasp strains that significantly reduced tumor phenotype penetrance (LgCam and GxHaw) and other strains of the same species that showed similar, though not significant trends (LgSA and GxUg). *L. guineaensis* strains caused significant increases in circulating host plasmatocytes (LgCam) and decreases in podocytes (LgSA) at 24 hours after wasp attack. This suggests that cells that might differentiate into lamellocytes during a regular immune response may not be activated due to *L. guineaensis* virulence. Following infection by the avirulent wasp LcNet, *D. melanogaster* mounts a successful immune response. In the $hop^{Tum\text{-}l}$ background, LcNet induces higher lamellocyte numbers, and flies attacked by LcNet continue to increase lamellocyte production up to 48 hours after attack. While LgCam and LgSA had no effect on lamellocyte numbers at any time point, comparison to

LcNet suggests that maintaining control lamellocyte numbers may be enough to suppress the fly immune response. In contrast, GxUg decreased the number of lamellocytes at 48 hours. Surprisingly, GxHaw caused no significant changes to hemocyte numbers despite decreasing tumor phenotype penetrance. At 24 hours after attack GxHaw induced equivalent Stat activity in hemocytes in comparison to LcNet and LcAtl. This suggests that GxHaw may evade the immune response by altering the function of lamellocytes rather than their abundance via some other unknown mechanism.

While we did identify trends relating tumor phenotype penetrance to hemocyte numbers (Figure 3.4), it is also evident that individual wasps employ very specific virulence strategies against *D. melanogaster*. These mechanisms of interference should be investigated further to better understand the ways that natural parasites may manipulate conserved signaling pathways involved in host immune responses. *Drosophila* and its parasitic wasps is an excellent model system for studying these interactions. Mutations in JAK/Stat pathway components and regulators have been associated with human lymphomas and leukemias. This knowledge has led to the use of JAK/Stat mutations as a diagnosis tool for detecting myeloproliferative neoplasms (MPNs) in patients (Chen, 2012). Much like the majority of human disease genes, the model genetic system *Drosophila melanogaster* can be exploited to study the highly conserved JAK/Stat signaling pathway *in vivo*.

CHAPTER 4

Discussion

Drosophila has been used as a model genetic system for decades. The tools of fly genetics allow studies, such as this one, to ask broad questions as well as investigate mechanistic details. This project's major aim was to better understand the activity of conserved signaling pathways in the context of immunity and hematopoiesis, as observations in the fly are applicable to mammalian systems (Valanne, 2011). One relevant example of how studies in the fly can translate to mammalian systems is the study of Toll signaling. Toll was first discovered as an essential component of fly development and was later implicated in *Drosophila* humoral immunity. The role of Toll signaling in the context of immunity is conserved in mammalian systems, a discovery that earned the 2011 Nobel Prize in Physiology and Medicine (Valanne, 2011). Our understanding of antimicrobial peptide (AMP) production via NfK-B signaling in the fly has radically changed our understanding of the mammalian immune system. Additionally, JAK/Stat hyperactivity in the fly can be used as a model of mammalian leukemia or lymphoma, as mutations in JAK/Stat pathway genes are often associated with these cancers of the blood (Chen, 2012). JNK signaling is also conserved in mammals and plays major roles in development, immunity, and disease. For example, mutations in JNK signaling components have been associated with malignant transformation of tumors (Ip, 1998). In the age of next generation sequencing and genomics, identifying conservation and homology between a well-studied system such as the fly and a less well-characterized mammalian system is a very useful tool for creating good, testable hypotheses regarding function.

This study investigated the mechanism of Toll, JAK/Stat, and JNK signaling in the context of lamellocyte differentiation in the fly cellular immune response. We found that Toll pathway activity is required in the lymph gland for successful melanotic encapsulation of parasitic wasps, whereas JAK/Stat and JNK signaling may be more important when activated in preexisting hemocytes. Our data suggest that, while JAK/Stat and JNK pathway genes can induce lamellocyte differentiation when ectopically expressed in hemocytes, knocking down these same genes did not affect the ability of flies to mount a successful melanotic encapsulation response. One explanation is that JAK/Stat and JNK signaling may be redundant in hemocytes. With new knowledge regarding tissue specificity of pathway activities, future studies will be aimed at investigation of crosstalk among Toll, JAK/Stat, and JNK signaling.

In addition to questions regarding pro-immune activity of Toll, JAK/Stat, and JNK, this study also investigated the ways in which pathogens may modulate host immune responses via action against conserved signaling. Specifically, we characterized the effects of parasitic wasp attack on JAK/Stat activity in *Drosophila* cellular immunity. We show that some wasps (LgCam, LgSA, GxUg, and GxHaw) may be targeting directly downstream of Stat activity, while other wasps may be acting farther upstream of JAK/Stat signaling to block the host defense.

It would be interesting to identify the particular components of wasp venom that may be acting directly on proteins involved in conserved signaling pathways. Because overexpression of Toll, JAK/Stat, and JNK in both fly and mammalian systems is

associated with hyperproliferation of cells, or cancer, it would be beneficial to identify compounds or proteins that have evolved in the wasp to effectively and specifically inhibit pathway activity. For example, this study suggests that some wasps, such as LgCam or GxHaw, may be directly inhibiting the activity of Stat targets. Identification of a specific venom protein responsible for this inhibitory action could provide information for treatments of human cancers associated with JAK/Stat signaling mutations. In conclusion, a better understanding of how Toll, JAK/Stat, and JNK signaling work together will allow us to also better understand wasp virulence strategies. The system of fly/wasp interactions is an excellent one for understanding how conserved signaling pathways can be modulated.

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