# ABSTRACT


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| Title of Document: | PHAGOCYTOSIS AND SIGNALING IN THE INNATE IMMUNE SYSTEM |
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The innate immune response provides broad spectrum defense through germline encoded components. Many aspects of innate immunity, such as the activation of NFκB transcription factors and phagocytosis, are highly conserved within the animal kingdom. The innate immune response of the cow, in particular, is important due to the cow's agricultural value. A major proportion of acute disease in domestic cattle is caused by Gram-negative bacteria, which produce the outer membrane component lipopolysaccharide (LPS). LPS binds to Toll-like receptor (TLR) 4 and activates multiple signaling pathways, which have been well-studied in humans, but not in ruminants. Human myeloid differentiation-factor 88 (MyD88) and TIR-domain containing adaptor protein (TIRAP) are critical proteins in the LPS-induced NFκB and apoptotic signaling pathways in humans. We demonstrated through the

expression of dominant negative constructs in bovine endothelial cells that both MyD88 and TIRAP activate NFκB in the cow. Additionally, bovine TIRAP was also shown to transduce LPS-induced apoptosis, indicating that multiple aspects of the TLR4-dependent signaling pathways are conserved between cows and humans. The model organism *Drosophila melanogaster*, was subsequently utilized to investigate the role of another branch of the innate immune response: phagocytosis. The extracellular fluid surrounding phagocytic cells in *Drosophila* has a high concentration of the amino acid glutamate. While glutamate has been well-characterized as a neurotransmitter, its effect, if any, on immune cells is largely unknown. We identified that a putative glutamate transporter in *D. melanogaster*, *polyphemus* (*polyph*), is critical to the fly's immune response. Flies with a disrupted *polyph* gene exhibit decreased phagocytosis of microbial-derived bioparticles but not of latex beads. Additionally, *polyph* flies show increased susceptibility to *S.aureus* infection, decreased induction of the antimicrobial peptide (AMP) Cecropin, increased melanization response, and increased ROS production. Glutamate transport has previously been shown to regulate the synthesis of the antioxidant glutathione. We demonstrate that a *polyph*-dependent redox system is necessary to maintain the immune cells' function against an infection. By utilizing two species, the cow and the fly, to study the innate immune system, we have gained unique and novel insights into NFκB activation and phagocytosis.

# PHAGOCYTOSIS AND SIGNALING IN THE INNATE IMMUNE SYSTEM

By

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# Dedication

To my husband, my mom, and my son.

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# Chapter 1: Introduction

## 1.1 Innate Immunity

The innate immune system serves as the body's first line of defense against pathogens. Its germline encoded receptors and signaling molecules respond to pathogen associated molecular patterns (PAMPs), providing a fast-acting broad-spectrum defense. Adaptive immunity, on the other hand, takes several days to become fully activated the first time it is challenged with a particular pathogen. Many animals rely entirely upon an innate immune system, as adaptive immunity only evolved within the jawed vertebrates (Abbas, 2003). The innate immune system may lack the diversity of the adaptive response but it is indispensable as it is not only the initial line of defense but also activates the adaptive branch (Abbas, 2003). For example, when dendritic cells are activated following phagocytosis, they produce the costimulatory molecules CD80 and CD86, which bind to CD28 on T cells causing the T cells to proliferate and produce cytokines (Lanier et al., 1995; McLellan et al., 1995). Innate immunity provides the basic defense of a barrier between the environment and the interior of the organism, preventing pathogens access to potential targets inside the body. Depending on the host, these barriers could include epithelial cells, mucus, and antimicrobial substances (Kimbrell and Beutler, 2001; Abbas, 2003). Once these mechanical and chemical barriers have been breached, the innate immune system can control the infection through both cellular and humoral means. Phagocytosis is a major part of the cellular response, while humoral immunity, depending on the host, is mitigated via the release of antimicrobial

peptides (AMPs), cytokines, oxygen- and nitrogen-reactive species, melanization, and the complement system.

### 1.1.1 Cellular Innate Immunity

Phagocytosis, the engulfment of molecules that are larger than 0.5μm (Kinchen and Ravichandran, 2008), is a vital process in immunity and in the development and growth of an organism. As an organism develops, tissue remodeling occurs during which cells undergo programmed cell death (PCD) and are then engulfed and removed via phagocytosis. For example, in mammalian embryonic development, PCD of the interdigital mesenchymal cells is necessary for individual digits form rather than one webbed structure (Ballard and Holt, 1968). PCD is necessary as flies without PCD do not survive to adulthood (White et al., 1994). Once the cells have undergone PCD, it is important to remove the dead cells and debris from the organism to allow fully functional development. For example, the failure to properly remove apoptotic debris has been linked to aging-related autoimmune complications (Aprahamian, 2008) and lupus pathology (Cohen, 2006). Although it has been shown that epithelial and microglial cells can play a limited role in phagocytosing dead cells, blood cells are the primary cells that remove apoptotic masses (Beyer et al., 2000; Gibbs et al., 2003). Transmission electron microscopy in late stage 12 *Drosophila* embryos shows hemocytes located around the brain and nervous tissue contain at least one apoptotic mass. By embryonic development stage 13, approximately 50% of *Drosophila* hemocytes have engaged in phagocytosis (Tepass et al., 1994).

During an infection, the primary role of phagocytosis is the removal and degradation of the invading microbes and infected cells. In mammals, the primary cells that

engage in phagocytosis are neutrophils, macrophages, and dendritic cells. Neutrophils are usually the first leukocyte to respond to infection as they are the most abundant circulating white blood cell. However because neutrophils are short-lived, it is the macrophage that becomes the predominant phagocyte during the later stages of infection (Abbas, 2003). Both macrophages and dendritic cells are professional antigen-presenting cells (APCs), serving as key links between the innate immune response and the adaptive (Abbas, 2003).

The fly is less complex, having only three types of hemocytes: lamellocytes, crystal cells and plasmatocytes. Lamellocytes encapsulate organisms too large to be phagocytosed and are mainly produced in response to parasitic wasps, such as *Leptopilina boulardi*, laying eggs in *Drosophila* larvae. Crystal cells provide humoral immunity through melanin byproducts, which will be addressed later. Plasmatocytes are phagocytic cells and are considered to be the equivalent to the mammalian macrophage due to similarity of their morphology, of their receptors, and of their kinetics of binding and cargo degradation (Abrams et al., 1992).

*Drosophila* hemocytes are produced in two phases, embryonic and larval, but cells from both lineages are present in the adult fly. *Drosophila* posess 700 plasmatocytes at the end of embryogenesis and more than 5000 present by pupariation, indicating that hematopoeisis is very active in the larval stage (Tepass et al., 1994; Lanot et al., 2001).The embryonic hemocytes can first be seen during late embryonic stage 10 or early stage 11 using an anti-peroxidasin antibody (Tepass et al., 1994). The

hemocytes are derived from the posterior portion of the procephalic mesoderm layer and then migrate throughout the embryo during stages 12-14 (Tepass et al., 1994). Larval hemocytes are produced in the primary lobes of the lymph gland in the third instar larvae (Lanot et al., 2001). All embryonic and larval prohemocytes first express the GATA factor homolog *serpent* (*srp*) and later the Friend of GATA (FOG) factor homolog *u-shaped* (*ush*) (Rehorn et al., 1996; Fossett et al., 2001; Fossett et al., 2003). Proliferation is regulated by the PVF2/PVR, Ras/Raf, and JAK/STAT pathways (Luo et al., 1997; Munier et al., 2002; Zettervall et al., 2004). The Toll pathway also plays a role in hematopoiesis, as mutants that activate Toll show an overproliferation of hemocytes (Qiu et al., 1998) and larvae have significantly fewer hemocytes when the two NFκB transcription factor homologs, *dif* and *dorsal*, are mutated (Matova and Anderson, 2006). A small subset of the Srp/Ush positive cells begin to downregulate Srp/Ush and upregulate the transcription factor *lozenge,* while the majority of the Srp/Ush cells begin to express *gcm* and *gcm-2* (Lebestky et al., 2000; Fossett et al., 2003). The Lozenge positive cells are crystal cell precursors and actively proliferate into crystal cells through signaling pathway involving Serrate and Notch (Lebestky et al., 2003). The *gcm* and *gcm-2* positive cells mature into plasmatocytes, a process which is dependent upon the transcription factor *pointed* in larval but not embryonic hematopoiesis. (Bernardoni et al., 1997; Lebestky et al., 2000; Alfonso and Jones, 2002). Under normal conditions, *Drosophila* produce very few lamellocytes. However, plasmatocytes can differentiate into lamellocytes upon a parasitic wasp infection through the upregulation of the transcription factors *charlatan* and *anterior open* and Serrate/Notch dependent upregulation of the early

B-cell factor homologue, *collier* (Krzemień et al., 2007; Márkus et al., 2009; Stofanko et al., 2010).

Once hemocytes have differentiated, they must migrate from the progenitor nodes to the rest of the animal, a process which is dependent upon VEGF signaling (Cho et al., 2002). The majority of hemocytes in the larvae were shown, by tracing their ingestion of India ink, to be circulating throughout the animal with approximately 1/3 remaining sessile. It is almost the opposite situation in the adult fly where most plasmatocytes are not freely circulating and are primarily interspersed in the fat body tissue, the equivalent of a mammalian liver (Elrod-Erickson et al., 2000; Lanot et al., 2001). The number of hemocytes sharply decreases during metamorphosis, resulting in approximately 1000-2000 blood cells in an adult animal (Lanot et al., 2001). Unlike mammals, where blood cells are constantly turned over and replaced, no hematopoiesis has been detected in adult *Drosophila*. Therefore *Drosophila* hemocytes are long-lived cells that must protect the fly for its entire lifespan. Interestingly, while female flies lose hemocytes as they age, male flies do not. However, the phagocytic capacity of the hemocytes in both sexes decreases with age (Mackenzie et al., 2011). This is consistent with the observation that the fly becomes more vulnerable to infection the older it becomes (Ramsden et al., 2008).

In the adult fly, the plasmatocyte is the predominant hemocyte as very few crystal cells or lamellocytes remain. In fact, when a relatively large foreign object such as a human hair was inserted into a *Drosophila* larva, large numbers of lamellocytes were

recruited to the scene forming a layer of cells with intercellular junctions sealing the hair from the hemolymph. However, when a hair was inserted into an adult animal, no lamellocytes were observed and the hair remained free of any host cellular defenses (Lanot et al., 2001). Therefore during the fly's adulthood, the plasmatocyte is the primary hemocyte available to fight infection.

Regardless of the organism and their particular type of phagocyte, the surveillance cell must be able to tell the difference between a fellow host cell and an invading microorganism. The receptors present on mammalian neutrophils and macrophages have been well-studied and defined. Although it has been a topic of interest, research on *Drosophila* phagocytic receptors has not been saturated (Philips et al., 2005).

Mammalian phagocytes can bind directly to microbes through mannose and scavenger receptors. Mannose receptors bind to products with sugar residues that terminate with either mannose or fucose, a signature specific to microorganisms, as mammalian sugars typically end in either sialic acid or N-acetylgalactosamine (Abbas, 2003). Scavenger receptors, which are found both on mammalian macrophages and fly plasmatocytes (Ramet et al., 2001), were originally defined as receptors that bind to acetylated-low density lipoproteins (LDL). However they are now known to bind to a variety of different ligands characterized as polyanionic (Goldstein et al., 1979). One of the first *Drosophila* scavenger receptors to be identified in phagocytosis was dSR-CI. It is needed to phagocytose both *Escherichia coli* and *Staphylococcus aureus* in S2 cells and its expression is sufficient to allow

CHO cells to bind to either bacteria (Ramet et al., 2001). Several of the mammalian scavenger receptors (CD14 and CD36) are cooperative binding proteins that facilitate Toll-like receptors (TLRs) binding to their respective ligands (Wright et al., 1990; Hoebe et al., 2005). A *Drosophila* homolog of CD36, Peste, was shown in an RNAi screen of the Drosophila phagocytic cell line S2 cells to be a phagocytosis receptor that recognizes both *Mycobacteria fortuitum* and *Listeria monocytogenes*. Researchers confirmed the RNAi results by showing that Peste conferred the ability to phagocytose *M. fortuitum* in HEK293 cells (Philips et al., 2005).

Another member of the CD36 family in *Drosophila*, Croquemort, is an important receptor for phagocytosing apoptotic host cells (Franc et al., 1996) and *S. aureus* (Stuart et al., 2005). Similarly, the Ced-1 homologue, Draper, was originally found in an S2 RNAi screen to be a receptor for apoptotic cell engulfment by hemocytes (Manaka et al., 2004) but has more recently been identified as a phagocytic receptor for both *E. coli* and *S.aureus* (Cuttell et al., 2008; Hashimoto et al., 2009). Integrin βv is a third receptor previously shown to bind to apoptotic cells that also plays a role in *S.aureus* phagocytosis (Shiratsuchi et al., 2012). The three receptors are not completely redundant in their role in phagocytosing *S.aureus*, as Croquemort and Draper bind to *S.aureus* lipoteichoic acid, while Integrin βv binds to *S.aureus* peptidoglycan. Draper contains a conserved motif CCxGY, which it shares with two other *Drosophila* phagocytic receptors: Eater and Nimrod C1. Both Eater and Nimrod C1 contain EGF-like repeats and are required for optimal phagocytosis of either *S.aureus* or *E.coli* (Kocks et al., 2005; Kurucz et al., 2007).

Opsonization is an aspect of phagocytosis present in mammalian systems where molecules enhance phagocytosis by serving as a binding adaptor between the target cell and phagocytes. This process can be mediated by antibodies by the Fab region of the antibody binding to an antigen on the pathogen's surface and the Fc segment then recruiting phagocytes expressing an Fc receptor (Abbas, 2003). Recently, the *Drosophila* antimicrobial peptide (AMP), Cecropin, has been demonstrated to have opsonin-like properties. Cecropin can bind to live *E.coli* and make the bacteria more readily available to Eater-mediated phagocytosis (Chung and Kocks, 2011). Although Eater can bind to Gram-positive and Gram-negative cell wall components, it has very low binding to any live Gram-negative bacteria without the addition of either Cecropin or detergent. As Cecropin is a cationic peptide, known to disrupt membranes (Sato and Feix, 2006), it is reasonable to hypothesize that it disrupts the outer membrane present in Gram-negative species so that PRRs such as Eater may bind to the cell wall.

In mammals, opsonization can also occur via the alternative complement pathway; when C3 is cleaved to form C3a and C3b, C3b goes through a conformational change which exposes a thioester bond and allows it to bind covalently to the cell surface of a pathogen (Mullereberhard et al., 1966; Sim et al., 1981; Pangburn et al., 1983). C3b can then be cleaved into iC3b, which recruits phagocytes to the targeted cell (Takizawa et al., 1996). Although *Drosophila* do not have direct complement homologs, they do have complement-like proteins, thioester-containing proteins

(TEPs). As their name suggests, TEPs include a thioester bond that would allow a mammalian complement molecule to bind to bacterial surfaces. Similar to complement proteins in mammals, TEPs play a role in opsonization in *Drosophila*, and are upregulated following a bacterial challenge (Lagueux et al., 2000). Five TEPs were originally found in the fly by sequence comparison, however only TEPs I-IV were found to be expressed as cDNAs. A sixth gene was found but it is more commonly known as Mcr rather than TEPVI as it is missing the crucial thioester bond that defines the group (Lagueux et al., 2000). Through an RNAi screen of S2 cells, Mcr was found to be involved in the phagocytosis of the fungus *Candida albicans*, but not *E. coli* or *S. aureus*. Stroschein-Stevenson et al. further showed that similar to complement molecules, Mcr is secreted and can bind directly to the fungal cell surfaces. Given Mcr's apparent involvement in phagocytosis, researchers then explored the possible phagocytic properties of the other TEPs through RNAi experiments in S2 cells. They found that TEPII is involved in *E. coli* phagocytosis whereas TEPIII is important in the uptake of *S. aureus* (Stroschein-Stevenson et al., 2006). Curiously, TEPII has also been shown to undergo alternative splicing within its variable region (Lagueux et al., 2000).

Another phagocytic receptor that can generate multiple splicing patterns is Down Syndrome cell adhesion molecule (Dscam) (Schmucker et al., 2000; Watson et al., 2005). Dscam was originally identified for its role in neuronal development (Schmucker et al., 2000) but it was later shown to be expressed on the surface of hemocytes and secreted into the hemolymph (Neves et al., 2004). Through RNAi

experiments in mosquitoes, Dscam was identified as a phagocytosis receptor for bacteria (Dong et al., 2006). Because of its alternative splicing patterns, its extracellular immunoglobulin domains, and its expression in hemocytes, researchers have hypothesized that Dscam could possibly be a component of an adaptive immune branch that, as of now, has not been identified in *Drosophila* (Agaisse, 2007).

There is currently no evidence that *Drosophila* have anything equivalent to the clonal expansion of lymphocytes or antibody production seen in the adaptive immune response of mammals. However, the *Drosophila* immune system can become more responsive with subsequent exposure to a pathogen (Pham et al., 2007); a trait normally associated with the adaptive immune response. When flies were first injected with a non-lethal dose of *Streptococcus pneumoniae* followed by an injection of a lethal dose, they were less susceptible than flies that were mock primed with a PBS injection. The priming response lasted at least two weeks and was dependent upon phagocytosis, but not AMP induction. Curiously, the priming response was not seen against most pathogens and appeared to be limited to a few microbial species. The few species that were able to induce a priming response did so through specific adaptation of the immune response, as they could only protect against subsequent infection of the same pathogenic species. Overall, this indicates that the *Drosophila* immune system has adaptive properties in limited circumstances, but that it appears to be distinct from a mammalian adaptive response where infection by most microbes primes the animal for future infections of the same microbe.

One further category of phagocytic receptors in *Drosophila*, are the peptidoglycan recognition proteins (PGRPs). The PGRPs have variable expression patterns and functions (Werner et al., 2000) with their most familiar role being receptors for the AMP inducing signaling pathways (Choe et al., 2002; Gobert et al., 2003; Takehana et al., 2004). PGRP-LC is expressed on the hemocyte cell surface (Werner et al., 2000; Gottar et al., 2002). It was discovered to play a role in upregulating AMPs in response to Gram-negative infections concurrently by three laboratories, two that used an *in vivo* mutant approach (Choe et al., 2002; Gottar et al., 2002) and another that used an *in vitro* RNAi approach (Ramet et al., 2002). Ramet et al., through their *in vitro* data in S2 cells, also showed that PGRP-LC is necessary for optimal levels of phagocytosis of *E. coli* (Ramet et al., 2002). PGRP-SC1a, on the other hand, was discovered through *in vivo* means to be required for the phagocytosis of *S. aureus* but not *E. coli* or *Bacillus subtilus*, (Garver et al., 2006). The PGRPs are most closely related to mammalian NODs (nucleotide-binding oligomerization domain containing proteins), which also bind to bacterial peptidoglycan products but do so within the cytoplasm (Werner et al., 2000; Chamaillard et al., 2003; Inohara et al., 2003). Interestingly, NODs are exclusively localized to the cytosol, yet they are known to recognize extracellular microbes such as *S.aureus*, through a mechanism that is not entirely clear. The apparent paradox was partially answered when the SLC15A transporter, Yin, was identified in a large scale *Drosophila* phagosome proteome screen (Stuart et al., 2005). When Yin was ectopically expressed in HEK293T cells, it amplified NFκB activation in a NOD2 dependent way (Charrière et al., 2010). The human SLC15A transporter PEPT2, was found to be expressed in mammalian

macrophages and showed similar activation of HEK293T cells that was seen with Yin. Both transporters share a common ligand: muramyl peptide, a product formed by the degradation of *S.aureus* in the phagolysosome. Therefore, SLC15A transporters are present in both *Drosophila* and mammalian phagosomes and transport bacterial byproducts into the cytosol where intracellular receptors bind to them.

Although it is likely that there are still more receptors to be identified, the cellular process of phagocytosis and phagosome maturation are well-studied processes with many known components. Once an extracellular particle has been recognized and bound by a receptor on the phagocyte, the plasma membrane of the phagocytosing cell must form the phagocytic cup in order to begin engulfment of the targeted particle (Swanson, 2008). The plasma membrane begins to unfold in order to cover the surface area of the particle (Herant et al., 2005). If the particle is too large for the plasma membrane to handle, intracellular vesicles can move to the site of phagocytosis to provide extra membrane (Petty et al., 1981). During phagocytic cup formation, the plasma membrane deviates from its normal structure and begins to push pseudopod tips around the target causing membrane invagination (Swanson, 2008). In *Drosophila* phagocytes, most phagocytosis occurs via the zipper mechanism (Pearson et al., 2003), the process used in FcR-mediated phagocytosis in mammalian macrophages (Griffin et al., 1976). The zipper phagocytosis model is derived from the observation that particles that were coated with ligand only on one side, were bound but not ingested. Only particles with ligand distributed evenly over the surface area were able to induce spreading of the cell membrane around the entire particle

and subsequent engulfment (Griffin et al., 1976). This engulfment results in a tightly formed phagosome where the engulfed particle determines the shape of the phagocytic compartment (Tollis et al., 2010).

In contrast, the phagosome formed during macropinocytosis is not necessarily in direct contact with the particle and its shape is independent of the molecule(s) that is has engulfed. Macropinosomes form spontaneously from naturally occurring ruffles on the plasma membrane to engulf extracellular fluid and particles. Their formation is independent of any ligand binding and they can range in size from 0.5 to 5.0 μm in diameter (Swanson, 1989). Macropinocytosis plays only a minor role in the uptake of *S.aureus* particles in *Drosophila* macrophages (Pearson et al., 2003) but is strongly involved in invasion and intracellular replication of both *Salmonella typhimurium* and *Legionella pneumophila* in mammalian macrophages. *S. typhimurium* increases macrophage plasma membrane ruffling via the PhoPc virulence locus in order to enter the cell via macropinocytosis (Francis et al., 1993; Alpuche-Aranda et al., 1994). If it is engulfed by the zipper mechanism, the bacteria is able modify the tight phagosome, making it more spacious until it morphologically resembles a macropinosome. Once inside the spacious vacuole, *S.typhimurium* can persist and replicate (Alpuche-Aranda et al., 1994). Similarly, *L.pneumophila* induces macropinocytosis in the mammalian macrophage via the bacterial Dot/Icm system in order to avoid detection and replicate (Watarai et al., 2001).

Clathrin, a triskelion molecule that binds to itself to form pentagon and hexagon shaped structures, plays a minor role in the phagocytosis of some molecules (Crowther and Pearse, 1981; Tse et al., 2003). The clathrin polymers assemble to form a soccer ball-like lattice resulting in a spherical structure (Crowther and Pearse, 1981) around the phagocytic cup to stabilize it. Interestingly, *L.monocytogenes* modulates clathrin through the surface protein InlB in order to successfully invade HeLa cells, indicating that clathrin plays an important role in some infections (Veiga and Cossart, 2005). In addition to clathrin, the formation of the phagocytic cup in both mammalian and *Drosophila* macrophages is heavily dependent upon actin polymerization as both cell types exhibit decreased phagocytosis with the addition of cytochalasin D (Pearson et al., 2003). Normally, globular-actin (G-actin) monomers are bound to ATP and associate at the barbed end of an actin filament, which is termed filamentous-actin (F-actin). Two F-actin polymers rotate around each other in a double helix to form a microfilament. F-actin has increased ATPase activity as compared to G-actin and as ATP is hydrolyzed to form ADP, the association between actin monomers becomes weaker. ADP-bound monomers then dissociate from the pointed end of the actin filament. In this way, the filament is always moving or "treadmilling," as monomers are constantly recruited to the barbed end and then subsequently lost from the pointed end of the filament. Cytochalasin D is a fungal toxin that prevents actin polymerization by blocking actin monomers from either associating or dissociating from the barbed end of actin filaments (Dominguez and Holmes, 2011).

Phagocytic cup formation also requires the synthesis of branched actin filaments, a process dependent upon the Arp2/3 complex (Amann and Pollard, 2001). This complex, which is comprised of seven proteins including actin related proteins (Arp) 2 and 3, binds with high affinity to the pointed end of actin filaments and crosslinks them end to side at a 70º angle (Mullins et al., 1998). Two proteins, Wiskott-Aldrich Syndrome protein (WASp) and Suppressor of cAR (Scar) interact with the p21 subunit of the Arp2/3 complex (Machesky and Insall, 1998). Consistent with its name, WASp was originally identified as the protein responsible for the X-linked recessive immunodeficiency, Wiskott Aldrich Syndrome, in which subjects present with symptoms such as eczema and recurrent infections (Derry et al., 1994). Symons et al. discovered that WASp interacts with the Rho protein CDC42 and regulates actin polymerization (Symons et al., 1996). Scar, on the other hand, was originally identified in the amoeba, *Dictyostelium discoideum* (Bear et al., 1998). Scar mutants were found to have decreased F-actin and abnormal cytoskeleton formation at rest and during chemotaxis. Both proteins are required for efficient dendritic nucleation of actin filaments through their interaction with the Arp2/3 complex (Machesky et al., 1999). Experiments in *Drosophila* confirmed that these interactions are necessary for phagocytosis. When the *Drosophila* Scar homolog, D-Scar, was disrupted via a P-element, macrophages showed a 60% decrease in phagocytosis. Phagocytosis was also inhibited when *Drosophila* S2 cells were treated with RNAi against the WASp homolog, D-WASp. Furthermore, phagocytosis was also decreased in mutants of the Profilin homolog, Chickadee (Pearson et al., 2003). Profilin is an endogenous inhibitor of actin polymerization. It binds and sequesters G-actin in order to prevent

spontaneous actin nucleation. Taken together, actin remodeling is a highly conserved process that is required for phagocytosis in many species.

However, it is not entirely clear how active a role actin plays in forming the phagocytic cup. If actin plays an active role, then actin treadmilling would be the driving force in the formation of the phagocytic cup. Conversely, actin could be acting in a passive manner to prevent the plasma membrane that has been recruited to the cup from returning to its normal relaxed conformation. In a computer simulated model, phagocytosis of small molecules was able to occur with actin playing a passive role. However, an actin-driven simulation increased the kinetics of small molecule uptake and was required for the uptake of larger (radius >2.5 μm) particles (Tollis et al., 2010). This indicates that actin is not necessarily required to actively drive all phagocytic events, but it likely makes the process more efficient.

Some pathogens have evolved to manipulate actin rearrangement to their advantage. The intracellular pathogen *S.typhimurium*, uses six effector molecules: SopE, SopE2, SopB, SipA, SipC, and SptP to first induce actin polymerization by activating Rho GTPases, nucleating actin polymerization, and stabilizing actin filaments. The bacteria then downregulates Rho protein activity by promoting their GTPase activity in order to restore the host cell homeostasis (Zhou and Galán, 2001). As the bacteria prefer to grow inside the cell, they must carefully control the process to preserve the integrity of the host cell. Extracellular pathogens, on the other hand modulate host cell actin in order to avoid phagocytosis. *Clostridium botulium*, *Vibrio*

*parahaemolyticus*, *Histophilus somni*, and *Yersinia enterocolitica* use C3, VopS, IbpA, and YopT respectively to modulate Rho family GTPases in order to disrupt the actin cytoskeleton and avoid engulfment by phagocytes (Didsbury et al., 1989; Fueller and Schmidt, 2008; Worby et al., 2009; Yarbrough et al., 2009).

Once the phagocytic cup has formed around its target, Dynamin, a GTPase, recruits Rab5 to the early endosome, through an interaction with the phosphoinositide 3-kinase VPS34 (Kinchen et al., 2008). Rabs are small Ras-like GTPases that regulate general membrane trafficking and play an important role in regulating phagosome maturation. Rabs cycle between being GTP-bound (active) and GDP-bound (inactive). This cycle is regulated by two types of proteins: guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs activate GDP-bound Rabs, by exchanging their GDP for a GTP. GAPs on the other hand amplify a GTP-bound Rab's native GTPase activity, resulting in GDP-Rab (Barr and Lambright, 2010). The Rabs also cycle between being membrane-bound or free in the cytosol. This cycle is regulated by two other classes of proteins: GDP dissociation inhibitor (GDIs) and GDI displacement factor (GDF). Rabs become membrane-bound through the prenylation of cysteine residues at their carboxy termini (Kinsella and Maltese, 1992). If the Rab is GDP bound, a GDI will bind to the prenylated residues, thus preventing the Rab from being membrane bound (Garrett et al., 1994; Shapiro and Pfeffer, 1995). GDI can become dislodged if the GDP-Rab binds to GDF (Sivars et al., 2003), which then exposes the prenylated residues on the Rabs allowing it to be inserted into the membrane.

Rab5 is an important regulator of phagosome maturation and must be converted to a GTP bound form for maturation to progress past the early endosome (Bucci et al., 1992). Early endosomal autoantigen 1 (EEA1) is a Rab5 effector protein that is recruited to the phagosome through a FYVE domain (McBride et al., 1999). This domain binds to phosphatidylinositol (3)-phosphate (PtdIns(3)P), which is in high concentration in the endosomal membrane due to the presence of the PI3K, VPS34 (Fratti et al., 2001). EEA1 is a long coiled coiled tethering protein (Simonsen et al., 1998), which connects Rab5-positive endosomes to t-SNAREs, such as syntaxin-13, in order to mediate fusion of endosomal membranes (McBride et al., 1999). *Mycobacterium tuberculosis* arrests phagosome maturation in part by inhibiting the recruitment of EEA1 to the early phagosomal membrane (Fratti et al., 2001) indicating that the protein plays a pivotal role in regulating maturation.

The phagosome undergoes multiple fusions with membrane bound compartments each carrying distinct protein components specific to the stage of vesicle. This is required for successful maturation from an early phagosome to a late phagosome to a phagolysosome. Rab7 replaces Rab5 during the late endosomal stage and thus serves as a marker for this stage of maturation (Rink et al., 2005). Rab2 is also required for efficient phagosome maturation, although its role is not as clear. While Rab5 and Rab7 show clear temporal association with a particular maturation stage, Rab2 appears to colocalize with phagosomes containing Rab5, Rab7, and the lysosomal marker LAMP, indicating that it is present throughout maturation (Lu et al., 2008;

Mangahas et al., 2008). Rab2 may be playing a role in moving vesicles from the ER/Golgi to the phagosome, which would explain how the GTPase can regulate maturation while appearing to be constantly present on the phagosome.

The homotypic fusion and vacuole protein sorting (HOPS) complex was originally identified to play a role in endosomal maturation (Seals et al., 2000; Rink et al., 2005). The complex is comprised of VPS11, VPS16, VPS18, VPS33, VPS39, VPS41, and VPS-45 (Kinchen and Ravichandran, 2008). One of the components, VPS-39, is a Rab7 GEF (Wurmser et al., 2000), which is required in the endocytic pathway for Rab5 positive endosomes to mature into Rab7 endosomes (Rink et al., 2005). Somewhat surprisingly, in the phagocytic pathway, the HOPS complex was found to function downstream of Rab7 in the late phagosome (Kinchen et al., 2008). In addition to acting as a Rab7 GEF, HOPS also mediates vacuole fusion, as both VPS33 and VPS44 directly interact with SNAREs (Peterson et al., 1999; Seals et al., 2000). In *Drosophila*, a *vps16B* (*fob*) mutant showed normal uptake of bacteria, but were unable to transition from Rab7 positive phagosomes to the phagolysosome (Akbar et al., 2011). Phagosome maturation is important to the fly's survival, as flies with mutant *fob* demonstrated increased susceptibility to an *E.coli* infection.

As the phagosome matures it becomes more and more acidic through a vacuolar ATPase pump (Lukacs et al., 1990). The pump is comprised of the cytoplasmic $V_1$ complex, which hydrolyzes ATP to ADP, and a membrane bound $V_0$ complex, which transports protons from the cytoplasm into the phagosome (Beyenbach and

Wieczorek, 2006). The pH continues to decrease until the vesicle reaches a pH of approximately 5.5 or less (Lukacs et al., 1991). Markers of the highly acidic phagolysosome include the lysosome-associated membrane proteins (LAMPs) (Lewis et al., 1985; Viitala et al., 1988) and the Psidin protein in *Drosophila*, which is required for efficient degradation of bacteria (Brennan et al., 2007). The drop in pH activates proteins, such as cathepsins, that assist in the degradation of the cargo as well as enzymes that produce reactive oxygen species (ROS) (Lennon-Dumenil et al., 2002). Before complete degradation occurs, the cargo dissociates from its receptor so that the receptor can be recycled back to the plasma membrane where it is available to begin the phagocytosis process again (Maxfield and McGraw, 2004).

ROS production in the phagolysosome is crucial to the degradation of engulfed particles. Due to the toxic nature of ROS, its' production in phagocytes is highly regulated. After exposure to microbes, both neutrophils and macrophages undergo oxidative burst (Abbas, 2003), which is enhanced by the presence of cytokines such IFN-γ or TNF-α (Murray et al., 1985; Philip and Epstein, 1986; Denis and Chadee, 1989). During an oxidative burst, the membrane bound enzyme, NADPH oxidase, transfers an electron to $O_2$, yielding superoxide anion ($O_2^-$). $O_2^-$ can directly kill microbes by oxidizing electron transport molecules in the microbe's respiratory chain. Human patients with chronic granulomatous disease (CGD) have a genetic mutation in their NADPH oxidase complex. This disease is characterized by recurrent infections by fungi, Gram-positive, and Gram-negative bacteria, indicating that the immune system is highly dependent upon the oxidative burst (Kume and Dinauer,

2000). The NADPH oxidase enzyme is actually a multi-subunit complex made up of $gp91^{phox}$ (NOX), $p22^{phox}$, $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and Rac. $gp91^{phox}$ and $p22^{phox}$ form the membrane bound catalytic core of the enzyme, called cytochrome b558. However, this core is not active until it associates with the other subunits, which act as regulators of the enzyme (Nauseef, 2004). The process of converting $O_2$ to $O_2^-$ is called "oxidative burst" because it rapidly consumes oxygen while making approximately 0.5 mM of $O_2^-$ per second. This rate of production would lead to an $O_2^-$ concentration of approximately 50 µM in a phagosome with a pH of 7.4 and 2 µM at pH 4.5 (Slauch, 2011). Superoxide dismutase can convert $O_2^-$ to hydrogen peroxide, $H_2O_2$, which can readily diffuse through membranes to modify methionine and cysteine residues (Caldwell and Tappel, 1964; Wu et al., 1998). Oxidation of these amino acids, leads to inactivation of enzymes such as tyrosine phosphatases (Lee et al., 1998a). $H_2O_2$ can further be converted to even more highly reactive species such as hydroxyl radicals (OH•), hypochlorite ($OCl^-$), and hypobromite ($OBr^-$), which can cause damage through free radical chain reactions, chlorination of tyrosines, and oxidation of enzymes (Lambeth, 2004). Nitric oxide (NO) is produced in an independent reaction where arginine and oxygen are modified by inducible nitric oxide synthase (iNOS). NO can then combine with $O_2^-$ to form the highly reactive molecule peroxynitrite (ONOO-), which can induce lipid peroxidation and tyrosine modification among other things (Radi et al., 1991; Beckman, 1996) .

Studies in *Drosophila* have also found that NADPH oxidase plays a role in immunity outside of phagocytes. When the expression of a *Drosophila* NADPH oxidase,

dDuox, was downregulated, flies became more susceptible to gut infections introduced by feeding (Ha et al., 2005). Interestingly, even though the gut is not a sterile environment, the induction of ROS was tightly regulated and not induced in the presence of commensal bacteria. Negative regulation of dDUOX was dependent upon MAP Kinase Phosphatase-3 (MPK3) (Ha et al., 2009).

Flies also produce antimicrobial ROS through melanization. In contrast to oxidative burst-derived ROS, which primarily occurs in phagocytes, melanization is, for the most part, initiated by a specialized *Drosophila* cell, the crystal cell (Rizki et al., 1985). The transcription factors Lozenge and Serpent regulate crystal cell expression of the zymogen prophenoloxidase (PPO) (Ferjoux et al., 2007). As PPOs have no secretion signal, they are released from crystal cells by rupturing the plasma membrane (Rizki et al., 1985; Bidla et al., 2007). Crystal cell lysis is dependent upon the JNK pathway, the *Drosophila* TNF-α homolog Eiger, and Rho signaling (Bidla et al., 2007). Experiments in multiple arthropod species have indicated that once in the hemolymph, PPO is cleaved to form the active phenoloxidase (PO) by the serine protease prophenoloxidase activating enzyme (PPAE) (Lee et al., 1998b; Satoh et al., 1999; Kanost et al., 2004). PO then converts tyrosine into melanin, producing multiple reactive antimicrobial species in the process (Zhao et al., 2007). There are three *Drosophila* PPOs; PPO1/ ProPO54/DoxA1 and PPO2/ProPO45 are expressed in crystal cells, whereas PPO3/ProPO59/DoxA3 appears to be expressed in another specialized *Drosophila* hemocyte, the lamellocyte (Irving et al., 2005). PPO1 is mutated in the *Drosophila* mutant *Black cells* (*Bc*) causing constitutive melanization

and nonfunctional crystal cells (De Gregorio et al., 2002; Gajewski et al., 2007) . PO is also involved in the coagulation response, which creates a clot following infection or wounding (Karlsson et al., 2004). PO is associated with the clot but is not required for its initial formation (Scherfer et al., 2004; Bidla et al., 2007). Instead it plays a role in hardening the clot to make it more effective in closing a wound or sealing invading microbes from the hemolymph (Bidla et al., 2005). Conversely, the hemocyte associated protein, Hemolectin, is required for the formation of the seminal soft clot but is not needed for the subsequent hardening or melanization (Bidla et al., 2007).

Currently, it is still unclear what signaling mechanisms lead to PPO activation. Neither bacterial nor fungal infections affect PPO transcription (De Gregorio et al., 2001; Irving et al., 2001) but many processes initiate PPO cleavage, including wounding, dying hemocytes, and bacterial, fungal, and parasitoid wasp infections (Ligoxygakis et al., 2002b; Leclerc et al., 2006; Tang et al., 2006). Activation of PPO depends upon two CLIP proteases, a class of serine proteases that have an N-terminal regulatory domain and three disulfide bonds (Castillejo-López and Häcker, 2005; Leclerc et al., 2006; Tang et al., 2006). Overexpression of either of the CLIP proteases MP1or Sp7/MP2/PAE1 leads to constitutive melanization. Downregulation of the proteases leads to decreased PPO cleavage and melanization, and increased susceptibility to infection (Tang et al., 2006; Ayres and Schneider, 2008). MP1 appears to act downstream of Sp7 and therefore could be the *Drosophila* PPAE, however there is no direct evidence showing cleavage of PPO by MP1. There is at

least one additional member in the protease pathway that separates the two CLIP enzymes, as Sp7 cannot cleave MP1 (Tang et al., 2006).

The ROS byproducts that are released into the hemolymph are equally toxic to host cells as they are to the microbes at which they are directed. Therefore, the fly carefully controls the melanization response in two ways. First, the PO-dependent clot that is formed around the microbe physically separates the toxic molecules from the general hemolymph (Bidla et al., 2009). Additionally, the crystal cells also release serine protease inhibitory Serpin (Spn) proteins into the hemolymph, which negatively regulate the pathway. Three Serpins have been identified in *Drosophila* to inhibit melanization: Spn27A, Spn28D, and Spn77B. Spn27A shows broad and strong inhibition of melanization throughout the fly; loss of Spn27A results in general uncontrolled melanization (De Gregorio et al., 2001; De Gregorio et al., 2002; Ligoxygakis et al., 2002b). Spn28D and Spn77Ba are more tissue specific, with Spn28D acting primarily in the cuticle and Spn77Ba localizing to the trachea (Scherfer et al., 2008; Tang et al., 2008). The Toll pathway also regulates the melanization pathway, as activated Toll upregulates Spn27A, Spn28D, MP1, and MP2 (De Gregorio et al., 2001). Additionally, mutating the serpin *necrotic*, which is a negative regulator of Toll, leads to constitutive melanization (Levashina et al., 1999).

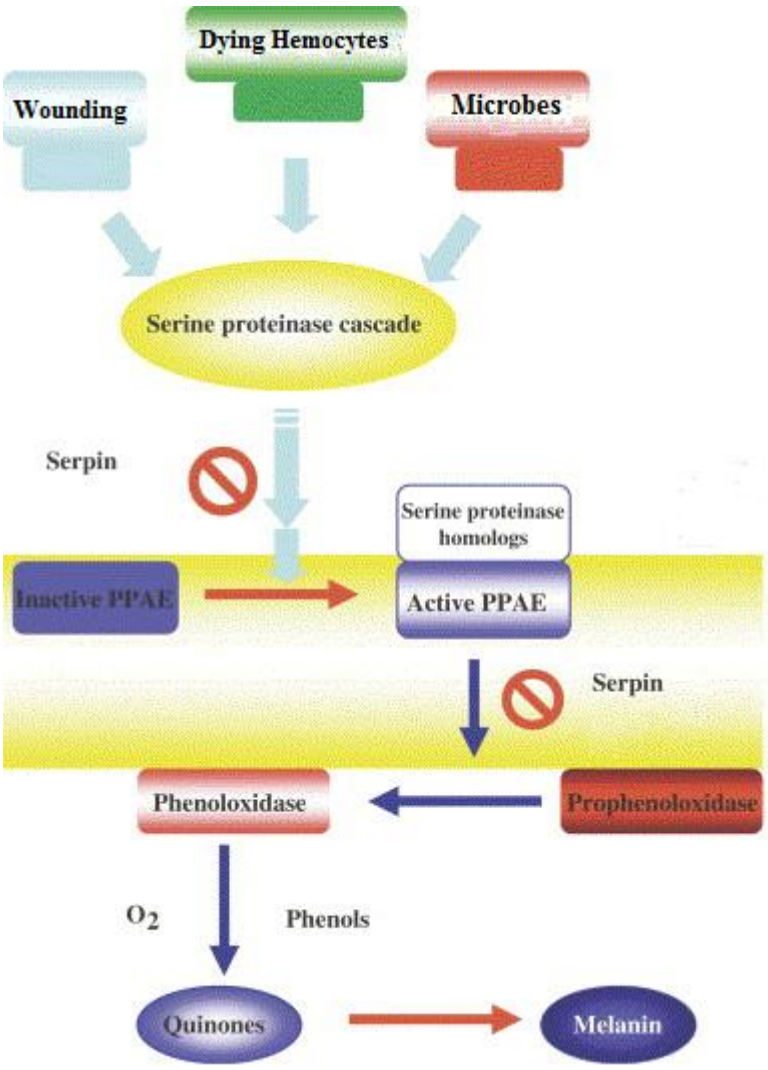



**Figure 1-1 (adapted from(Jiravanichpaisal et al., 2006): Crystal cells mediate melanization**. Wounding, dying hemocytes, and microbial products initiate a serine protease cascade which cleaves inactive PPAE into active PPAE. Prophenoloxidase is released by lysed crystal cells and is cleaved by PPAE to yield phenoloxidase. Phenoloxidase then synthesizes quinones releasing reactive byproducts in the process. The protease reactions are inhibited by Serpin proteins.

The cellular response is an important part of the immune system during an infection. There are a host of bacterial infections, such as *S.aureus*, *Serratia marcescens*, *Micrococcus luteus*, and *Enterococcus faecalis*, that are lethal to flies with decreased phagocytosis but a normal AMP response (Kocks et al., 2005; Nehme et al., 2011). Blocking phagocytosis in *immunodeficiency* (*imd*) flies makes them vulnerable to normally non-pathogenic *E. coli* infections compared to *imd* flies with wild-type phagocytosis (Elrod-Erickson et al., 2000). However, many bacteria have developed ways to avoid death within the phagolysosome either by escaping the phagosome before it fuses with the lysosome (*L.monocytogenes*) (Portnoy et al., 1989) or by halting its maturation (*M.tuberculosis*) (Philips, 2008). Therefore, it is critical that there is a humoral response to reinforce the organism's defense against infection.

### 1.1.2 Humoral Innate Immunity

Humoral innate immunity includes antimicrobial peptides, cytokines, and the complement system. All of these effectors require host recognition of the invading microbe, which initiates a signaling cascade and results in the release of host defense molecules. The host uses pattern recognition receptors (PRRs) to recognize PAMPs, which are conserved patterns unique to microorganisms. PRRs are another ancient branch of the innate immune response as many PRR signaling cascades have been conserved from plants to arthropods to mammals (Beutler and Rehli, 2002; Abbas, 2003). For these reasons, *Drosophila* is a useful model organism to study and gain insight into human immunology. The most notable example of a discovery in *Drosophila* leading to progress in the mammalian immunity field is the story of the

protein Toll. Toll was originally found in 1985 in *Drosophila* to be a maternal effect protein necessary for dorsal-ventral embryonic development. In loss of function *Toll* mutants, the female flies laid eggs that developed into completely dorsalized larvae, whereas gain of function *Toll* mutants led to completely ventralized larvae. This led the researchers to name the gene the German word for "weird" or "fantastic" (Anderson and Nusslein-Volhard, 1984; Anderson et al., 1985). It was not until 1995 when Lemaitre et al. found that loss-of-function mutants in the Toll pathway no longer expressed an antifungal peptide Drosomycin while gain-of-function Toll led to constitutive expression of the AMP, that Toll was forever linked to innate immunity (Lemaitre et al., 1996). This paper led to two labs discovering that human homologs of Toll existed (Medzhitov et al., 1997; Rock et al., 1998) and that expression of a constitutive mutant in mammalian cells activated NFκB (Medzhitov et al., 1997). Further research has since found 10 functional Toll-like receptors (TLR) in humans (Kawai and Akira, 2007; Kawai and Akira, 2010). The importance of TLR signaling to infections was clearly defined when Poltorak et al. discovered that the C3H/HeJ mice were resistant against endotoxic shock but highly susceptible to Gram-negative infections because of a mutation in their intracellular signaling domain of TLR4 (Poltorak et al., 1998). This observation began the interesting task of characterizing the TLRs and their roles in innate immunity.

In flies, there are nine Toll genes. Of them, Toll-1 is the most important for the induction of AMPs, while two other Tolls (-5 and -9) play minor roles (Imler et al., 2000; Tauszig et al., 2000; Ooi et al., 2002). Whereas, mammalian TLRs bind to

PAMPs, Toll's ligand is the host protein Spätzle. Spätzle is secreted as an inactive precursor and is cleaved through a serine protease cascade, which is initiated by PRRs binding to PAMPs. *Drosophila* PRRs can differentiate between Gram-positive and – negative bacteria peptidoglycan, by the third residue of the peptide crosslinks. Most peptidoglycan from Gram-positive bacteria contains a lysine residue, whereas peptidoglycan present in Gram-negative bacteria and *Bacilli spp.* primarily incorporates meso-diaminopimelic acid (DAP) at this position (Schleifer and Kandler, 1972). The PRRs GNBP-1, PGRP-SA, and PGRP-SD are secreted and are important to differing degrees for binding Gram-positive bacterial peptidoglycan. GNBP-1 cleaves peptidoglycan into muropeptides allowing PGRP-SA to bind to them with higher affinity (Wang et al., 2006). According to Wang et al., different bacterial species initiate the formation of different peptidoglycan binding complexes. For example while *M.luteus* peptidoglycan binds to only PGRP-SA and GNBP-1, *S.aureus* needs those two proteins as well as PGRP-SD to mount an optimal host response (Wang et al., 2008). Regardless of the combination, when peptidoglycan binds to these proteins, the serine protease ModSP is activated (Gobert et al., 2003); (Michel et al., 2001); (Bischoff et al., 2004); (Buchon et al., 2009). Alternatively, ModSP activation can also be triggered by GNBP-3 binding to fungal β-(1,3)-glucan (Buchon et al., 2009). ModSP then activates the serine protease Grass (Buchon et al., 2009), which then activates Spätzle processing enzyme (SPE) (Kambris et al., 2006; El Chamy et al., 2008). (SPE) is directly responsible for Spätzle's cleavage (Jang et al., 2006) at an arginine residue to create the active ligand Spätzle C-106 (Weber et al., 2003), which can then bind to Toll. Fungi and Gram-positive bacteria can also

activate SPE through an alternative serine protease cascade involving the protease Persephone (Ligoxygakis et al., 2002a; El Chamy et al., 2008).

Once Toll has bound to cleaved Spätzle and undergone dimerization, it recruits *Drosophila* MyD88 (dMyD88) through a TIR-TIR interaction (Tauszig-Delamasure et al., 2002). dMyD88 recruitment to the receptor is also dependent upon a C-terminal phosphoinositide binding domain (Marek and Kagan, 2012). Like its mammalian homolog, dMyD88 contains a death domain (DD). The Toll-dMyD88 complex recruits the DD-containing protein Tube, which then recruits the kinase Pelle (an IL-1 receptor associated kinase homolog) (Galindo et al., 1995; Towb et al., 1998). Pelle/IRAK-1 Interacting protein (Pellino) positively regulates the pathway at this step, although its molecular function is currently not clear (Grosshans et al., 1999; Haghayeghi et al., 2010). Nonetheless, activated Pelle then leads to the degradation of an IκB homolog (Cactus) (Galindo et al., 1995; Reach et al., 1996). Cactus degradation is dependent upon it first being phosphorylated (Grosshans et al., 1994) by a kinase that has yet to be identified. It is possible that Pelle, itself, phosphorylates Cactus, as it was the only kinase identified to be required for Cactus phosphorylation from a large scale RNAi screen of all predicted *Drosophila* kinases (Huang et al., 2010). However, no evidence has shown that Pelle directly phosphorylates Cactus *in vivo*.

Cactus degradation releases the NFκB homolog Dif allowing it to translocate into the nucleus (Belvin et al., 1995; Bergmann et al., 1996; Wu and Anderson, 1998) and

upregulate the production of AMPs such as Drosomycin, Defensin, and Metchnikowin (Irving et al., 2001). The Friend of GATA factor U-shaped, the Toll Activation Mediating Protein (TAMP), and the G-protein coupled receptor kinase all act either with Cactus or downstream of it to positively regulate the Toll pathway but the mechanisms by which they do so are still unknown (Valanne et al., 2010). Although it was originally thought that Drosomycin was exclusively produced by the Toll pathway, it has recently been found that the response to a bacterial infection is often more complicated. For example, different bacteria species will trigger a unique AMP profile produced by synergistic interactions between both pathways (Tanji et al., 2007).

The AMP pathway initiated by Gram-negative bacterial peptidoglycan was named the IMD pathway because the RIP1 homolog was the first protein to be identified in the pathway (Lemaitre et al., 1995). Two PGRPs have been implicated as receptors for Gram-negative bacteria peptidoglycan in the IMD pathway, PGRP-LC and –LE (Takehana et al., 2002; Kaneko et al., 2006). PGRP-LC is a membrane protein with three splice variants, PGRP-LCx, PGRP-LCy, and PGRP-LCa, which each contain a different extracellular domain. The function of the PGRP-LCy variant is, as of yet, unknown. PGRP-LCx can either homodimerize to detect polymeric peptidoglycan or heterodimerize with PGRP-LCa to detect monomeric TCT peptidoglycan fragments (Werner et al., 2003; Kaneko et al., 2004). PGRP-LE, on the other hand, is found to be both intracellular and secreted. In its extracellular capacity, it relies upon PGRP-LC as a co-receptor, but when it is intracellular, it can act in an independent manner

to sense smaller peptidoglycan fragments that have been endocytosed (Kaneko et al., 2006). Once either PGRP has bound to its ligand, it recruits IMD, which then recruits *Drosophila* Fas associated death domain protein (dFADD) through homotypic death domain binding (Naitza et al., 2002). dFADD then binds to the Drosophila homolog of Caspase-8, DREDD through their death effector domains (Hu and Yang, 2000; Leulier et al., 2002; Zhou et al., 2005). Once recruited, DREDD cleaves IMD revealing a binding site for the ubiquitin E3-ligase DIAP2 (*Drosophila* Inhibitor of Apoptosis 2) (Paquette et al., 2010). This cleavage and subsequent interaction is crucial to the signaling pathway given that when the DIAP2 binding site in IMD is mutated, NFκB activation is severely limited. The E2 ligases, Effete, Bendless (Ubc13 homologue), and Uev1a, then polyubiquinate IMD through a K63-linkage resulting in the activation of *Drosophila* TGF- activated kinase (dTAK1) (Zhou et al., 2005; Paquette et al., 2010). The activation of NFκB by non-degradative ubiquitination is very similar to the mechanism used in mammalian systems (see later). Activated dTAK1 then in turn activates Drosophila IKK (Lu et al., 2001; Silverman et al., 2003), which is comprised of two subunits, the immune response deficient 5 (Ird5) kinase (IKKβ homolog) and the regulatory subunit Kenny (IKKγ homolog) (Rutschmann et al., 2000; Lu et al., 2001). IKK directly phosphorylates the NFκB homolog, Relish (Silverman et al., 2000). Relish contains its own IκB domain, which conceals its nuclear localization signal and keeps it in the cytoplasm. Once phosphorylated, the IκB domain becomes cleaved, allowing the Rel homology domain (RHD) to translocate to the nucleus and upregulate AMP production (Stoven et al., 2000; Stoven et al., 2003a). The identity of the enzyme responsible for Relish

cleavage is still unclear. The caspase DREDD can physically interact with Relish (Stoven et al., 2003b) but direct cleavage has never been shown. On the other hand, DREDD has been shown to directly cleave IMD and the cleavage sites in IMD and Relish are identical (Paquette et al., 2010). Further experimentation is needed to clarify DREDD's role in the IMD signaling pathway.

The IMD signaling pathway is under tight control with many proteins acting to negatively regulate it. The receptor PGRP-LC is inhibited by PGRP-SC1/2 and PGRP-LB, which both enzymatically degrade peptidoglycan and thus prevent receptors from binding to it (Mellroth et al., 2003; Bischoff et al., 2006; Zaidman-Rémy et al., 2006). PGRP-LF inhibits PGRP-LC more directly by binding to the receptor and preventing contact with its ligand (Persson et al., 2007; Maillet et al., 2008; Basbous et al., 2011). The protein Pirk is upregulated transcriptionally by IMD signaling, it then acts in a negative feedback loop to bind PGRP-LC and IMD to prevent further signaling (Kleino et al., 2008). Plenty of SH3s (POSH) is another negative regulator upregulated by IMD signaling. It acts as an E3 ligase to target TAK1 for proteasomal degradation (Tsuda et al., 2005). The Drosophila Fas associating factor 1 homolog, Caspar, inhibits the cleavage of Relish. *caspar* mutant flies constitutively express the AMP Diptericin and are less susceptible to bacterial infection (Kim et al., 2006). On the other hand, flies without the negative regulator cylindromatosis tumor suppressor gene (CYLD) show increased susceptibility, indicating the complexity of IMD pathway signaling. CYLD is associated with skin tumors in humans and acts as a deubiquitylating enzyme. Its function is conserved in

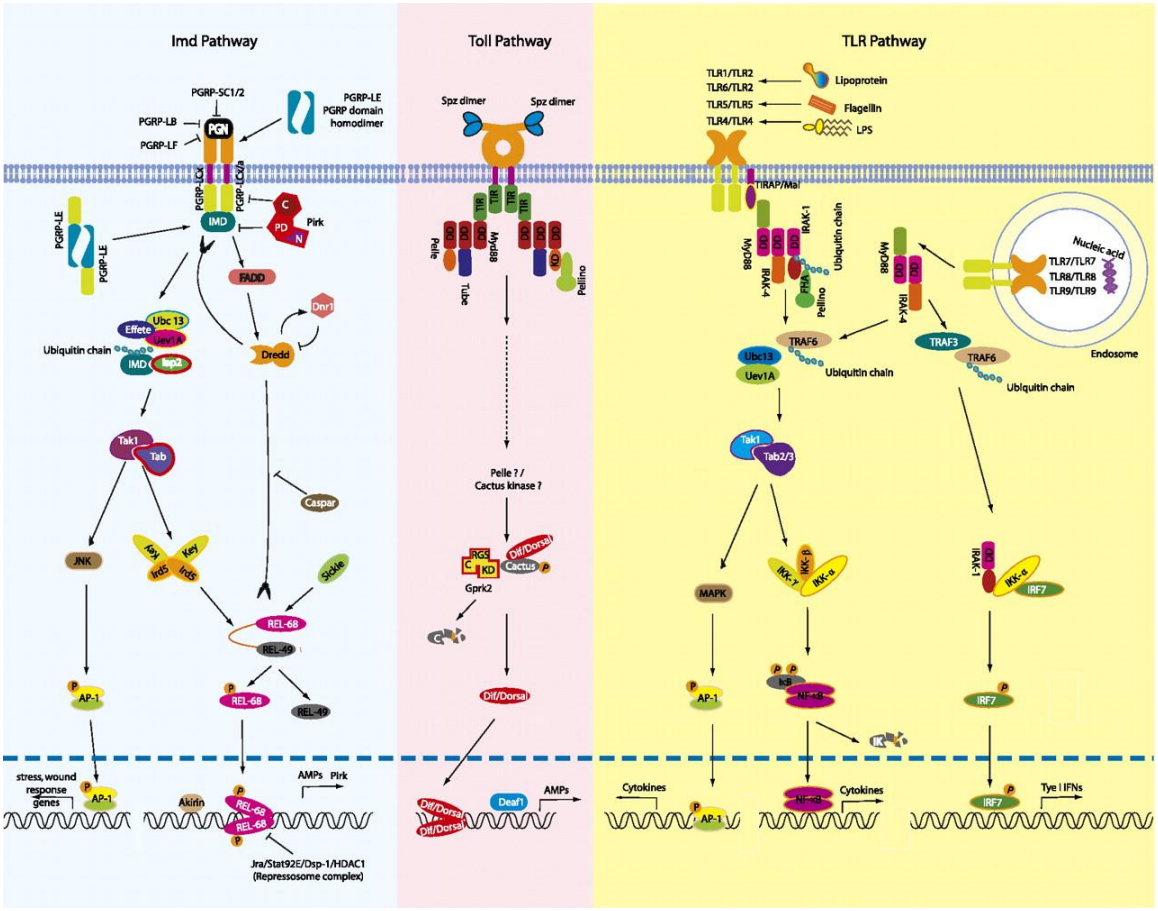



**Figure 1-2: (adapted from Valanne et al. 2011) There are distinct similarities between the mammalian TLRs, and *Drosophila* Toll and IMD signaling pathways.** Both the TLR and the Toll pathway utilize homodimerization of TIR domains to recruit their adaptors which then recruit a serine-threonine kinsae through death domain interactions. Phosphorylation and ubiquitination of an IκB family member releases an NFκB protein and allows it to translocate to the nucleus. Both the TLR4 and the IMD pathway employ the non-degradative K63-linked ubiquitination involving TAK1 to activate downstream pathways.

*Drosophila*, however the mechanism by which it negatively affects IMD signaling is unclear (Tsichritzis et al., 2007). Another protein involved in removing polyubiquitin chains, dUSP36/Scny, acts as a negative regulator by decreasing K63 linked polyubiquitin and promoting K48 linked polyubiquitin on IMD, thus leading to its degradation by proteasome (Thevenon et al., 2009).

Both Toll and IMD signaling primarily occur in the *Drosophila* fat body, which is similar to the mammalian liver. However, other tissues such as the tracheal epithelia, phanryx, reproductive tract, gut, and phagocytes can also play a more minor localized role in producing AMPs (Samakovlis et al., 1990; Tzou et al., 2000; Liehl et al., 2006). It is not clear if the signaling in the local AMP responses is the same as what is known in the systemic response. One difference in regulation is that an intestinal specific homeobox protein, Caudal, negatively regulates AMP induction by commensal bacteria. During a gut infection, the high bacterial load overcomes Caudal repression and allows AMPs to be produced (Ryu et al., 2008).

Like the IMD pathway in *Drosophila*, the mammalian TLR pathways also employ membrane entrenched pattern recognition receptors. Each TLR directly binds to a different PAMP. TLR4, for example, binds with high affinity to the Gram-negative outer membrane associated molecule, lipopolysaccharide (LPS). As Gram-negative bacteria invade, divide, and die, they shed their LPS coating into host tissue and fluids (Mackowiak, 1984) where it binds to LPS-binding protein (LBP), forming a complex that then binds to CD14 (Schumann et al., 1990; Dunzendorfer et al., 2004;

Lloyd and Kubes, 2006; Sauter et al., 2007). This heterotrimeric complex then allows the lipid A tail of LPS to bind to TLR4 with the help of MD2 (Sauter et al., 2007) on the surface of macrophages, neutrophils, dendritic cells, endothelial cells, and mucosal epithelial cells (Abbas, 2003; Kawai and Akira, 2007).

In human hosts, the binding of LPS to its receptor (TLR4) causes a variety of cellular responses including an increase in apoptosis and NFκB-induced gene expression (Harlan et al., 1983a; Harlan et al., 1983b; Kawai and Akira, 2007). Although, apoptosis and NFκB are both necessary to induce a proper immune response in the host, they are also each harmful to the host in their own way. An increase in apoptosis causes a decrease in the number of leukocytes present and fighting at the local infection. Although apoptosis is inevitable for most leukocytes, at the height of infection it is important for them to be prevalent in the infected tissues. In fact, an increase in lymphocyte-programmed cell death during sepsis increases the mortality rate of the patients (Hotchkiss et al., 1999b). Apoptosis is an energy-dependent, non-inflammatory form of cell death induced by the proteolytic caspase cascade and characterized by cell shrinkage, nuclear condensation, and membrane blebbing (Cohen, 1997). Although apoptosis does not engage an inflammatory response, it plays a role in multiple organ failure in septic patients (Hotchkiss et al., 1999a). Sepsis causes an increase in apoptosis in lymphocytes and induces PCD in other host cells such as mucosal epithelial cells (Coopersmith et al., 2002) and endothelial cells (Zhou et al., 2004). Endothelial cells are of particular interest as they are one of the first cell types to encounter a septic microorganism as they line the bloodstream. LPS,

devoid of any other microbial product, causes endothelial cell death. The death is a result of LPS directly interacting with the cell, as death is not prevented by blocking extracellular death receptors such as Fas and TNF receptor-1(Choi et al., 1998) or by blocking new protein synthesis, which would be necessary if the process was mediated by cytokines (Harlan et al., 1983a).

The signaling pathway that transmits apoptotic signals from LPS /TLR4 binding is not as well studied as the pathway leading to NFκB activation, but several of the components are known. Once TLR4 has bound to its ligand through its extracellular leucine rich repeat (LRR) domain, it becomes homodimerized (Toshchakov et al., 2011). The receptor's intracellular Toll/IL-1R (TIR) domain is then able to recruit the small molecule, TIR containing adaptor protein (TIRAP)/MyD88 adaptor-like (Mal), through a TIR-TIR interaction (Fitzgerald et al., 2001; Horng et al., 2001). It has recently been discovered that TIRAP contains a PIP2-binding domain in its N-terminus that allows it to bind to the plasma membrane. This binding interaction is necessary for TIRAP-mediated LPS-induced signaling to occur, given that when cells are depleted of PIP2 through the use of a *S.typhimurium* PI phosphatase, SopB, LPS-induction of NFκB was inhibited (Kagan and Medzhitov, 2006). TIRAP acts as an adaptor to recruit MyD88 to the complex through another TIR-TIR interaction (Kagan and Medzhitov, 2006). Although MyD88 can bind to other TLRs' TIR domains directly, it is believed that TIRAP is a necessary adaptor between MyD88 and TLR4 because both of their TIR domains are predicted to be electropositive, while TIRAP's is predicted to be electronegative (Dunne et al., 2003). Zhande et al.

have shown that FADD, a well-known regulator of cell death, can be recruited to MyD88 (Aliprantis et al., 2000) through an interaction with IRAK-1 (Zhande et al., 2007), a serine-threonine kinase originally identified for its role in IL-1R signaling. All three molecules (FADD, MyD88, and IRAK1) contain death domains (DD) and use these domains to facilitate their interactions with one another (Zhande et al., 2007). FADD's recruitment to the activated TLR4 intracellular complex both allows FADD to successfully initiate pro-apoptotic signals and sequesters IRAK1 (Zhande et al., 2007), effectively down-regulating NFκB activation (see later). FADD interacts with pro-Caspase-8 through mutual death-effector domains leading to its dimerization, cleavage, and subsequent activation (Muzio et al., 1996). Caspase-8 is an initiator caspase that when activated, begins the caspase cascade, ultimately resulting in the cell undergoing apoptosis (Salvesen and Abrams, 2004). The molecule FLIP was long believed to be an inhibitor of this process as its long form is very similar to Caspase-8, in that it also contains a DED domain, a caspase domain, and is similar in size. However, its caspase domain contains a mutation, rendering it inactive (Irmler et al., 1997). All of these properties, along with *in vitro* data, indicate that it acts as an endogenous dominant-negative for the cell and protects against apoptosis by sequestering FADD (Bannerman et al., 2001; Bannerman et al., 2004). These findings were challenged when it was discovered that FLIP/Caspase-8 heterodimers are proteolytically active and their formation is preferred over Caspase-8 homodimers (Chang et al., 2002; Micheau et al., 2002). Subsequent research seems to be consistent with the first hypothesis suggesting that FLIP's role is anti-apoptotic. Notably, the viral serpin CrmA, which blocks enzymatic activity of Caspase-8

homodimers but not Caspase-8/FLIP heterodimers, can block Caspase-8 mediated apoptosis (Oberst et al., 2011).

Although apoptosis plays an important role in septic mortality, the cytokine release that results from LPS binding to TLR4 is responsible for the uncontrolled inflammatory response in septic patients (Danner et al., 1991). These molecules contribute to overall inflammation, pain, fever, and swelling, leading to both host discomfort and tissue destruction. TNFα in particular plays an important role in the inflammatory response as mice without the TNFα receptor are resistant to septic shock, but unable to fight off a localized infection (Pfeffer et al., 1993). The process of LPS-induced activation of NFκB-dependent genes has been well-characterized in humans and depends on a complicated and often redundant signaling pathway. The primary signaling pathway begins as previously described with TIRAP associating with the intracellular TIR domain of TLR4 through its own TIR domain. However, in order for LPS stimulation to result in NFκB activation, both TLR4 and TIRAP require several modifications. First, both TLR4 and TIRAP become tyrosine phosphorylated. Chen et al. found that when human peripheral monocytes were treated with genistein, a tyrosine phosphorylase inhibitor, it inhibited both TLR-4 phosphorylation and LPS-induced IL-8 production (Chen et al., 2003). Further research by Medvedev et al. indicates that Lyn is the responsible tyrosine kinase (Medvedev et al., 2007). TIRAP's tyrosine phosphorylation at amino acid residues 86 and 187 by Bruton's tyrosine kinase (Btk) was also found to be necessary for inducing NFκB-driven luciferase activity in HEK293 cells following treatment with LPS (Gray et al., 2006).

Additionally, TIRAP must be cleaved by Caspase-1 to produce the activated N-terminal 21 kD fragment. TIRAP with a mutated cleavage site (D198A) was able to bind Caspase-1, but could not be cleaved or induce NFκB-driven luciferase (Miggin et al., 2007). If all of these modifications have occurred then MyD88 is recruited to the complex (Medzhitov et al., 1998).

The TLR-4/TIRAP/MyD88 complex recruits and activates IRAK4 (a serine-threonine kinase) (Burns et al., 2003). Activated IRAK4 then transphosphorylates IRAK1 (Li et al., 2002), which then autophosphorylates until it becomes hyperphosophorylated (Kollewe et al., 2004). This allows TNF-α receptor associated factor (TRAF) 6 to be recruited to the complex (Cao et al., 1996; Kollewe et al., 2004) where it undergoes non-degradative polyubiquitination (Deng et al., 2000). TIRAP contains a TRAF-6 binding domain, and the two molecules are capable of direct interaction (Mansell et al., 2004). It has therefore been hypothesized that TIRAP can signal directly to TRAF-6 bypassing MyD88 and IRAKs. TRAF-6 contains both a TRAF and a RING domain (Cao et al., 1996). The latter functions as an E3 ubiquitin ligase and when TRAF6 associates with the E2 ubiquitin conjugating complex Ubc13/Uev1, it becomes polyubiquinated through a K63 linkage (Deng et al., 2000). TRAF6 then oligomerizes through its TRAF domain, which allows it to then form a complex with TAK1, TAB1, and TAB2 (Jiang et al., 2002). Recruited TAK1 then activates IKK and MAPKs (Sato et al., 2005) (Wang et al., 2001). The ERK1/2, p38, and JNK MAPK pathways are all induced by LPS, and lead to the phosphorylation of Elk1, ATF2, cJun, and CREB, which then regulate cell growth, survival, and cytokine

induction (Chang and Karin, 2001; Guha and Mackman, 2001; Brown et al., 2011). IKK is made up of two catalytic subunits IKKα and IKKß and one regulatory subunit IKKγ/NEMO (Yamaoka et al., 1998; Zandi et al., 1998). The IKKß subunit phosphorylates IκB, targeting it for ubiquitination and subsequent proteasome degradation (Chen et al., 1995; Mercurio et al., 1997). Once NFκB has been released from the inhibitory factor IκB, it translocates into the nucleus (Baeuerle and Baltimore, 1988) and binds to DNA sequences to initiate the production of inflammatory cytokines such as TNFα (Goldfeld et al., 1990), IL-1β (Hiscott et al., 1993), and IL-6 (Kannabiran et al., 1997). IκBζ/MAIL/INAP, a member of the IκB family, is only induced by LPS in a MyD88-dependent manner and appears to be required to maximize the induction of IL-6, IL-12b, and Csf2 (Yamamoto et al., 2004).

A secondary pathway activates a delayed NFκB response, which is initiated when TLR4 moves from the plasma membrane to an endosomal compartment (Kagan et al., 2008). It involves two other TIR-domain containing molecules: TIR domain containing adaptor inducing IFN-β (TRIF) and TRIF related adaptor molecule (TRAM). TRIF is also known as TIR domain-containing adaptor molecule-1 (TICAM1) and TRAM is also known as TICAM2. These molecules were originally discovered to activate the transcription factor IRF3, which upregulates the IFN-β response (Oshiumi et al., 2003; Yamamoto et al., 2003a; Yamamoto et al., 2003b). However, they also play a redundant role in activating NFκB albeit with delayed kinetics. TRAM, similar to TIRAP, acts as an adaptor molecule that links TRIF to

TLR4 (Oshiumi et al., 2003). Like the other adaptor molecules, TRIF (Yamamoto et al., 2003a) and TRAM (Oshiumi et al., 2003) both contain TIR domains, which is how TRAM binds to TLR4 and to TRIF. Similar to TIRAP, TRAM must be recruited to the plasma membrane in order to be recruited to TLR4, which is dependent upon a myristoylation site found between amino acid residues 1 to 8 (Rowe et al., 2006). Also similar to TIRAP, TRAM is phosphorylated, but it is at a serine instead of a tyrosine. TRAM becomes phosphorylated at S16 after LPS activation (McGettrick et al., 2006) by protein kinase ε (Castrillo et al., 2001). Once TRIF is recruited through its association to TRAM, it binds to RIP1 via its C-terminus and to TRAF3 and 6 through its N-terminus (Yamamoto et al., 2002; Sato et al., 2003; Jiang et al., 2004; Meylan et al., 2004; Häcker et al., 2006). Overexpression of either the TRIF N-terminus or C-terminus results in NFκB target induction indicating that either RIP1 or TRAF6 can activate NFκB. However, only overexpression of the C-terminus results in the induction of IFNβ, consistent with TRIF mediating IRF signaling via TRAF3. TRAF3 associates with TBK1 (TANK Binding Kinase-1) and IKKi (Guo and Cheng, 2007), both of which are required for the dimerization and translocation of IRF3 (Fitzgerald et al., 2003; Hemmi et al., 2004). TRIF/IRF3 signaling is also important in LPS tolerance, a phenomenon where cells respond less vigorously to a second LPS challenge after being preconditioned with a small primary dose. Cells undergoing tolerance display a lower expression of inflammatory cytokines TNFα, IL-6, and IL-8 and a higher expression of TLR4 negative regulators (Piao et al., 2009).

Because the overproduction of inflammatory cytokines has such severe consequences, the TLR4 signaling pathway is under tight regulation. Many steps of negative regulation are involved in keeping the TLR4 pathway in check. The most upstream inhibitor is soluble TLR4 (sTLR4), which is a soluble variant of TLR4 without an intracellular TIR domain. It acts to sequester ligand from the membrane bound TLR4 thus decreasing intracellular signaling (Iwami et al., 2000). The TIR-containing adaptor molecule, SARM, also acts as a negative regulator. SARM is the only TIR adaptor protein that does not lead to NFκB activation when overexpressed (Liberati et al., 2004). Carty et al. found that SARM specifically inhibits the TRIF/TRAM pathway and not TIRAP/MyD88 (Carty et al., 2006). The TIR containing proteins SIGIRR and ST2L, on the other hand, specifically prevent MyD88 from being recruited to TLR4 (Brint et al., 2004; Qin et al., 2005).

SOCS1 (Suppressor of Cytokine Signaling-1) targets TIRAP through its PEST domain, resulting in TIRAP becoming ubiquitinated at amino acid residues 15 and 16. Interestingly, TIRAP must first be phosphorylated by Btk in order to bind to SOCS1 (Mansell et al., 2006). TRIAD3 (Triad Domain containing protein-3) also inhibits the pathway through ubiquitination. It has a multitude of targets including TIRAP, TRIF, and RIP1 (Fearns et al., 2006). A short splice variant of MyD88, MyD88s, inhibits the next step of the MyD88-dependent signaling pathway. It is missing the intermediary sequence between the TIR domain and the DD and is upregulated when cells are exposed to pro-inflammatory molecules (Janssens et al., 2002). It acts as an endogenous dominant-negative, binding TIRAP but inhibiting recruitment of IRAK4,

resulting in a non-phosphorylated and therefore non-activated IRAK1 (Burns et al., 2003). TOLLIP has also been shown to negatively affect IRAK1 activation and has thus been considered an inhibitory molecule (Zhang and Ghosh, 2002). However, an *in vivo* study using TOLLIP null mice showed that the mutant mice had a lower induction of IL-6 and TNFα when challenged with LPS (Didierlaurent et al., 2006) indicating that TOLLIP's role may be more complicated than previously thought. TOLLIP may act as a chaperone protein that allows IRAK1 to be recruited to the signaling complex with its kinase activity turned off. The data indicates that TOLLIP's role within the signaling pathway may be highly dependent on its concentration. The inhibitory function of IRAKM, on the other hand, has been confirmed both *in vitro* and *in vivo*. Kobayashi et al. found that IRAKM$^{-/-}$ mice produced a significantly higher amount of proinflammatory molecules and that HEK293 cells expressing IRAKM did not form the IRAK1/TRAF6 complex (Kobayashi et al., 2002). IRAKM expression is significantly upregulated after LPS treatment of cells and plays a role in endotoxin tolerance (Liu et al., 2008) indicating its importance in maintaining the fine balance between pro- and anti-inflammation. IRAK2c and 2d are two more negative regulators from the IRAK family that when overexpressed also lead to decreased LPS-induced NFκB signaling (Hardy and O'Neill, 2004). Lastly, A20 and CYLD negatively regulate the pathway by removing K63-linked polyubiquitin chains. A20 is a Zn finger-containing protein that was originally found to be upregulated by TNFα. It removes polyubiquitin chains from TRAF6, thus preventing it from activating TAK1 (Heyninck and Beyaert, 1999; Boone et al., 2004; Wertz et al., 2004). A20 may also inhibit IRF3 dimerization by

interacting with TBK1 and IKKi (Saitoh et al., 2005). As previously reviewed, both *Drosophila* and mammalian CYLD negatively regulate NFκB signaling. While the target(s) of *Drosophila* CYLD deubiquitination are so far unknown, mammalian CYLD has been shown to regulate IKK activation by inactivating TRAF6 and preventing its recruitment of TAK1 (Kovalenko et al., 2003; Reiley et al., 2007; Jin et al., 2008).

## 1.2 Glutamate Transport

### 1.2.1 In the CNS

Glutamate is an important excitatory neurotransmitter in both mammals (Fonnum, 1984; Niciu et al., 2012) and *Drosophila (*Jan and Jan, 1976; Chase and Kankel, 1987; Nässel, 1996). Excitotoxicity caused by increased extracellular glutamate concentrations is a major cause of neuronal death in neurodegenerative diseases such as stroke, ALS, and Parkinson's disease (Bruijn et al., 2004; Blandini, 2010; Kostandy, 2012). Therefore, glutamate transport is highly regulated within the central nervous system. Intracellular [glutamate] in cells within the mammalian CNS is approximately 10mM (Carlson et al., 1989; McMahon and Nicholls, 1991), while the extracellular [glutamate] is approximately 2μM (Baker et al., 2002). This steep gradient is maintained by the $X^-_{AG}$ $Na^+/K^+$ dependent transporters also known as excitatory amino acid transporters (EAATs), which transport glutamate into the cell along with three co-transported $Na^+$ ions and one counter-transported $K^+$ ion (Zerangue and Kavanaugh, 1996). In mammals, there are five EAATs expressed in various cell types related to the CNS. EAAT1/GLAST/SLC1A3 and EAAT2/GLT-1/SLC1A2 are primarily expressed in glial cells (Pines et al., 1992; Storck et al.,

1992; Phillis et al., 2000); EAAT3/EAAC1/SLC1A1 is expressed in neurons (Kanai and Hediger, 1992); EAAT4/SLC1A6 is expressed in the cerebellum (Fairman et al., 1995); and EAAT5/SLC1A7 is expressed in retinal neurons (Arriza et al., 1997). Interestingly, when the neuronal transporter EAAT3 is deleted in mice, they display normal motor skills and no spontaneous seizures although they do experience increased brain atrophy with aging (Peghini et al., 1997; Aoyama et al., 2006). EAAT1 and 2, on the other hand appear to play a strong role in clearing extracellular glutamate from the CNS. EAAT1 deficient mice have decreased motor function and increased susceptibility to brain injury (Watase et al., 1998). Mice without EAAT2 are even more severely affected, experiencing a significantly increased extracellular glutamate concentration that correlates with spontaneous seizures (Tanaka et al., 1997).

*Drosophila*, on the other hand, appear to express only two EAATs, dEAAT1 and dEAAT2 (Seal et al., 1998; Besson et al., 1999; Kawano et al., 1999). They are both glial-specific transmembrane proteins but only dEAAT1 transports glutamate with any efficiency, as dEAAT2 only shows high binding affinity to aspartate (Seal et al., 1998; Besson et al., 2000). dEAAT1 deficient flies exhibit behavioral symptoms similar to those seen in the EAAT2 mice, including decreased motor defects and hypoactivity. These phenotypes are rescued by the addition of either riluzole, which decreases extracellular glutamate, or melatonin, which acts as an antioxidant. Additionally, expression of human EAAT2 could compensate for a lack of dEAAT1 in the fly (Rival et al., 2004). Thus, it appears that dEAAT1 functions in a similar

manner as human EAAT2 and that the behavioral phenotypes seen in dEAAT1 deficient flies are mediated by an increase in extracellular glutamate and oxidizing molecules.

Glutamate excitotoxicity is a multifaceted process in which an overstimulation of neuronal glutamate receptors leads to a toxic level of intracellular $Ca^{2+}$ resulting in cell death (Valdovinos-Flores and Gonsebatt, 2012). There are several types of glutamate receptors on neurons which result in an increase in intracellular $Ca^{2+}$ levels. N-methyl D-Aspartate (NMDA) and α amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are ionotropic glutamate receptors that become permeable to $Na^{+}$, $K^{+}$, and $Ca^{2+}$ ions when bound to glutamate. AMPA receptors can also move $Zn^{2+}$ when activated. Metabotropic glutamate receptors (mGluRs) on the other hand are 7 transmembrane G-protein coupled receptors (Valdovinos-Flores and Gonsebatt, 2012). When mGluR1 binds glutamate, it signals to its associated $G_q$ protein to activate phospholipase C, which cleaves $PIP_2$ to release $IP_3$ (Lin et al., 1997). Receptors on the ER bind to $IP_3$ causing its $Ca^{2+}$ stores in the lumen to be released back into the cytosol (Periasamy and Kalyanasundaram, 2007). *Drosophila* possess NMDA-type and AMPA ionotropic glutamate receptors as well as metabotropic GluRs glutamate receptors in their CNS. DNMDAR-I and DNMDAR-II are ionotropic receptors that possess high sequence similarity to mammalian NMDARI and NMDAR2A-D, respectively (Ultsch et al., 1993; Völkner et al., 2000). The *Drosophila* CNS also expresses DGluR-IA and DGluR-IB, which show high similarity to mammalian AMPA glutamate receptors (Völkner et al.,

2000), although DGluR-IA shows preferential binding to kainate rather than AMPA (Ultsch et al., 1992). Finally, the DmGluRA receptor, which is expressed in the CNS of the late *Drosophila* embryo, binds to mGluR3-specific agonists (Parmentier et al., 1996). Although, it is unclear whether these *Drosophila* receptors lead to the same signaling pathways that occur in mammalian cells, *Drosophila* neurons are sensitive to glutamate excitotoxicity, which appears to be $Ca^{2+}$ dependent (Daniels et al., 2011; Wu et al., 2011).

An increase in cytosolic $Ca^{2+}$ following glutamate binding to its receptors is normal as $Ca^{2+}$ is an essential signaling molecule that regulates various vital cellular processes such as the axonal growth and synaptic signal strength (Ghosh and Greenberg, 1995). However, overstimulation of glutamate receptors leads to excessive intracellular $Ca^{2+}$ levels which then causes constitutive activation of destructive enzymes such as calpain proteases, neuronal nitric oxide synthase (nNOS), and NADPH oxidase (NOX) (Hardingham, 2009). Products derived from NOS and NOX, such as NO and ROS species are directly deleterious to the cell. The cell normally produces a high concentration (2-3mM) of the antioxidant, glutathione (GSH) (Rice and Russo-Menna, 1998), which reduces rogue oxidative molecules. However, GSH production is directly dependent upon glutamate transport and therefore is inhibited by high extracellular glutamate concentrations (Rimaniol et al., 2001).

GSH is a tripeptide comprised of L-glutamate, L-cysteine, and L-glycine. It is synthesized in the cytosol through two enzymatic steps, the first of which is catalyzed by γ-glutamylcysteine synthetase (GCL) (Mandeles and Block, 1955) . GCL attaches L-glutamate to L-cysteine through a non-canonical linkage to form γ-glutamyl-cysteine. The covalent bond is formed between the amine group from the cysteine backbone and the carboxyl group from the glutamate sidechain (Valdovinos-Flores and Gonsebatt, 2012). GSH synthetase (GSS) then catalyzes the addition of L-glycine to γ-glutamyl-cysteine to the carboxy terminus of the cysteine with a normal peptide bond, producing the tripeptide GSH (Snoke and Bloch, 1955). GSH synthesis is limited by the availability of L-cysteine within the cell (Shih et al., 2006) meaning that an increase of intracellular L-cysteine leads to an increase in GSH.

Cells can absorb L-cysteine directly via $Na^+$ -independent neutral amino acid L-system transporters (Shanker and Aschner, 2001). However, under aerobic conditions most extracellular cysteine is in the oxidized dimer form, cystine (Wang and Cynader, 2000), which cannot be transported by L-system transporters (Shanker and Aschner, 2001). Instead, many cells in the CNS use the $X^-_c$ antiporter to exchange an intracellular glutamate for an extracellular cystine molecule (Bannai, 1986; Cho and Bannai, 1990). Once cystine is inside the cytosol, it is reduced to cysteine and can then be used to synthesize GSH. The direction of the antiporter is concentration dependent (Bannai, 1986). If the EAATs are functioning properly, then the glutamate concentration within the cell will be approximately 1000 fold higher than the extracellular concentration, thus driving the $X^-_c$ antiporter in the canonical direction.

However, if the EAATs are no longer able to maintain the glutamate gradient, the $X^-_c$ antiporter will begin to pump cystine out of the cell as it brings glutamate in. The $X^-_c$ antiporter is a disulfide-linked heterodimer of $X_{CT}$/SLC7A11 and the glycoprotein 4F2hc/SLC3A2/CD98 (Sato et al., 2000). There are five Drosophila genes predicted to encode an $X_{CT}$ subunit but only one, *genderblind* (gb), has been functionally characterized to act as a cystine/glutamate antiporter (Augustin et al., 2007). *gb* deficient larvae have lower glutamate levels in their hemolymph (Piyankarage et al., 2008) and behavioral abnormalities (Grosjean et al., 2008). The $X_{CT}$ subunit confers specificity and although it is expressed in a variety of mammalian CNS cells such as astrocytes, microglia, and glioma cells, it is not present in mature neurons (Cho and Bannai, 1990; Piani and Fontana, 1994). Accordingly, cultured neuronal cells increase GSH synthesis when L-cysteine, but not L-cystine, is added to media (Kranich et al., 1996). Thus regulation of GSH synthesis in neuronal cells is even more complicated than it is in other CNS cell-types.

As astrocytes express the $X_{CT}$ subunit, they regulate GSH synthesis in the manner previously described. They release approximately 10% of their intracellular GSH stores into the extracellular milieu where it is quickly bound by γ-glutamyl transpeptidase (γ- GT) (Anderson et al., 1989; Kranich et al., 1996). γ-GT catalyzes the production of the dipeptide L-cystyl-glycine from GSH and L-glycine. The dipeptide is then cleaved into L-cysteine and L-glycine by the ectopeptidase ApN on the surface of the neurons (Dringen et al., 2001). This creates a local increase in the concentration of extracellular L-cysteine, which is quickly pumped into the neuron

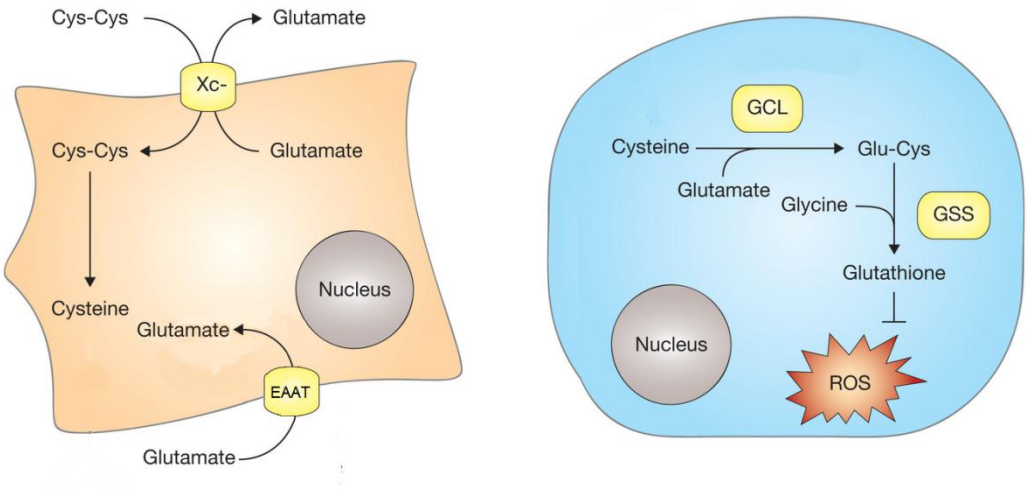



**Figure 1-3 (adapted from (DeBerardinis, 2012): Glutamate transport in the CNS.** (Left panel) EAATs present on astrocytes, glial cells, or macrophages transport glutamate from outside the cell to inside creating a glutamate gradient, which drives the $X_c^-$ transporter to transport cystine (Cys-Cys) into the cell, while exporting glutamate. Cys-Cys becomes reduced to cysteine once inside the cell. (Right panel) GCL (γ-glutamylcysteine synthetase) catalyzes the addition of cysteine to glutamate to form the dipeptide Glu-Cys, which then combines with glycine to form glutathione. The latter reaction is catalyzed by GSS (glutathione synthetase). Glutathione then acts as an antioxidant by reducing reactive oxygen species present in the cytosol.

via L-system transporters before it is oxidized to L-cystine. Thus GSH synthesis in neurons is dependent upon GSH synthesis in CNS support cells, which is in turn dependent upon tight regulation of glutamate and cystine transport. Excess extracellular [glutamate] overstimulates neuronal glutamate receptors which increases intracellular [$Ca^{2+}$] resulting in an increase in toxic molecules such as ROS. The neuron is unable to increase production of the antioxidant glutathione because it is dependent upon GSH synthesis from CNS support cells, which is inhibited by excess extracellular [glutamate]. Therefore, oxidative damage only increases within the cell leading to eventual death. Given that neurons are vital to an organism's physical and mental health and are not normally regenerative, their death leads to severe consequences. Glutamate induced excitotoxicity of neurons contributes to a multitude of serious neurodegenerative illnesses including ALS and Parkinson's Disease. It is less widely known that glutamate transport in immune cells has been implicated in human disease.

### 1.2.2. In the Immune System

Given the importance of glutamate transport in the nervous system, it is clear how misregulation of glutamate can lead to neurodegenerative diseases. Evidence indicates that immune cells can also play a role in glutamate transport and that this can also lead to neurological disease. For example, more than 10% of AIDS patients develop HIV-associated dementia (HAD) (Sacktor et al., 2001), a disease characterized by cognitive and motor dysfunction resulting from nerve death in the basal ganglia and the cerebral cortex (Aylward et al., 1993; Thompson et al., 2005).

Interestingly, HIV is unable to effectively infect neurons, but can infect macrophages circulating in the CNS (Budka et al., 1987; Eilbott et al., 1989). Mammalian macrophages express both EAATs and the $X^-_c$ antiporter, indicating that they are capable of transporting glutamate in a manner similar to glial cells (Rimaniol et al., 2000; Rimaniol et al., 2001). Several studies have discovered that HAD-associated nerve death is dependent on macrophage mitochondrial glutaminase (Zhao et al., 2004; Erdmann et al., 2007; Tian et al., 2008). This enzyme deaminates glutamine to glutamate and is elevated in HIV-infected macrophages. The elevation in glutamate concentrations results in the macrophages releasing glutamate into the extracellular milieu, leading to glutamate-associated toxicity in the surrounding nerve tissue.

Glutamate also plays a role in the immune system outside of the CNS, although the mechanisms through which it does so are unclear. HIV-infections cause an increase in glutamate levels in the plasma, which correlates with a decrease in intracellular cysteine and glutathione concentrations in circulating monocytes (Dröge et al., 1987; Eck et al., 1989; Ferrarese et al., 2001). Plasma glutamate levels are also elevated in patients with a wide range of other diseases including ALS (Iwasaki et al., 1992a), epilepsy (Janjua et al., 1992), and breast cancer (Ollenschläger et al., 1989). All three of the phagocytic mammalian lymphocytes: neutrophils, macrophages, and dendritic cells release glutamate upon stimulation with PAMPs such as LPS, formyl-methionyl-leucyl-phenylalanine, and Staphylococcal enterotoxin A (Rimaniol et al., 2001; Collard et al., 2002; Pacheco et al., 2006). Glutamate released from neutrophils bound to metabotrophic glutamate receptors on endothelial cells leads to an increase

in permeability (Collard et al., 2002). Dendritic cells, on the other hand, mediate the adaptive immune response through glutamate signaling. When dendritic cells are in contact with T cells they release glutamate through the $X^-_c$ transporter which then inhibits T cell activation by binding to the constitutively present mGluR5. After antigen presentation has occurred, the T cell then upregulates mGluR1, which when bound to glutamate stimulates T cell proliferation (Pacheco et al., 2006).

Finally, glutamate transport also plays a role in a genetic disorder where glutathione reductase is mutated, which results in defective neutrophils (Roos et al., 1979). Glutathione reductase is the enzyme that converts oxidized GSSG to reduced GSH. Without it, the redox system becomes skewed towards the oxidized state and is less able to reduce any free reactive species. Neutrophils from individuals with this genetic disorder have decreased glutathione reductase activity, which leads to increased levels of hydrogen peroxide. One study discovered a small impairment of these deficient neutrophils to kill *S.aureus* (Roos et al., 1979), however the effect of glutamate upon the functional properties of the phagocyte itself has not been well studied.

## 1.3 Significance

The cow and the fly are both interesting and useful systems in which to study bacterial infections because each provides its own particular advantage. The cow is particularly sensitive to Gram-negative infections, specifically to LPS (Berczi et al., 1966). Although human endothelial cells are protected against apoptosis when treated directly with LPS (Harlan et al., 1983b), bovine endothelial cells are not (Harlan et

al., 1983a). It is possible that this difference in sensitivity is at least in part due to a difference in TLR4 signaling. Several differences in this pathway in the bovine pathway indicate that this hypothesis is highly likely. For example, although human FADD downregulates NFκB activation in endothelial cells (Bannerman et al., 2002c), bovine FADD has no homologous effect (Szperka et al., 2005). Bovine FADD is however still functional as it retains the mechanism to upregulate LPS-induced apoptosis (Szperka et al., 2005). Another difference is that bovine endothelial cell FLIP mRNA is present at a concentration that is approximately 7-19 times lower than human endothelial cell FLIP; this decreased concentration of FLIP is correlated with a higher caspase activity when treated with LPS (Szperka et al., 2006). It is possible that more differences exist between human and bovine TLR4 signaling, and that by understanding why cows are so sensitive, it will be possible to safeguard human patients against Gram-negative toxicity.

Flies, on the other hand, are curiously insensitive to LPS but mount a strong immune response when exposed to peptidoglycan (Leulier et al., 2003; Kaneko et al., 2004). Additionally, *D.melanogaster* is a well-characterized animal model with numerous genetic and molecular tools available. They are particularly useful for studying glutamate transport as they have only one EAAT with a high affinity for glutamate. Additionally, multiple *in vivo* techniques have been developed in order to study the phagocytic capacity of the *Drosophila* macrophage. Therefore, *Drosophila* is an ideal model organism to study the effect that glutamate transport has on phagocytosis. This dissertation utilizes *Drosophila* as an *in vivo* tool, to further elucidate phagocytosis in

a useful biological context. The discovery of mammalian TLRs is credited to work previously done in *Drosophila*, and further characterization of the downstream signaling pathways has shown clear conservation between species (Gay and Gangloff, 2007). If researchers were to fully understand bacterial infections and their relation to glutamate transport, novel techniques could be developed to more effectively fight infections. This dissertation uses both cows and flies to further characterize how the immune system responds to bacterial infections; specifically using the bovine system to explore the TLR4 signaling pathways and the *Drosophila* model to further characterize how glutamate affects the phagocytosis of bacteria.

# Chapter 2: Functional Characterization of Bovine TIRAP and MyD88 in Mediating Bacterial Lipopolysaccharide-induced Endothelial NFκB Activation and Apoptosis

## 2.1 Abstract

Mastitis is a prevalent disease in dairy cows. Gram-negative bacteria, which express the pro-inflammatory molecule lipopolysaccharide (LPS), are responsible for the majority of acute clinical cases of mastitis. Previous studies have identified differential susceptibility of human and bovine endothelial cells (EC) to the pro-inflammatory and injury-inducing effects of LPS. The Toll-like receptor (TLR) 4 signaling pathway, which is activated by LPS, has been well-studied in humans, but not in ruminants. Human myeloid differentiation-factor 88 (MyD88) and TIR-domain containing adaptor protein (TIRAP) are critical proteins in the LPS-induced NFκB and apoptotic signaling pathways. To assess the role of the bovine orthologs of these proteins in bovine TLR4 signaling, dominant-negative constructs were expressed in bovine EC, and LPS-induced NFκB activation and apoptosis evaluated. The results from this study indicate that bovine MyD88 and TIRAP play a functional role in transducing LPS signaling from TLR4 to downstream effector molecules involved in NFκB activation and that TIRAP promotes apoptotic signaling.

## 2.2 Introduction

In humans, septic shock is one of the most common causes of death in intensive care units and is a major cause of morbidity and mortality worldwide (Parrillo et al., 1990;

Pinner et al., 1996). In cows, mastitis remains among one of the most prevalent diseases (Wells et al., 1998). Mastitis is also one of the most costly diseases of food production animals with worldwide economic losses associated with this disease estimated to approach $35 billion annually (Wellenberg et al., 2002). Systemic complications associated with mastitis include the development of septic shock. Gram-negative bacteria are responsible for ≥50% of the cases of septic shock in humans (Dahmash et al., 1993a; Zanetti et al., 1997a) and the majority of the acute clinical cases of mastitis in cows, which are correspondingly the most likely cases of mastitis to develop into septic shock (Erskine and Eberhart, 1991; Ziv, 1992).

A highly pro-inflammatory component expressed by all Gram-negative bacteria, lipopolysaccharide (LPS), has been implicated in the pathogenesis of septic shock and the deleterious inflammatory responses associated with acute clinical mastitis (Erskine and Eberhart, 1991; Ziv, 1992; Opal, 2007). The vascular endothelium is a key host target of LPS and the pathogenesis of certain complications associated with septic shock, including the development of vascular leak syndromes, has been attributed to LPS-induced endothelial dysfunction and/or injury (Grandel and Grimminger, 2003; Vallet, 2003).

Endothelial recognition of and responses to LPS are mediated, in part, by LPS binding protein (LBP) and CD14, which facilitate the presentation of the lipid A portion of LPS to MD2 and the leucine-rich repeat domains of Toll-like receptor (TLR) 4 (Henneke and Golenbock, 2002; Dunzendorfer et al., 2004; Dauphinee and

Karsan, 2006). TLR4 signaling is propagated by the recruitment of adaptor molecules to its Toll-IL-1 receptor (TIR) domain. Two known adaptor molecules, myeloid differentiation factor 88 (MyD88) (Medzhitov et al., 1998) and TIR domain-containing adaptor protein (TIRAP), also known as MyD88-adaptor-like (Mal) (Fitzgerald et al., 2001; Horng et al., 2001; Horng et al., 2002), facilitate TLR4-induced NF-κB activation. Once MyD88 has been activated, it recruits and activates IL-1 receptor activated kinase (IRAK) 4, a serine-threonine kinase that then trans-phosphorylates IRAK1 (Fitzgerald et al., 2004; Kawai and Akira, 2007; Nishiya et al., 2007). IRAK1 then autophosphorylates becoming hyperphosphorylated IRAK1, which causes TNF-α receptor activated factor-6 (TRAF6) to oligomerize and polyubiquinate, allowing it to activate the IκB kinase (IKK) complex. The IKK complex, which is made up of IKK α, β, and γ, is then capable of phosphorylating IκB, which causes it to be ubiquitinated and undergo proteosomal degradation. Following IκB degradation, NFκB is able to translocate from the cytoplasm to the nucleus and promote the induction of cytokines and adhesion molecules, including IL-6, IL-8, TNF-α, and E-selectin (Tak and Firestein, 2001).

In addition to their role in NFκB activation, TIRAP and MyD88 have established roles in mediating LPS-induced apoptotic signaling in human EC (Bannerman et al., 2002a; Bannerman et al., 2002b). Another protein, FAS-associated death domain protein (FADD), has been demonstrated to promote LPS-induced apoptotic signaling in both human and bovine EC (Choi et al., 1998; Szperka et al., 2005). FADD is an adaptor molecule that is able to recruit pro-caspase-8 through homotypic death

effector domain (DED) binding. Once pro-caspase-8 is bound, it is able to undergo self-cleavage, which initiates the activation of caspases, resulting in apoptosis (Boldin et al., 1996; Cohen, 1997). At the present time, the nature of the interaction of TIRAP, MyD88 and FADD in promoting caspase activation in response to LPS remains to be clearly defined.

Cows are exquisitely sensitive to LPS (Berczi et al., 1966). Interestingly, bovine EC are more sensitive to the pro-inflammatory and injurious effects of LPS than human EC. In response to LPS, injury-independent permeability is induced to a greater extent in bovine versus human endothelial monolayers (Bannerman and Goldblum, 1999). Further, bovine EC are directly sensitive to the apoptosis-inducing effects of LPS, whereas human EC are resistant (Harlan et al., 1983a; Harlan et al., 1983b; Bannerman and Goldblum, 2003). This latter finding has been attributed to differential expression of the anti-apoptotic protein, FLICE-like inhibitory protein (FLIP) (Bannerman et al., 2004; Szperka et al., 2006). Finally, human FADD has been demonstrated to inhibit LPS-induced NFκB activation, whereas the bovine ortholog lacks this downregulatory effect (Bannerman et al., 2002c; Szperka et al., 2005; Zhande et al., 2007). Thus, differences in both the responses and signaling mediators involved in promoting these responses have been identified between human and bovine EC.

Recently, bovine TIRAP and MyD88 were cloned and sequenced (Connor et al., 2006). Unlike their human orthologs, the functional roles of these proteins in

mediating LPS responses in bovine EC have yet to be ascertained. Because differences between bovine and human EC response to LPS have been reported, and human, but not bovine FADD, is able to downregulate NFκB activation, it cannot be simply assumed that the functions of TIRAP and MyD88 are conserved across species. Thus, the objective of the current study was to use dominant-negative constructs of these proteins to functionally characterize the role of bovine TIRAP and MyD88 in mediating bovine TLR4 NFκB and pro-apoptotic signaling.

## 2.3 Results and Discussion

### 2.3.1 Expression of TIRAP and MyD88 D/N constructs

By virtue of its direct recruitment to TLR4, TIRAP is one of the most proximal intracellular signaling molecules involved in LPS signaling. TLR4 interaction with TIRAP is mediated through reciprocal binding of TIR domains contained within each protein. A proline to histidine mutation at amino acid 125 (P125H) in the TIR domain of human TIRAP has been reported to impair its ability to bind to the TIR domain of TLR4 (Horng et al., 2001). Correspondingly, expression of human TIRAP constructs containing this mutation function to inhibit signaling mediated by endogenous TIRAP in a dominant-negative manner and impair the ability of LPS to induce NFκB activation and apoptosis (Fitzgerald et al., 2001; Bannerman et al., 2002a). MyD88 is another proximal LPS signaling molecule that is recruited to the TLR4 intracellular signaling complex via its interaction with TIRAP. MyD88 contains two distinct protein binding domains, a TIR domain and a death domain. Expression of the human TIR domain of MyD88 has been demonstrated to block TLR4 mediated NFκB activation and apoptosis in a dominant negative fashion (Medzhitov et al., 1998; Bannerman et al., 2002b).

Bovine TIRAP and MyD88 possess the predicted protein binding domains necessary for their participation in TLR4-mediated signaling and demonstrate 81% and 86% conservation, respectively, with their human orthologs at the amino acid level (Connor et al., 2006). To determine whether bovine TIRAP and MyD88 mediate LPS-induced signaling in bovine EC, a retroviral infection system was used to stably transfect bovine pulmonary artery cells (BPAEC) and bovine aortic endothelial cells

(BAEC) with vector, or vector containing cDNA encoding either TIRAP with a P136H mutation or the TIR domain alone of MyD88. The P136H mutation in bovine TIRAP corresponds with the P125H mutation of human TIRAP that has been reported to ablate its function. RT-PCR (data not shown) and Western blot analysis confirmed expression of the FLAG-tagged D/N constructs in both cell types (Figure 2-1).

### 2.3.2 Expression of TIRAP and MyD88 D/N constructs inhibit LPS-induced NFκB activation

To evaluate the functional roles of TIRAP and MyD88 in promoting NFκB signaling, BPAEC's expressing either vector alone or vector encoding TIRAP or MyD88 D/N constructs were treated with media or LPS (100 ng/ml) for 6 h and assayed for NFκB activity (Figure 2-2). Expression of the TIRAP D/N completely inhibited LPS-induced NFκB activation, whereas the MyD88 D/N construct inhibited ~18% of this response. TNF-α is another well-described activator of NFκB in EC (Beg et al., 1993; Madge and Pober, 2001), however, distinct transmembrane receptors and associated proximal intracellular signaling molecules mediate TNF-α- and LPS-induced NF-κB activation. To rule out that expression of either the MyD88 or TIRAP D/N construct non-specifically inhibit NFκB activation, BPAEC expressing these constructs were also stimulated for 6 h with TNF-α (100 ng/ml) (Figure 2-2). Consistent with the predicted roles of bovine TIRAP and MyD88 in mediating signaling elicited by LPS, but not TNF-α, expression of either D/N construct demonstrated no effect on TNF-α-induced NFκB activation.

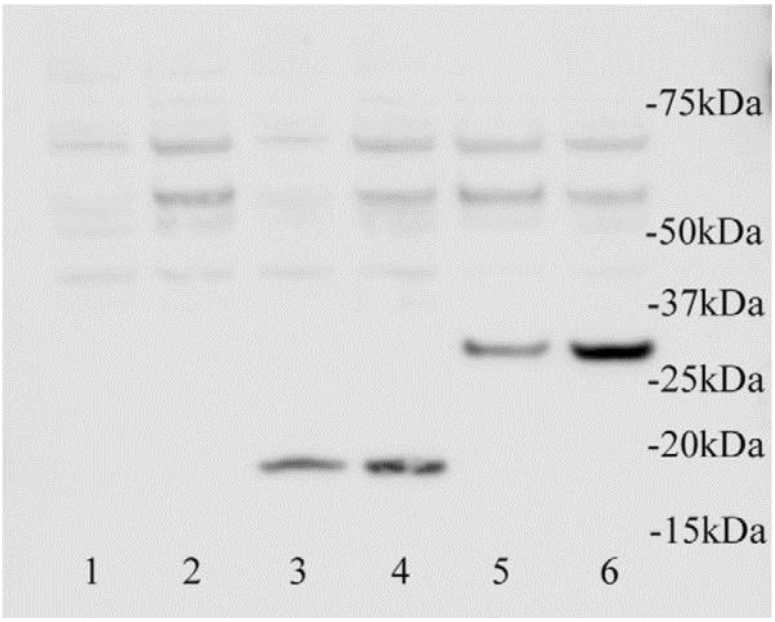



**Figure 2-1: Expression of TIRAP and MyD88 dominant-negative (D/N) constructs in bovine aortic (BAEC) and pulmonary artery (BPAEC) endothelial cells.** BAEC (lanes 1, 3, 5) and BPAEC (lanes 2, 4, 6) were stably transfected with either vector alone (lanes 1, 2), or vector containing cDNA encoding either the TIR domain of MyD88 (lanes 3, 4) or full-length TIRAP containing $P_{136}$ mutated to $H_{136}$ (lanes 5, 6). An anti-FLAG antibody was used to detect the expression of the FLAG-tagged constructs. Molecular mass (in kDa) is indicated.

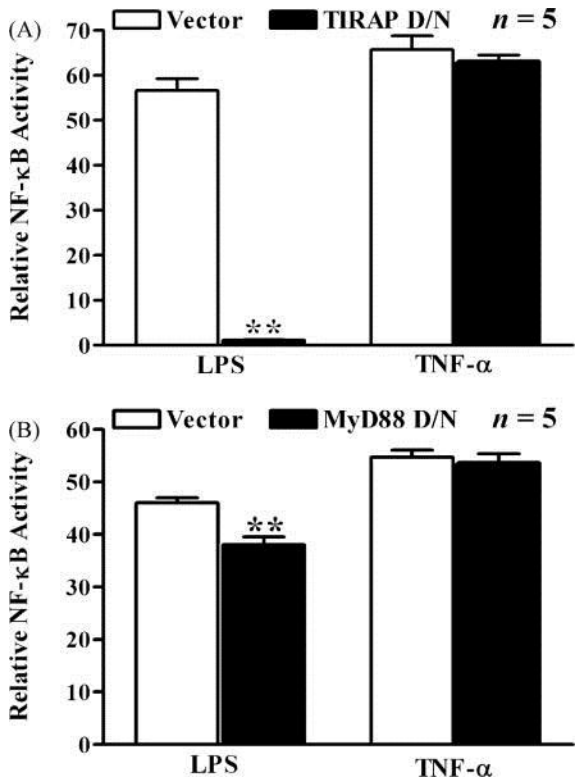



**Figure 2-2: Effect of the expression of bovine TIRAP or MyD88 dominant-negative (D/N) constructs on LPS-induced NFκB activation in bovine pulmonary artery endothelial cells (BPAEC).** BPAEC stably transfected with either vector (A, B), or vector encoding for TIRAP (A) or MyD88 (B) D/N constructs were treated with media, LPS (100 ng/ml), or TNF-α (100 ng/ml) for 6 hours, and NFκB activity assayed. Vertical bars represent mean (±S.E.) NFκB activity relative to media controls. **Significantly ($p < 0.01$) decreased compared to BPAEC expressing vector alone and exposed to identical treatment.

### 2.3.3 Expression of TIRAP and MyD88 D/N constructs inhibit LPS-induced expression of E-selectin

The assay used to measure NFκB activity (Figure 2-2) relied on adenoviral transfection of EC with a reporter construct encoding the luciferase gene under the control of a pure NFκB promoter. The advantage of this assay is that it allows for the sole measurement of NFκB-dependent signaling. However, this assay does not preclude the possibility that bovine TIRAP and MyD88 were restricted in their participation in LPS-induced NFκB-dependent gene expression under the artificial conditions of expression of an exogenously-induced reporter construct. To rule out this possibility, the effect of expression of the D/N constructs on expression of endogenous E-selectin, which is regulated by NFκB (Tak and Firestein, 2001), was studied (Figure 2-3). BPAEC expressing either vector alone or vector encoding TIRAP or MyD88 D/N constructs were treated with media, LPS (100 ng/ml), or TNF-α (100 ng/ml) for 8 h and assayed for E-selectin expression by ELISA (Figure 2-3). The TIRAP D/N construct completely inhibited LPS-induced E-selectin expression, whereas, the MyD88 D/N construct inhibited this response by ~20%. Neither D/N construct had any demonstrable effect on TNF-α-induced E-selectin expression. These results investigating the NFκB-dependent expression of an endogenous protein are in agreement with those investigating NFκB-dependent expression of an exogenously introduced reporter construct (Figure 2-2).

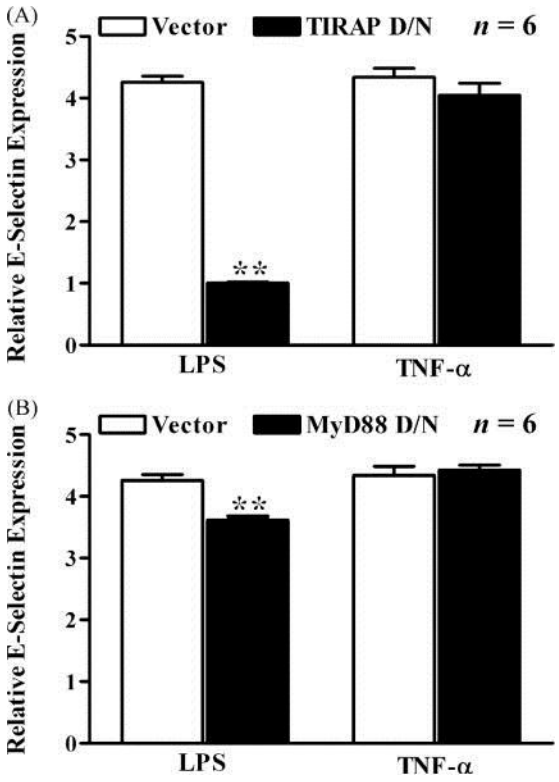



**Figure 2-3: Effect of the expression of bovine TIRAP or MyD88 dominant-negative (D/N) constructs on LPS-induced expression of E-selectin in bovine pulmonary artery endothelial cells (BPAEC).** BPAEC stably transfected with either vector (A, B), or vector encoding for TIRAP (A) or MyD88 (B) D/N constructs were treated with media, LPS (100 ng/ml), or TNF-α (100 ng/ml) for 8 hours, and assayed by ELISA for E-selectin expression. Vertical bars represent mean (±S.E.) E-selectin expression relative to media controls. **Significantly ($p < 0.01$) decreased compared to BPAEC expressing vector alone and exposed to identical treatment.

EC are notoriously difficult to transfect and retroviral-mediated infection systems have been successfully used to express constructs of interest in this cell type. We have established with this system and subsequent selection with puromycin that ~99% of the selected EC express the construct of interest (Szperka et al., 2006). To preclude that the observed effects of the D/N constructs are the result of where they were stably integrated into the cell genome rather than a result of their D/N function when expressed, several wells of infected EC were selected for and then pooled. Thus, the pooled EC from the different infections contain constructs of interest inserted in different sites of the genome. To further rule out any site-specific integration effects and to evaluate whether these D/N constructs exert their effect on bovine EC derived from a different anatomical source, the retroviral infection system was used to stably transfect BAEC with vector, or vector containing cDNA encoding either the TIRAP or MyD88 D/N constructs (Figure 2-1). These EC were then treated with media, LPS (100 ng/ml), or TNF-α (100 ng/ml) for 8 h and assayed for E-selectin expression by ELISA (Figure 2-4). Similar to the results with the BPAEC, the TIRAP D/N construct completely inhibited LPS-induced E-selectin expression, whereas, the MyD88 D/N construct only partially blocked this response. TNF-α-induced E-selectin expression was unaffected by expression of either D/N construct.

### 2.3.4 Expression of the TIRAP D/N construct inhibits LPS-induced apoptosis

In addition to their roles in mediating NFκB activity, both human TIRAP and MyD88 have been demonstrated to promote LPS-induced apoptotic signaling in human EC (Bannerman et al., 2002a; Bannerman et al., 2002b). Differences in the mechanisms by which LPS induces apoptosis in human versus bovine EC have been implied by

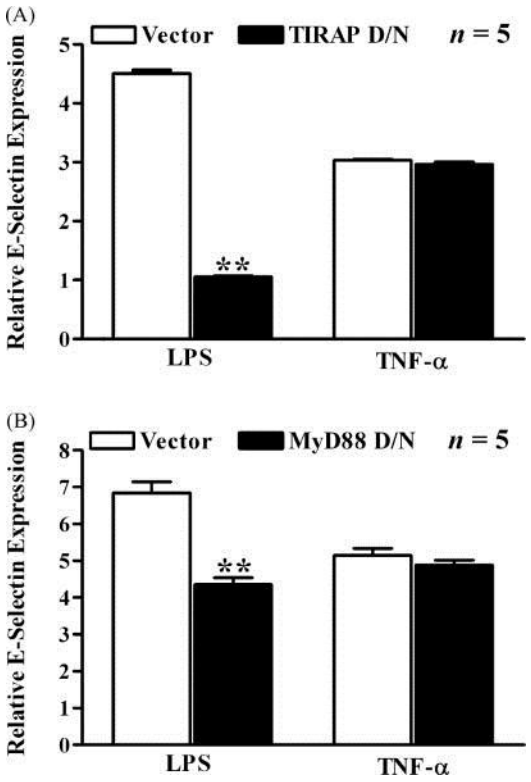



**Figure 2-4: Effect of the expression of bovine TIRAP or MyD88 dominant-negative (D/N) constructs on LPS-induced expression of E-selectin in bovine aortic endothelial cells (BAEC).** BAEC stably transfected with either vector (A, B), or vector encoding for TIRAP (A) or MyD88 (B) D/N constructs were treated with media, LPS (100 ng/ml), or TNF-α (100 ng/ml) for 8 hours, and assayed by ELISA for E-selectin expression. Vertical bars represent mean (±S.E.) E-selectin expression relative to media controls. **Significantly ($p < 0.01$) decreased compared to BAEC expressing vector alone and exposed to identical treatment.

findings that demonstrate that bovine EC are directly sensitive to LPS-induced killing, whereas, human EC are only rendered sensitive to this effect when gene expression is inhibited (Bannerman and Goldblum, 2003). This differential sensitivity has been attributed, in part, to differential expression of the FLICE-like inhibitory protein (FLIP), which inhibits LPS-induced caspase activation (Szperka et al., 2006). However, whether the bovine orthologs of the TLR4 signaling molecules that promote pro-apoptotic signaling in human EC have a similar function in bovine EC has remained unknown.

To investigate whether bovine TIRAP and MyD88 are able to promote LPS-induced pro-apoptotic signaling, BPAEC and BAEC expressing vector alone or one of the D/N constructs were treated with media or LPS (100 ng/ml) for 12 h and assayed for caspase activity. The TIRAP D/N construct completely inhibited LPS-induced apoptosis in both cell types (Figure 2-5), whereas the MyD88 D/N construct had no effect (data not shown). Expression of the TIRAP D/N construct had no effect on staurosporine-induced apoptosis, demonstrating the specificity of this construct to specifically inhibit LPS-induced apoptotic signaling and ruling out that this construct globally inhibited apoptosis.

Because the activation of initiator caspases sets off an amplifying caspase cascade leading to apoptosis, it may not be surprising that the MyD88 D/N construct, which could only partially inhibit NF-κB activation, had no inhibitory effect on LPS-induced caspase activation. Although a detailed time course was not performed, it's

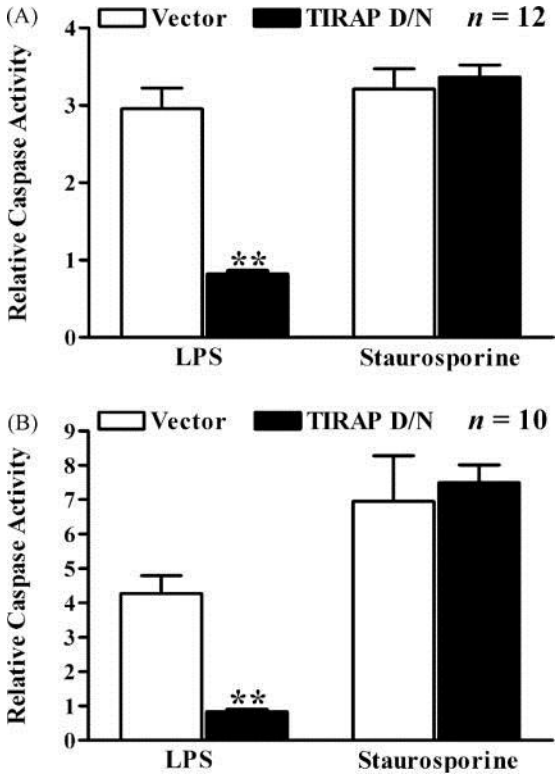



**Figure 2-5: Effect of the expression of a bovine TIRAP dominant-negative (D/N) construct on LPS-induced caspase activation in bovine pulmonary artery (BPAEC) and aortic (BAEC) endothelial cells.** BPAEC (A) and BAEC (B) stably transfected with either vector or vector encoding for a TIRAP D/N construct were treated with media, LPS (100 ng/ml), or staurosporine (1 μM) for 12 hours, and assayed for caspase activity. Vertical bars represent mean (±S.E.) caspase activity relative to media controls. **Significantly ($p< 0.01$) decreased compared to cells expressing vector alone and exposed to identical treatment.

possible that expression of the MyD88 D/N construct delayed the onset of initial caspase activation. It has also been reported that TIRAP contains a TRAF6 binding site and that it may be capable of promoting LPS-induced signaling independently of MyD88 (Mansell et al., 2004). This may also explain why the MyD88 D/N construct only partially inhibited LPS-induced NFκB activation whereas the TIRAP D/N construct completely inhibited this response as well as LPS-induced caspase activation.

In conclusion, the current study has established through the expression of D/N constructs that bovine TIRAP and MyD88 promote LPS-induced NFκB activation in bovine EC. Further, this study has established a role for bovine TIRAP in promoting LPS-induced apoptotic signaling in bovine EC. To our knowledge, this is the first study to establish a functional role for bovine TIRAP in mediating responses to LPS in any bovine cell type. Thus, despite reported differences in the sensitivity of human and bovine EC to LPS, the function of TIRAP, which is the most proximal intracellular TLR4 signaling molecule, is conserved across species. If the differential species-specific sensitivity to LPS is due to differences in the signaling pathway, the data presented here suggest that these difference occur downstream of TIRAP and MyD88.

## 2.4 Materials and methods

### 2.4.1 Materials

Highly purified LPS extracted from *Escherichia coli* serotype 0111:B4 was purchased from List Biological Laboratories, Inc., (Campbell, CA). Recombinant human TNF-α and staurosporine were obtained from R&D Systems, Inc., (Minneapolis, MN) and Sigma Chemical Co., (St. Louis, MO), respectively.

### 2.4.2 Cell culture

Bovine aortic (BAEC) and pulmonary artery (BPAEC) endothelial cells (generous gifts of Dr. L.M. Sordillo, Michigan State University, East Lansing, MI and Dr. C.J. Czuprynski, University of Wisconsin, Madison, WI) were cultured in Ham's F12K medium (Irvine Scientific Sales Co., Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), heparin (100 μg/ml), insulin (10 μg/ml), transferrin (5 μg/ml) (Sigma Chemical Co.), HEPES (20 mM), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Cambrex BioScience Walkersville, Inc., Walkersville, MD). The Phoenix amphotropic packaging cell line (ATCC, Manassas, VA) was cultured in Dulbecco's Modified Eagle's Medium (Sigma Chemical Co.) supplemented with 10% heat inactivated FBS, non-essential amino acids (1x) (Cambrex BioScience Walkersville, Inc.), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were cultured at 37$^{o}$C in the presence of 5% $CO_2$.

### 2.4.3 Cloning and generation of TIRAP and MyD88 dominant-negative (D/N) constructs

Primers were designed to amplify sequence encoding amino acids 152-296 of bovine MyD88 (NM_001014382), which corresponds only to the TIR domain of the full-length protein. The forward primer ($^{5'}$CCGGAATTCGACCCCCTAGGGCAAAAGCCCGAG$^{3'}$) and the reverse primer ($^{5'}$CCGCTCGAGCTATCAGGGCATGGACAGGGCCTTGGC$^{3'}$) also included sequence encoding 5' *EcoRI* (*gaa ttc*) and 3' *XhoI* (*ctc gag*) restriction sites to enable subsequent insertion of the construct into the pCR3.V64 Met FLAG vector (gift from Dr. Jurg Tschopp, Institute of Biochemistry of the University of Lausanne, Switzerland). PCR amplification was performed using 2 μl of first-strand cDNA, which was generated from RNA isolated from bovine mammary epithelial cells, iQ Supermix (Bio-Rad Laboratories, Hercules, CA), and the primers described above. The cycling conditions were 95˚C for 3 min followed by 35 cycles of the following: 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 1 min.

To generate the TIRAP d/n construct, primers were designed to amplify the entire coding region of bovine TIRAP (NM_001039962). The forward primer ($^{5'}$CCGGAATTCATGGCATCATCAACCTCC$^{3'}$) and the reverse primer ($^{5'}$CCGCTCGAGCTATCAGCCAAGGGTCTGCAG$^{3'}$) also included sequence encoding 5' *EcoRI* and 3' *XhoI* restriction sites. PCR amplification was performed with these primers as described above. Subsequently, $P_{136}$ in the construct was mutated to $H_{136}$ using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, Inc., La Jolla, CA) using the following primers:

$^{5'}$CTTCGCGACGCCACCCATGGTGGCGCCATCGTG$^{3'}$ and

$^{5'}$CACGATGGCGCCACCATGGGTGGCGTCGCGAAG$^{3'}$.

The PCR products for both the MyD88 and TIRAP D/N constructs were digested with *EcoRI* and *XhoI*, and ligated into the pCR3.V64 Met FLAG vector. This vector contains a 5' *BamHI* site followed by a coding region for an initiator methionine, a FLAG tag (DYKDDDDK), and a spacer of two amino acids (EF) immediately before the *EcoRI* - *XhoI* PCR product insertion site. Bacteria were transformed with the ligation reactions and clones from bacteria containing appropriate sized inserts were fully sequenced. Correct clones were then subcloned into the *BamHI* and *XhoI* restriction sites of the pBMN-IRES-PURO retroviral expression vector (kindly provided by Dr. Gary Nolan, Stanford University, Stanford, CA and subsequently modified by Dr. Kyle Garton, University of Washington, Seattle, WA).

### 2.4.4 Stable expression of TIRAP and MyD88 dominant-negative (D/N) constructs

High-titer retrovirus was prepared from the Phoenix amphotropic packaging cell line as previously described (Szperka et al., 2006). Briefly, 24 μg of endotoxin free-expression plasmid were diluted with dd$H_2O$ in a final volume of 1.314 ml in a 5 ml polystyrene tube. After a gentle vortex, 186 μl of 2 M $CaCl_2$ were added to each tube, followed by another gentle vortex. Using a pasteur pipette, 1.5 ml of 2x HEPES-buffered saline (HBS) were added to the tube followed by aggressive bubbling for 10 s. 100 mm culture plates containing Phoenix cells at ~75% confluence were spotted with 10 μl of chloroquine (1,000x) and the plasmid/$CaCl_2$/HBS solution gently added.

The medium in the plates was changed at 10 and 24 hours post-transfection. At 48 hours post-transfection, the media supernatants containing viral particles were collected and filtered through 0.45 μm Millex-HV filters (Millipore Corp., Bedford, MA), aliquotted into 15 ml polypropylene tubes, and stored at -80˚C.

For infection of BAEC and BPAEC, cells were seeded into 6-well plates and grown until reaching ~80% confluence. The growth medium was replaced with 2.5 ml of retroviral supernatant supplemented with 8 μg/ml of polybrene and 10 mM of HEPES, and the plate centrifuged for 2 h at 1,430 x *g*. An additional 2.5 ml of growth media were added to each well and the cells were then incubated for 10 h (5% $CO_2$, 37 $^{o}$C), after which the retroviral-containing supernatant was replaced with normal growth medium. Twenty-four hours post-infection, growth media was supplemented with 2 μg/ml puromycin (Sigma Chemical Co.) to select for cells stably expressing the construct of interest. To confirm expression, cDNA, which was generated from RNA isolated from stably infected EC using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA), was amplified using a FLAG specific forward primer ($^{5'}$TGGATTACAAAGACGATGACG$^{3'}$) and the construct specific reverse primers described above. The resulting product was sequenced in both directions using the CEQ8000 automated DNA sequencer and DTCS Quickstart chemistry (Beckman Coulter, Fullerton, CA). Western blotting was performed as previously described with 1:2,000 diluted anti-FLAG antibody conjugated to horseradish peroxidase (Szperka et al., 2006) to further confirm expression of the constructs in the EC.

### 2.4.5 NFκB-luciferase assay

Sequence from the pNiFty-Luc2 vector (InvivoGen Corp., San Diego, CA), which encodes five NFκB sites, the proximal E-selectin promoter, and luciferase, was subcloned into a shuttle vector. This composite, engineered promoter is NFκB specific and lacks the AP1/CREB site found in the endogenous E-selectin promoter. The entire expression cassette encoding the reporter construct was transferred into an adenovirus genome vector and virus expressing the construct was generated commercially by Vector Biolabs, Inc. (Philadelphia, PA).

To assay for NF-κB-dependent luciferase expression, EC were seeded into 96-well black, clear bottom plates and grown to confluence. EC were then exposed for 24 hours to 5 x $10^6$ PFU/ml of adenovirus diluted in EC culture media. The adenovirus was aspirated from each well, and EC were subsequently exposed to media alone (Ham's F12K medium supplemented with 5% heat inactivated FBS, 20mM HEPES, and 0.5% BSA), or media containing LPS or TNF-α. Luciferase activity was measured using the Bright-Glo Luciferase Assay System (Promega Corp., San Luis Obispo, CA) and a Veritas microplate luminometer (Turner Biosystems Inc., Sunnyvale, CA) according to the manufacturer's instructions and protocol.

### 2.4.6 E-selectin enzyme-linked immunosorbent assay (ELISA)

EC were seeded into 96-well plates and cultured until confluent. Following treatment, cells were washed twice with RPMI-1640 media supplemented with 2.5% bovine calf serum and fixed for 10 min with 0.5% glutaraldehyde in PBS at room temperature. Monolayers were washed and incubated for 1 h at 37 $^{o}$C with anti-E-

selectin (CD-62E) rabbit polyclonal antibodies (NeoMarkers, Inc., Fremont, CA) diluted 1:1,000 in wash buffer. The cells were washed twice and incubated as above with horseradish peroxidase-conjugated goat anti-rabbit antibodies (BD Biosciences Corp.) diluted 1:2,000 in wash buffer. The wells were then washed five times with PBS and 100 μl of 3,3',5,5'- tetramethylbenzidine (TMB) substrate solution (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was added to each well. The reaction was stopped by the addition of 100 μl of 2 M $H_2SO_4$ and the absorbance read at 450 nm on a microplate reader (Bio-Tec Instruments, Inc., Winooski, VT). A background correction reading at 565 nm was subtracted from the 450 nm absorbance readings.

### 2.4.7 Caspase assay

EC were seeded into 96-well plates and cultured until confluent. Following treatment, caspase activity was measured with a fluorimetric homogenous caspases assay according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). The plates were analyzed on a fluorescence plate reader (Bio-Tec Instruments, Inc.) at a 485 nm excitation and a 530 nm emission, and caspase activity expressed relative to a simultaneous medium control.

### 2.4.8 Statistical methods

A *t*-test was used to compare the mean responses between an experimental group and its respective control. All statistical analyses were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, Inc., San Diego, CA). A *p*-value of $<0.05$ was considered significant.

# Chapter 3: A Glutamate Dependent Redox System in Blood Cells is Integral for Phagocytosis in *Drosophila melanogaster*

## 3.1 Abstract

Glutamate transport is highly regulated as glutamate directly acts as a neurotransmitter and indirectly regulates the synthesis of antioxidants. Although glutamate deregulation has been repeatedly linked to serious human diseases such as HIV infection and Alzheimer's, glutamate's role in the immune system is still poorly understood. In this study, we find that a putative glutamate transporter in *Drosophila melanogaster*, *polyphemus* (*polyph*), plays an integral part in the fly's immune response. Flies with a disrupted *polyph* gene exhibit decreased phagocytosis of microbial-derived bioparticles but not of latex beads. *polyph* expression is specifically enriched in phagocytes, consistent with a role there. When infected with *S.aureus*, *polyph* flies show an increase in both susceptibility and bacterial growth, indicating that the gene is involved in resistance rather than tolerance. *polyph* expression by both the blood cells and the testes is required for the fly's humoral response. *polyph* flies exhibit a low induction of the antimicrobial peptide (AMP) Cecropin. We demonstrate that the induction of this AMP is vitally important for surviving an *S.aureus* infection. Consistent with previous data showing that glutamate transport plays a regulatory role in the synthesis of the major antioxidant glutathione, *polyph* flies produce more reactive oxygen species (ROS) as compared to wildtype when exposed to *S.aureus*. We examined the expression profile of two genes known to play a role in neuronal glutamate transport, and found that *genderblind* (*gb*), but not

*excitatory amino acid transporter 1* (*eaat1*), is expressed in blood cells. *gb* flies showed a decrease in phagocytosis and survival. When we expressed *eaat1* in *polyph* mutant blood cells, both phagocytosis and cecropin induction were rescued. Additionally, *polyph* flies have an overactive melanization response, which may exacerbate the flies' elevated ROS levels. In conclusion, we demonstrate that a *polyph*-dependent redox system is necessary to maintain the immune cells' function against an infection. Furthermore, our model is useful towards understanding the importance of glutamate transport in human disease.

## 3.2 Introduction

The innate immune system is the first line of defense against infection and is needed to induce an adaptive response. Its germline encoded pattern recognition receptors (PRRs) respond to a variety of pathogen-associated molecular patterns (PAMPs) (Abbas, 2003). Many aspects of innate immunity are highly conserved between mammals and the model organism, *Drosophila melanogaster*. For example, the discovery that the *Drosophila* protein Toll was involved in immune regulation led to the seminal discovery of Toll-like receptors in mammals (Lemaitre et al., 1996; Medzhitov et al., 1997; Poltorak et al., 1998). Additionally, the primary *Drosophila* blood cell, the plasmatocyte, is functionally equivalent to the mammalian macrophage (Abrams et al., 1992). Both of these specialized blood cells are principally phagocytes, which bind, engulf, and digest particles that are too large to ingest via endocytosis. The phagocytic process is paramount to a successful immune response during infection as it is both directly microbicidal and necessary for antigen presentation in vertebrates (Savina and Amigorena, 2007).

Human macrophages express membrane-bound glutamate transporters, which regulate the concentration of glutamate across the plasma membrane (Rimaniol et al., 2000). Given that glutamate is a highly abundant excitatory neurotransmitter, its transport in and out of neurons has been studied in great detail (Fonnum, 1984; Omote et al., 2011; Niciu et al., 2012). In contrast, the role of glutamate transport in the innate immune response has been widely ignored. This is unfortunate as high levels of plasma glutamate are strongly connected to a variety of diseases including HIV infections (Ferrarese et al., 2001) and cancers (Dröge et al., 1987). Phagocytes also play a role in modulating glutamate concentration within the central nervous system during neurological diseases such as HIV-associated dementia (HAD) and amyotrophic lateral sclerosis (ALS) (Piani et al., 1993; Graves et al., 2004; Tian et al., 2008). In addition, treating macrophages with lipopolysaccharide, an outer cell membrane component of Gram-negative bacteria, upregulates the expression of glutamate transporters (Sato et al., 1995). Lastly, glutamate transport in blood cells may also play a role in the adaptive immune response, as the release of glutamate from dendritic cells regulates antigen presentation and subsequent T-cell proliferation (Pacheco et al., 2006).

There are three known types of glutamate transporters: excitatory amino acid transporters (eaats or $X_{AG-}$), cystine/glutamate antiporters ($X_{C-}$), and a more recently discovered sodium-dependent transporter ($X_{AG}$). Eaats transport extracellular glutamate into the cell using a sodium/potassium exchange system (Barbour et al., 1988). $X_{C-}$ transporters exchange a cystine molecule for a glutamate residue in a sodium-independent process. The direction of this exchange is concentration

dependent (Bannai, 1986). Normally, there is more glutamate inside the cell than outside with the inverse being true for cystine, thus causing the antiporter to exchange an intracellular glutamate for an extracellular cystine. Once inside the cell, cystine is reduced to cysteine, which is the limiting reagent in glutathione (GSH) synthesis (Bannai, 1986; Rimaniol et al., 2001). GSH acts as a major antioxidant in the cell by reducing reactive oxygen species (ROS) (Deneke and Fanburg, 1989). In the process GSH becomes oxidized into glutathione disulfide (GSSG), which can then be converted back to GSH via glutathione reductase and NADPH (Dafré et al., 1996). Both eaats and $X_{C^-}$ transporters are expressed on mammalian macrophages and have been shown to play a role in glutathione synthesis and thus ROS regulation (Watanabe and Bannai, 1987; Rimaniol et al., 2001). Yet, how they affect the functional properties of the macrophage and the overall immune response is not well known.

We identified a putative glutamate transporter, *polyphemus (polyph)*, which is expressed on *Drosophila* blood and testes cells and is essential for the overall immune function of the fly. The blood cells, or hemocytes, are the immune effector cells in *Drosophila*. They are comprised of three distinct cell types: lamellocytes, crystal cells, and plasmatocytes. Lamellocytes are large cells that encapsulate parasitoid wasp eggs (Rizki and Rizki, 1984) and play little role in septic bacterial infections. Crystal cells are involved in the melanization response, a process where ROS byproducts are released as tyrosine is converted to melanin (Rizki et al., 1985; Zhao et al., 2007). The ROS are directly antagonistic towards invading microbes and the melanin is incorporated into a clot to prevent microbes from escaping the

immediate area (Bidla et al., 2005). The plasmatocytes, the phagocytic cells, make up 95% of the blood cell population and therefore must take on the majority of the burden when defending the fly against infection (Lanot et al., 2001). When *polyph* is mutated, the flies demonstrate decreased immune-specific phagocytosis, decreased induction of the antimicrobial peptide (AMP) Cecropin, and increased susceptibility to infection. Expression of *polyph* by both the hemocytes and the testes affects the fly's immune response, indicating that peripheral tissues can also play a role in immunity. Consistent with its role as a putative glutamate transporter, *polyph* regulates the levels of ROS inside the cell. This indicates that the role of glutamate transport in *Drosophila* blood cells is conserved with what is known from mammalian cells. Importantly, we show that functional and tightly regulated glutamate transport within blood cells is necessary to induce the phagocyte driven immune response needed to survive an infection.

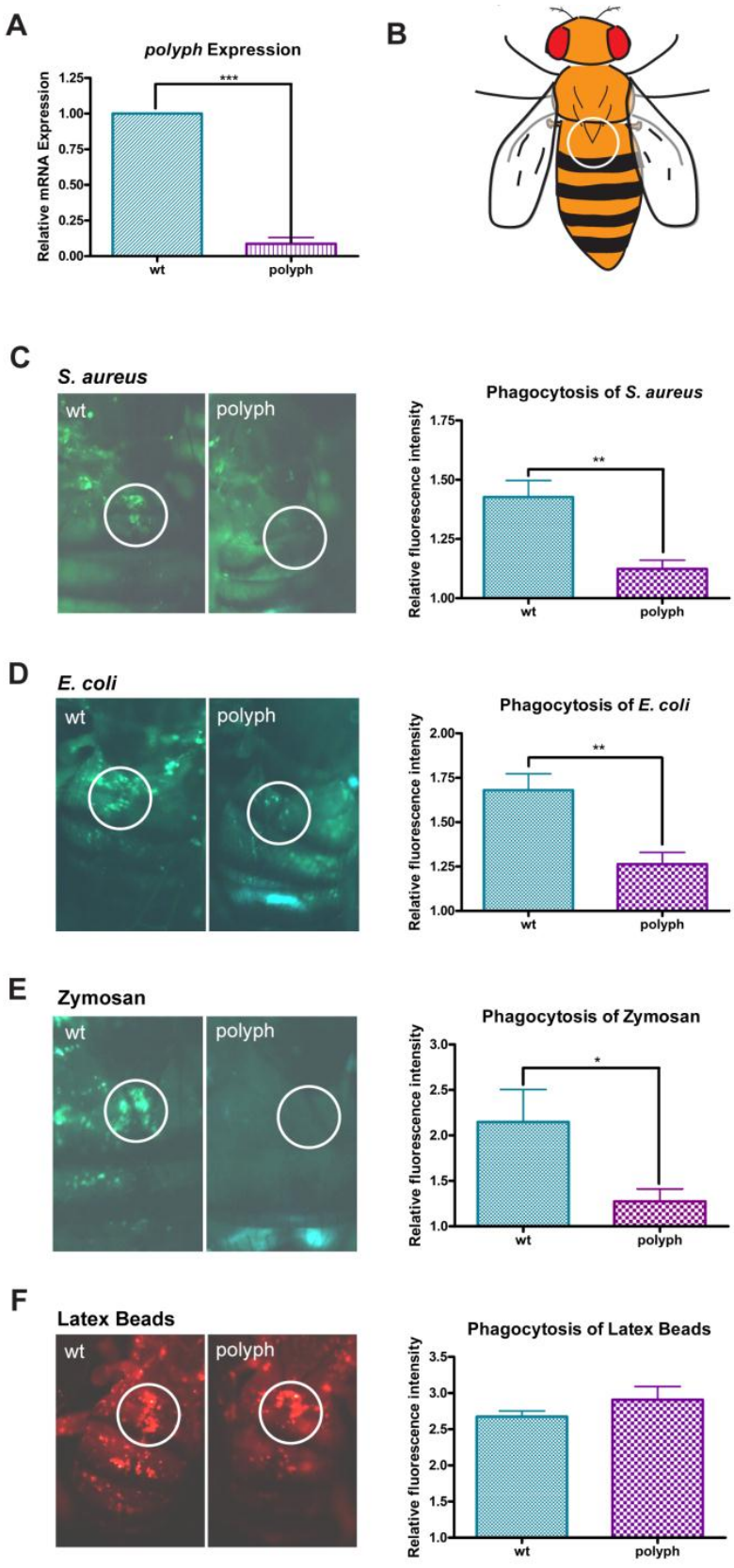
## 3.3 Results

### 3.3.1 The putative amino acid transporter, *polyph*, is required for phagocytosis of PAMP-containing molecules

We mapped a phagocytosis mutant to the right arm of the second *Drosophila* chromosome between the cytological markers 47C1 and 47F1. The line $w^{1118}$; Mi[ET1]CG12943$^{MB02238}$ has a Minos transposon inserted in a gene within this chromosomal interval and shows a defect in phagocytosis. The disrupted gene, CG12943, is 1,993 nucleotides long, contains seven exons, and is predicted to contain a single domain: an amino acid transmembrane transporter domain. The protein shows 21.1% amino acid similarity with *Drosophila* Eaat1. We named the gene

*polyphemus* (*polyph*) after the Cyclops in Greek mythology who failed to guard against Odysseus as *polyph* flies are similarly unable to defend themselves from an intruding microbe when infected. We were unable to make additional *polyph* alleles as it is very difficult to mobilize Minos insertions. Fortunately, we found that the transposon insertion on its own greatly decreased *polyph* expression (Figure 3-1A).

The phagocytic capacity of *polyph* flies was measured using the *in vivo* adult phagocytosis assay designed by Elrod-Erickson, et al. (Elrod-Erickson et al., 2000). Briefly, flies were injected with fluorescently labeled microbial bioparticles, incubated at room temperature to allow phagocytosis, and then injected with Trypan Blue, which quenches any fluorescence that has not been taken up by a cell. The phagocytosis is then visualized by looking through the cuticle in the dorsal vein area of the adult fly where large numbers of sessile phagocytes accumulate (Figure 3-1B). *polyph* flies were deficient in their ability to undergo phagocytosis of *S.aureus* (Figure 3-1C), *E.coli* (Figure 3-1D), and zymosan bioparticles (Figure 3-1E). The phagocytosis of latex beads was normal, indicating the mutant cells' general phagocytic machinery is not defective (Figure 3-1F). Instead, the phenotype appears to be specific to the phagocytosis of PAMP- containing molecules. As normal phagocytosis of latex beads was observed, it is unlikely that the decrease in phagocytosis of PAMPs is due to fewer blood cells in the mutant. To assess this, we


A
polyph Expression
Relative mRNA Expression
1.25
1.00
0.75
0.50
0.25
0.00
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wt
polyph
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S. aureus
wt
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Phagocytosis of S. aureus
Relative fluorescence intensity
1.75
1.50
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**
wt
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wt
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Phagocytosis of E. coli
Relative fluorescence intensity
2.00
1.75
1.50
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wt
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polyph
Phagocytosis of Zymosan
Relative fluorescence intensity
3.0
2.5
2.0
1.5
1.0
*
wt
polyph
F
Latex Beads
wt
polyph
Phagocytosis of Latex Beads
Relative fluorescence intensity
3.5
3.0
2.5
2.0
1.5
1.0
wt
polyph

**Figure 3-1: *polyph,* a putative amino acid transporter, is required for microbial phagocytosis.**

(A) Comparison of *polyph* transcript levels via qPCR in wildtype flies (wt) and flies containing a transposon insertion in the *polyph* gene (polyph). Relative expression was measured using *rp49* as an endogenous control. A pool of ten flies per genotype was used in each experiment. (B) Representation of the how the fly is visualized during the *in vivo* adult phagocytosis assay. The encircled area represents the area of the dorsal vein around which the sessile blood cells congregate. (C, D, E) Representative pictures depicting phagocytosis in wt and *polyph* flies of (C) fluorescein-labeled *S.aureus* bioparticles, (D) fluorescein-labeled *E.coli* bioparticles, (E), fluorescein-labeled zymosan bioparticles, or (F) red fluorescently labeled latex beads. n = 14-16 flies. Quantification follows. Experiments were performed in triplicate. Error bars, ±SE. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

used a hemocytometer to count blood cells and found that the mutant actually has significantly more blood cells (Figure 3-2). The fact that *polyph* flies phagocytose less even though they contain more blood cells, makes the phenotype even more striking.

### 3.3.2 The blood cells' phagocytic capacity is dependent upon their expression of *polyph*

As *polyph* flies exhibit a striking defect in their cellular immune response, we determined whether the gene and protein are expressed in blood cells. These cells can circulate in larvae, while in adult flies they have become mainly sessile and are difficult to separate from the surrounding tissue (Lanot et al., 2001). Therefore, the blood cell expression of *polyph* was measured by collecting lymph from *Drosophila* larvae and using qPCR to amplify the two predicted isoforms of *polyph*. *polyph-RA* and *RB* are identical in sequence except that *polyph-RA* has 48 additional base pairs in the coding region as compared to *RB*. Because, there is no sequence unique to *RB*, we used primers that recognized both transcripts to measure total *polyph* and primers unique to *polyph-RA* to specifically measure the longer transcript. The *Drosophila* expression database FlyAtlas did not examine *polyph* expression in the blood cells, but did report an enriched expression in the testes (Chintapalli et al., 2007), so possible sex differences were anticipated and males and females were evaluated separately.

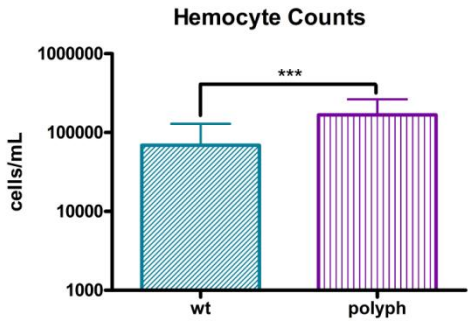



**Figure 3-2: *polyph* flies have more blood cells than wildtype flies.**

Single wt or *polyph* larvae were bled into 20µL PBS and 10µL of the hemolymph was loaded onto a hemocytometer. n = 20. Error bars, ±SD. ****p*<0.001.

In female larvae, the blood cells have an enriched *polyph* expression as compared to the carcass (Figure 3-3A). Males also have *polyph* expression in their blood cells, however they have similar levels present in their carcass presumably due to the high level of expression in the testes. Interestingly, in both sexes, the blood cells are primarily expressing *polyph-RA* as there is no difference between the total *polyph* and *polyph-RA* measured. Due to the sessile nature of the adult blood cells, adult *polyph* expression was only measured in whole animals. As expected, the male expression of the gene was higher than in females but neither sex showed significant upregulation of the gene when infected with *S.aureus* for six hours. Immunostaining of Flag-tagged Polyph protein confirmed that the putative amino acid transporter was indeed localized on the plasma membrane of blood cells, which is consistent with it playing a role in phagocytosis (Figure 3-3B). Dr. Aprajita Garg generously assisted in this experiment by immunostaining the cells.

After determining that blood cells are a major source of *polyph*, we expressed an RNAi construct against *polyph* specifically in blood cells and recapitulated the decrease in *S.aureus* phagocytosis that was seen in the original transposon mutant (Figure 3-4A). In addition, the decrease in phagocytosis could be rescued by expressing *polyph* specifically in blood cells (Figure 3-4B), confirming that the mutant's phagocytosis defect is indeed due to a lack of *polyph* expression in the blood cells.

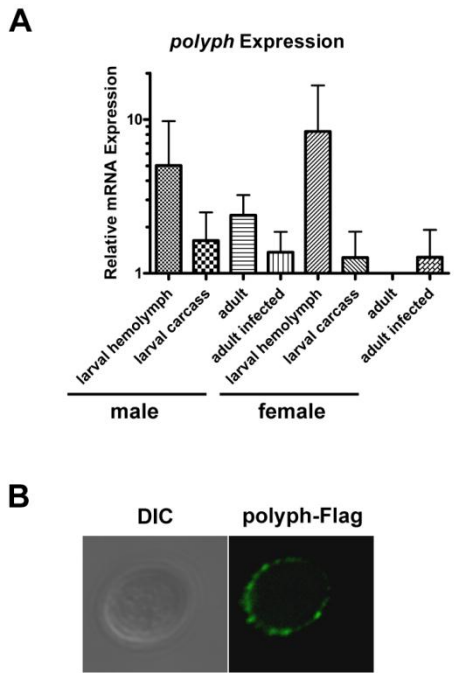



**Figure 3-3: *polyph* is expressed by blood and testicular cells. The protein localizes to the plasma membrane.**

(A) Distribution of *polyph-RA* expression in developmental stage, sex, and upon infection via qPCR. Relative expression was measured using *rp49* as an endogenous control. Hemolymph was collected by carefully rupturing the anterior end of the larvae into PBS in order to draw out the liquid. Everything that remained in the larvae after 1 minute of bleeding was considered carcass. Infected flies were injected with an overnight culture of *S.aureus* and collected at 6 hours postinfection. n = 3. (B) Blood cells collected from *hemlΔ*GAL4>UAS *polyph*Flag larvae were immunostained with an anti-Flag primary antibody, and GFP labeled secondary antibody. *hemlΔ*GAL4 is a blood cell specific driver. Cells were visualized via confocal microscopy and representative pictures are shown. Left image, DIC. Right image, GFP fluorescence. n = 20 cells. Error bars, ±SE.

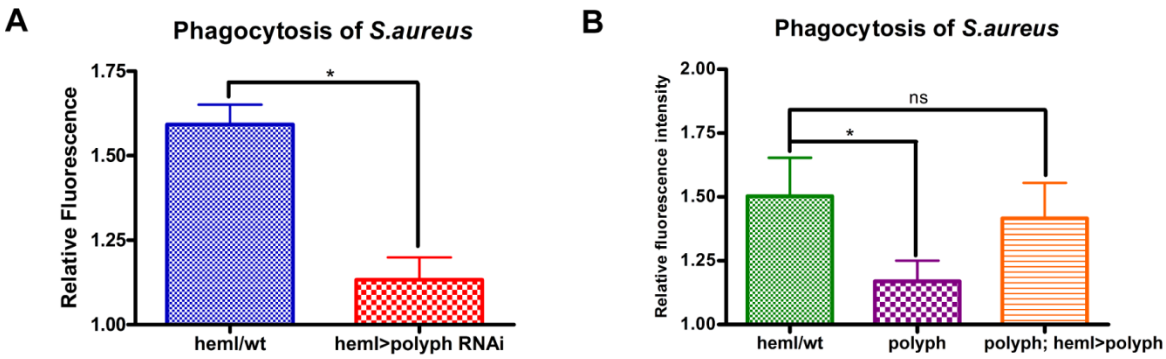



**Figure 3-4: Hemocyte expression of *polyph* is required for phagocytosis.**

Quantification of the phagocytosis of fluorescein-labeled *S.aureus* bioparticles in (A) *heml*/wt and *heml>polyph* RNAi flies and (B) *heml*/wt, *polyph*, and *polyph; heml>polyph* flies. n = 16-20 flies. Error bars, ±SE. *$p<0.05$, ns = not significantly different.

### 3.3.3. *polyph* flies have decreased resistance to an *S.aureus* infection

Both male and female *polyph* flies showed increased susceptibility to *S.aureus* infection (Figure 3-5A-B), which could be rescued by expressing the *polyph* transcript specifically in blood cells (Figure 3-5E). The critical role *polyph* plays in blood cells when the fly is trying to survive an *S.aureus* infection was further confirmed by expressing RNAi against the gene in blood cells (Figure 3-5D). The increase in bacterial load in *polyph* flies as compared to wildtype, indicates that the gene is involved in resistance instead of tolerance (Figure 3-5C). Blood cell-specific expression of the gene could rescue the bacterial growth indicating that *polyph* is required in the blood cells to control the growth of *S.aureus* during an infection (Figure 3-5F).

*polyph* flies do not appear to be generally weak as they are not more susceptible to wounding with PBS (Figures 3-5A-B), an *E.coli* infection (Figure 3-6A), or being kept at 29°C (Figure 3-6B). The fact that *polyph* shows a defect in *E.coli* phagocytosis yet is able to survive an infection, is consistent with previous data showing that upon injection, *E.coli* is efficiently killed by antimicrobial peptides (AMPs) (Schneider et al., 2007; Marek and Kagan, 2012). Therefore, most mutants with cellular defects only show increased susceptibility to *E.coli* after they are put into backgrounds with extremely limited AMP production. Although Polyph is predicted to be an amino acid transporter, it is unlikely that the protein is affecting fly

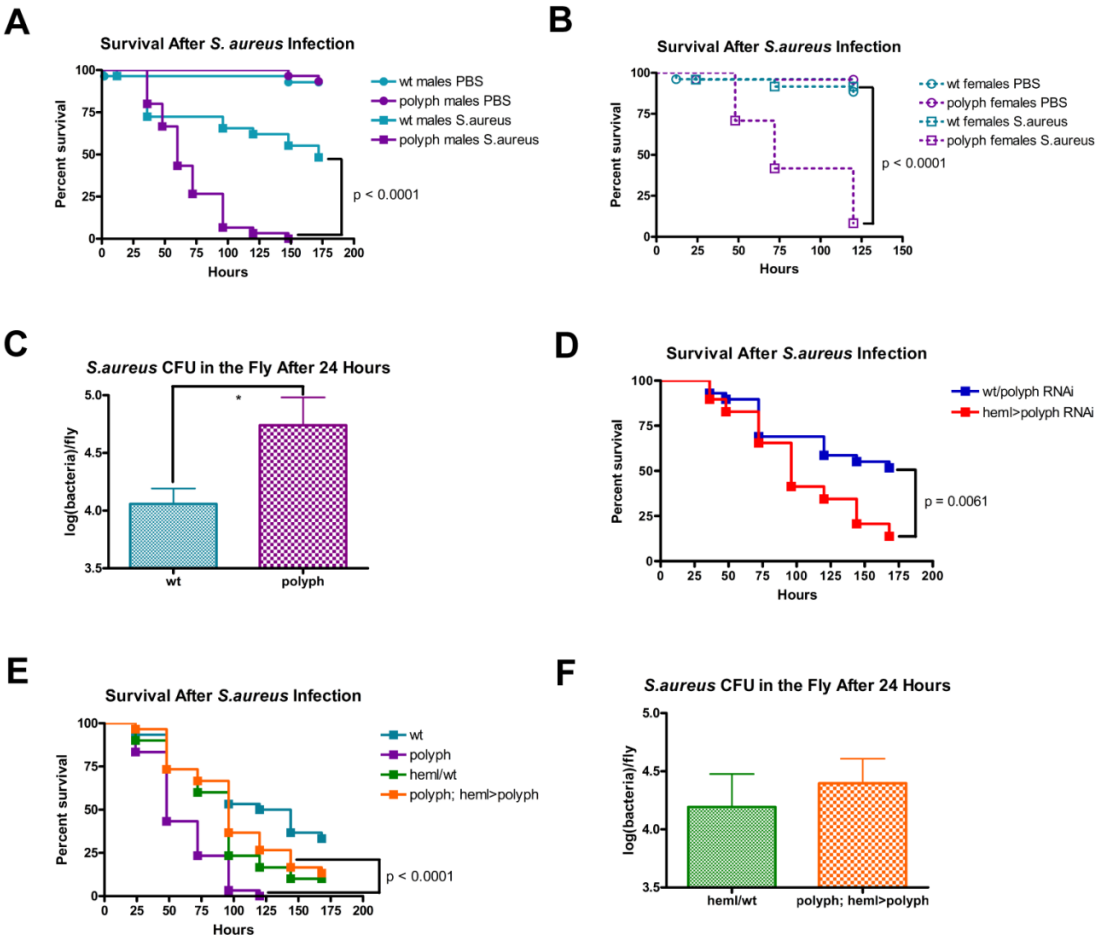



**Figure 3-5: *polyph* flies have decreased resistance against an *S.aureus* infection.**

Representative survival curves of male (A) and female (B) wt and *polyph* flies after injection of *S.aureus* (OD 0.5). n = 24-30 flies. Experiments were performed in triplicate. (C) Comparison of the *S.aureus* (OD 1.0) recovered in wt and *polyph* flies 24 hour postinfection. Bacterial load was measured in 5-6 individual flies per genotype in each experiment. More than five replicate experiments were performed. Representative survival curves of (D) wt/*polyph* RNAi and heml>*polyph* RNAi flies and (E) wt, *polyph*, *heml*/wt, and *polyph; heml>polyph* flies after injection of *S.aureus* (OD 0.5). Groups consisted of equal numbers of male and female flies. n = 28-30 flies. Experiments were performed in triplicate. (F)

Comparison of the *S.aureus* (OD 1.0) recovered in *heml*/wt and *polyph; heml>polyph* flies 24 hour postinfection. Bacterial load was measured in 5-6 individual flies per genotype in each experiment. Three replicate experiments were performed. No significant difference was found. Error bars, ±SE. $*p<0.05$

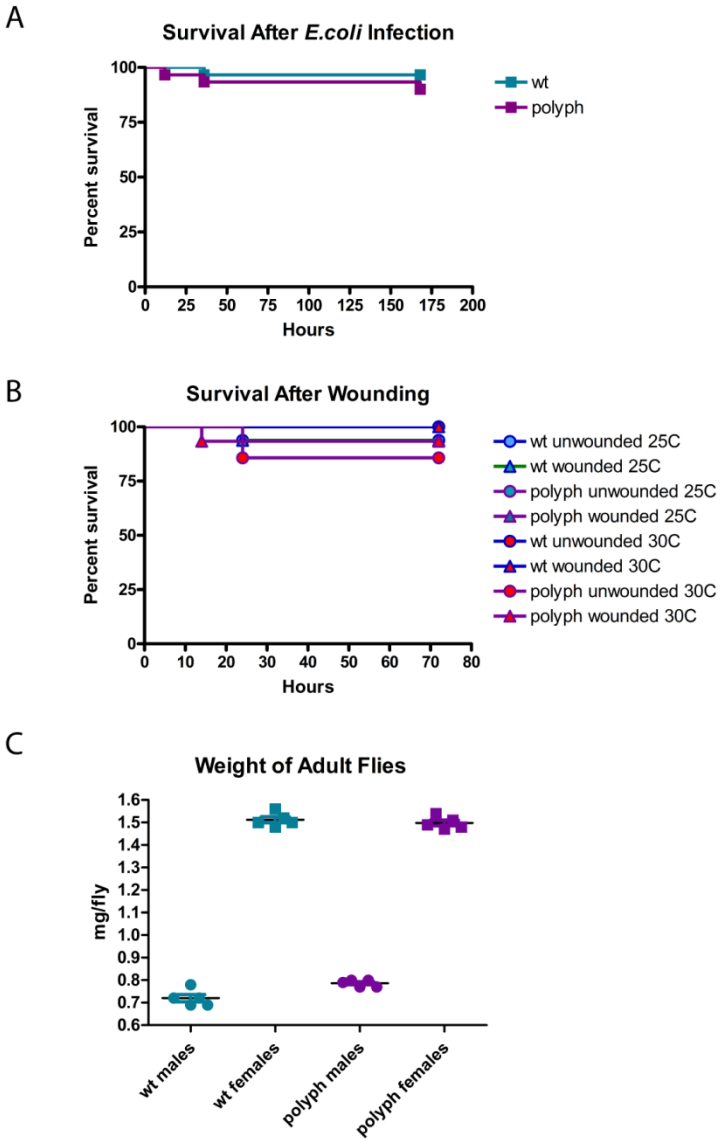



**Figure 3-6: *polyph* flies appear to be robust and normal sized.**

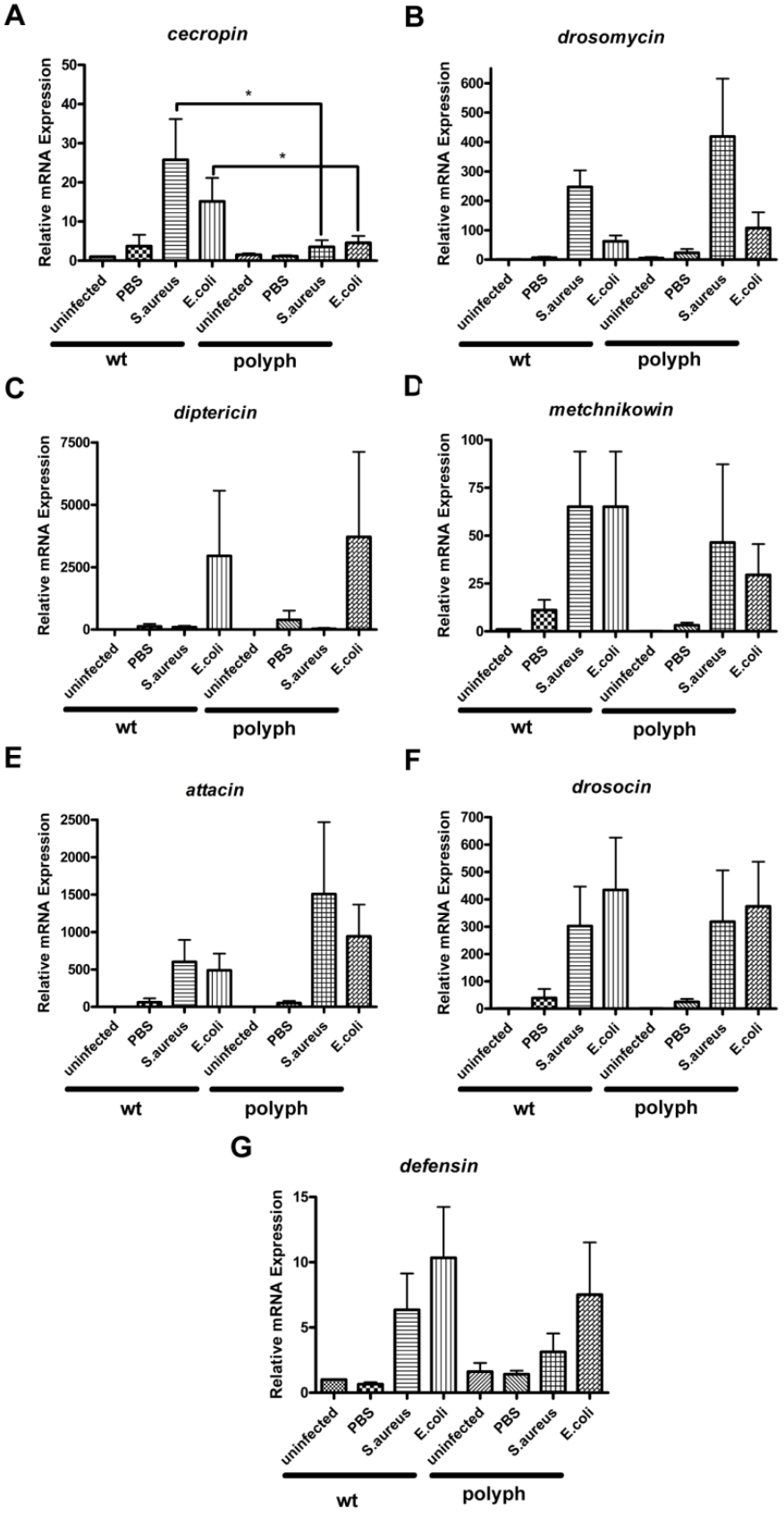
Representative survival curves of wt and *polyph* flies after (A) injection of *E.coli* (OD 50) and (B) no intervention or sterile wounding. Groups consisted of equal numbers of male and female flies. n = (A) 30 flies and (B) 12-16 flies. Experiments were performed in triplicate, no significant difference was found. Flies were either kept at the normal 25°C or at the stress-inducing temperature 29°C as noted. (C) Flies were collected from crosses of 10 females and 10 males. Five groups of 10 flies of each sex from each genotype were weighed on weigh paper. Weight was adjusted to mg/fly.

growth as its expression appears to be limited to the blood cells and testes (FlyAtlas and Figure 3-3A). We confirmed this by weighing the mutant flies and finding there was no difference in weight compared to wildtype (Figure 3-6C).

### 3.3.4 *polyph* plays a role in *cecropin* induction, which also contributes to the defense against *S.aureus*

The humoral response in *Drosophila* is mainly comprised of the production of seven classes of AMPs by the fat body (the *Drosophila* equivalent to the mammalian liver). Although the molecular mechanism is not known for all of the AMPs, some directly kill microbes or limit their growth. Recent studies in *Drosophila* have indicated that the blood cells may play a limited role in AMP production (Samakovlis et al., 1990; Brennan et al., 2007; Shia et al., 2009). To determine whether *polyph* plays a role in the humoral response, AMP induction upon infection was measured. Six of the AMPs: *drosomycin, diptericin, metchnikowin, attacin, drosocin,* and *defensin,* showed no difference in induction in *polyph* flies as compared to wildtype when infected with either *S.aureus* or *E.coli* (Figures 3-7B-G). Interestingly, the induction of the AMP *cecropin,* was significantly less in *polyph* mutants in response to either *S.aureus* or *E.coli* (Figure 3-7A). Expressing an RNAi construct against *polyph* in hemocytes also led to a decrease in *cecropin* induction (Figure 3-8A.)

The *cecropin* induction was rescued by blood cell-specific expression of *polyph* (Figure 3-8B) indicating that the blood cells are either directly producing *cecropin* or signaling to the fat body to induce *cecropin,* or both. Previous work by Samakovlis et al. has shown that blood cells can serve as a minor source of *cecropin*


A
cecropin
Relative mRNA Expression
50
40
30
20
10
0
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*
uninfected
PBS
S.aureus
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PBS
S.aureus
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wt
polyph
B
drosomycin
Relative mRNA Expression
600
500
400
300
200
100
0
uninfected
PBS
S.aureus
E.coli
uninfected
PBS
S.aureus
E.coli
wt
polyph
C
diptericin
Relative mRNA Expression
7500
5000
2500
0
uninfected
PBS
S.aureus
E.coli
uninfected
PBS
S.aureus
E.coli
wt
polyph
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metchnikowin
Relative mRNA Expression
100
75
50
25
0
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attacin
Relative mRNA Expression
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2000
1500
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500
0
uninfected
PBS
S.aureus
E.coli
uninfected
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S.aureus
E.coli
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polyph
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Relative mRNA Expression
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600
500
400
300
200
100
0
uninfected
PBS
S.aureus
E.coli
uninfected
PBS
S.aureus
E.coli
wt
polyph
G
defensin
Relative mRNA Expression
15
10
5
0
uninfected
PBS
S.aureus
E.coli
uninfected
PBS
S.aureus
E.coli
wt
polyph

**Figure 3-7: *polyph* flies have a defect in *cecropin* induction**

The induction of *cecropin* (A), *drosomycin* (B), *diptericin* (C), *metchnikowin* (D), *attacin* (E), *drosocin* (F), and *defensin* (G) was measured in wt and *polyph* flies using qPCR after injection of one of the following: PBS, overnight culture of *S.aureus*, or overnight culture of *E.coli*. All AMPs were measured 6 hours postinfection with the exception of *drosomycin*, which was measured 24 hours postinfection. Relative expression was measured using *rp49* as an endogenous control. Pools of 2-10 flies with an equal distribution of females and males were used in each experiment. Experiments were performed at least in triplicate. Error bars, ±SE. *$p<0.05$.

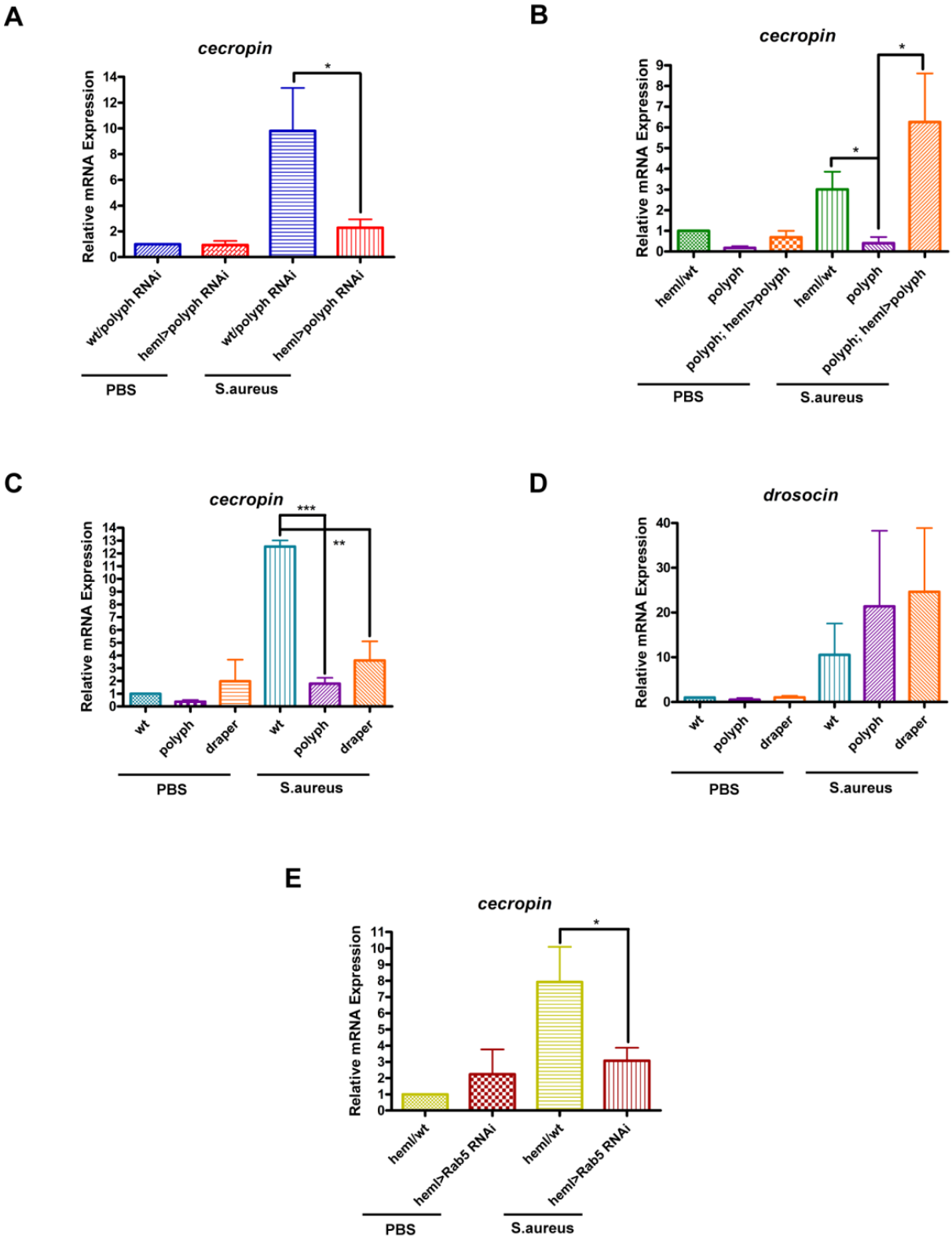
A
cecropin
Relative mRNA Expression
wt/polyph RNAi
heml>polyph RNAi
wt/polyph RNAi
heml>polyph RNAi
PBS
S.aureus
B
cecropin
Relative mRNA Expression
heml/wt
polyph
polyph; heml>polyph
heml/wt
polyph
polyph; heml>polyph
PBS
S.aureus
C
cecropin
Relative mRNA Expression
wt
polyph
draper
wt
polyph
draper
PBS
S.aureus
D
drosocin
Relative mRNA Expression
wt
polyph
draper
wt
polyph
draper
PBS
S.aureus
E
cecropin
Relative mRNA Expression
heml/wt
heml>Rab5 RNAi
heml/wt
heml>Rab5 RNAi
PBS
S.aureus

**Figure 3-8: Phagocytosis and subsequent phagosome maturation are required for *cecropin* induction.**

Using qPCR, the induction of *cecropin* in (A) wt/*polyph* RNAi and heml>*polyph* RNAi flies, (B) *heml/*wt, *polyph*, and *polyph; heml>polyph* flies, (C) wt, *polyph*, and *draper* flies, and (E) heml/wt and heml>*rab5* RNAi flies was measured 6 hours after injection with either PBS or overnight culture of *S.aureus*. (D) *drosocin* induction was also measured in wt, *polyph*, and *draper* flies 6 hours after injection with PBS or overnight culture of *S.aureus*. *hemlΔ*GAL4 is a blood cell specific driver. Relative expression was measured using *rp49* as an endogenous control. Pools of 2-10 flies with an equal distribution of females and males were used in each experiment. Experiment was performed in triplicate. Error bars, ±SE. **p*<0.05, ***p*<0.01, ****p*<0.001

(Samakovlis et al., 1990)]. There is less agreement in the scientific community concerning blood cell-to-fat body signaling. In one study, ablation of the blood cell population resulted in no effect on AMP induction (Defaye et al., 2009). The ablation may not have eliminated all of the blood cells and the few remaining cells may have been able to compensate. Also, the paper only looked at two AMPs: *defensin* and *diptericin*, which did not show any difference in this study as well (Figures 3-7C and G). Other studies have indicated that phagosome maturation in blood cells is required to signal to the fat body to produce a comprehensive AMP profile (Samakovlis et al., 1990; Brennan et al., 2007; Shia et al., 2009).

To address whether the induction of *cecropin*, specifically, requires phagocytosis, we measured *cecropin* expression in the well-established phagocytosis mutant *draper* (Cuttell et al., 2008; Hashimoto et al., 2009), and in flies expressing an RNAi construct against Rab5, an essential phagosome maturation GTPase (Bucci et al., 1992). Both *draper* (Figure 3-8C) and heml>*rab5* RNAi (Figure 3-8E) flies have significantly decreased *cecropin* induction, which is consistent with the hypothesis that phagocytosis maturation is required for full *cecropin* expression. As Draper is a membrane bound receptor that binds to *S.aureus* peptidoglycan (Hashimoto et al., 2009), *draper* mutant hemocytes have decreased uptake of the bacteria. Rab5 deficient cells are able to phagocytose particles but they are unable to progress past the early phagosome (Alvarez-Dominguez et al., 1996). As both proteins are required for *cecropin* induction following *S.aureus* infection, the signaling process involved in hemocyte-driven *cecropin* transcription requires that the bacteria be engulfed and

processed past early phagosome stage. It has recently been reported that Draper also plays a role in autophagy (McPhee et al., 2010); therefore, it is possible that *draper* mutants may have a generally decreased AMP response due to improper allocation of cellular resources within the fat body. We therefore measured the induction of another AMP *drosocin*, which has not previously been shown to be upregulated by hemocytes. *draper* flies demonstrated normal *drosocin* induction upon infection (Figure 3-8D), indicating that the decrease in *cecropin* induction in *draper* mutants is specific and is not generally affecting the humoral response.

Conversely, Cecropin has also been proposed to act as an opsonin (Chung and Kocks, 2011), which raises the possibility that *polyph* exhibits less phagocytosis because it makes less Cecropin. We therefore expressed an RNAi construct against *cecropin* using a driver specific to the fat body and blood cells (*yolk*-GAL4) and examined the flies' ability to phagocytose. *yolk>cecropin* RNAi flies had no defect in phagocytosis of *S.aureus* bioparticles (Figure 3-9A), indicating that the decreased cellular response to an *S.aureus* infection in *polyph* flies is not due to the impaired humoral response. However, yolk>*cecropin* RNAi flies did show a difference in susceptibility to an *S.aureus* infection as compared to wildtype (Figure 3-9B). The multiple classes of AMPs have often been assumed to be redundant, but we show that the loss of a single AMP class cannot always be compensated by the others. Our study indicates that Cecropin plays a contributing role in survival against a septic *S.aureus* infection and that the limited *cecropin* production in *polyph* may contribute to the mutant's increased susceptibility to the bacteria.

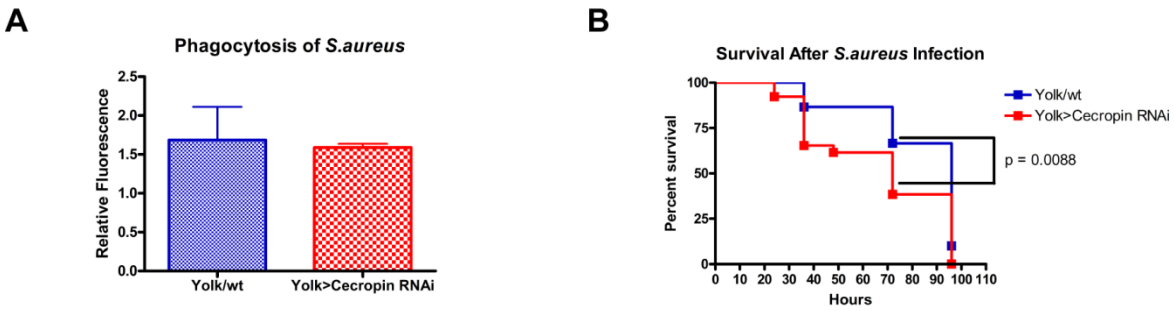


**Figure 3-9: Cecropin is required to survive an *S.aureus* infection.**

(A) Quantification of the phagocytosis of fluorescein-labeled *S.aureus* bioparticles in *yolk*/wt and *yolk>cecropin* RNAi female flies. *yolk*GAL4 is a driver active in female blood cells and fat body. n = 13-17 flies. No significant difference was found. (B) Representative survival curve of *yolk*/wt and *yolk>cecropin* RNAi female flies after injection of *S.aureus* (OD 0.1). n = 26-30 flies. Experiment was performed in duplicate.

### 3.3.5 *polyph* expressed in the testes also plays a role in the immune response

Although the fat body is the major contributor to AMP synthesis, other peripheral tissues such as blood cells or testes also play a role (Samakovlis et al., 1990; Junell et al., 2010). Of the seven classes of AMPs, Cecropin is the one that is the most highly regulated by the testes. While AMP induction in some peripheral tissues such as the blood cells or trachea, remain dependent upon the canonical Toll and Imd signaling pathways, the testes upregulate Cecropin independently of either of these pathways (Junell et al., 2010). Instead, the transcription factors Drifter/Ventral (Dfr/Vvl) and Caudal work synergistically to regulate Cecropin induction in the testes, which is amplified during an infection (Junell et al., 2010).

Because *polyph* is highly expressed in the testes in addition to the blood cells, we determined whether or not *polyph* expression in the testes affects *cecropin* induction. Expressing an RNAi construct against *polyph* wtih a testes-GAL4 driver, significantly decreased *cecropin* induction following infection (Figure 3-10A). Additionally, the expression of *polyph* in the testes of *polyph* mutants rescued *cecropin* induction to wildtype levels (Figure 3-10B), indicating that the *polyph* expression in the testes affects global *cecropin* levels. As we found that limiting *cecropin* induction during an *S.aureus* infection increased the flies' susceptibility, we then assayed the survival of *S.aureus* infected flies with decreased *polyph* transcript in their testes. Male flies expressing an RNAi construct against *polyph* in the testes were significantly more susceptible to *S.aureus* than their wild-type counterparts (Figure 3-11A).

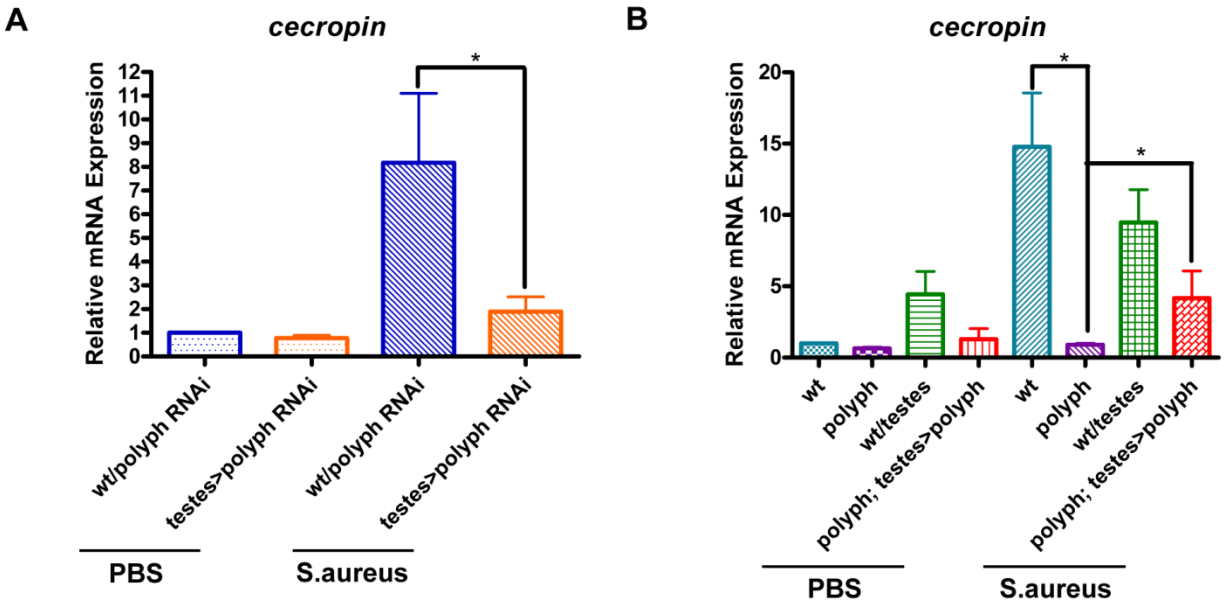


**Figure 3-10: Testes expression of *polyph* is required for *cecropin* induction.**

Using qPCR, the induction of *cecropin* in (A) wt/*polyph* RNAi and *testes*>*polyph* RNAi flies and (B) wt, *polyph*, *testes*/wt and *polyph; testes*>*polyph* flies was measured 6 hours after injection with either PBS or overnight culture of *S.aureus*. Relative expression was measured using *rp49* as an endogenous control. Only male flies were used in each experiment. Experiments were performed in triplicate. Error bars, ±SE. *$p<0.05$

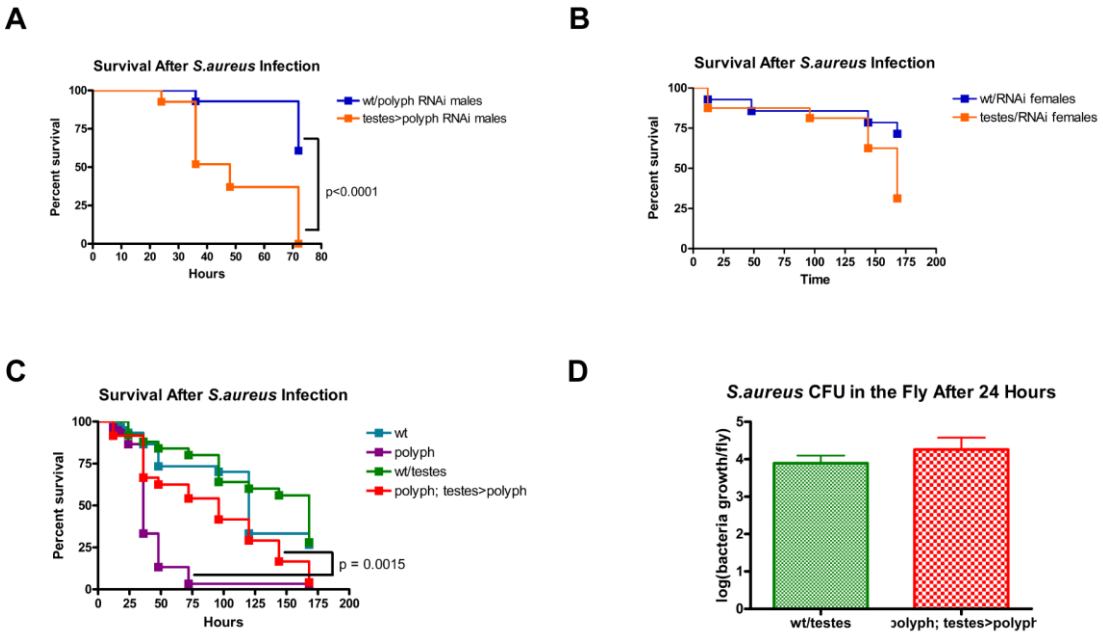


**Figure 3-11: Testes expression of *polyph* also contributes to survival following an *S.aureus* infection.**

Representative survival curves of (A-B) wt/*polyph* RNAi and testes>*polyph* RNAi flies and (C) wt, *polyph*, *testes*/wt, and *polyph; testes>polyph* flies after injection of *S.aureus* (OD 0.5). n = 28-30 flies. Experiments were performed in triplicate. (D) Comparison of the *S.aureus* (OD 1.0) recovered in *testes*/wt and *polyph; testes>polyph* flies 24 hour postinfection. Bacterial load was measured in 5-6 individual flies per genotype in each experiment. Three replicate experiments were performed. No significant difference was found. Only male flies were used in these experiments. Error bars, ±SE.

Additionally, expression of *polyph* in the testes could rescue the increased susceptibility and bacterial growth seen in the mutant (Figure 3-11C and D). As this GAL4 has been characterized to be expressed in the testes but not exclusively so, we also measured the survival of female flies expressing the RNAi construct with the testes-GAL4 driver. These female flies died slightly faster than their wildtype counterparts but not significantly so (Figure 3-11B.) Additionally, blood cells from larvae with testes-GAL4 driven GFP expression, expressed very minimal GFP as compared to blood cells from *heml*- GAL4; UAS GFP larvae (data not shown.) These data along with the knowledge that *polyph* is primarily expressed in the blood cells and the testes, indicate that the phenotypes measured using the testes-GAL4 driver are most likely due to expression in the testes.

However, it is unclear how important testes expression of *polyph* is to phagocytosis. Although a decrease in *polyph* expression in the testes does not lead to a decrease in phagocytosis (Figure 3-12A), expression of *polyph* in the testes of the mutant does rescue the phagocytosis phenotype (Figure 3-12B). The simplest explanation for the discrepancy is that the assay has low sensitivity. Due to the *in vivo* nature of the assay and the fact that it requires injection of highly concentrated bioparticles, it is difficult to measure a subtle decrease in phagocytosis. For example, the lab has been unable to see a significant difference in phagocytosis when approximately 50% of the hemocyte population has been ablated (communication with J. Robalino). Therefore it may be that the difference in phagocytosis is subtle when the *polyph* transcript is reduced via RNAi and that it therefore is not measurable with the current

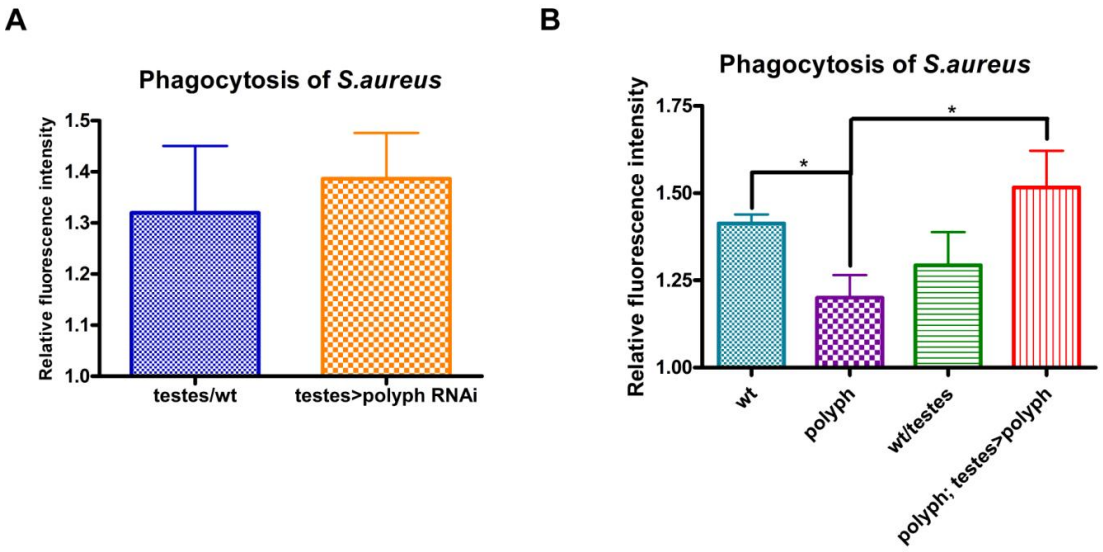


**Figure 3-12: Testes expression of *polyph* rescues phagocytosis defect.**

Quantification of the phagocytosis of fluorescein-labeled *S.aureus* bioparticles in (A) *testes/*wt and *testes>polyph* RNAi flies and (B) wt, *polyph*, *testes/*wt and *polyph; testes>polyph* flies. n = 8-18 flies. Error bars, ±SE. **p*<0.05

technique. On the other hand, if expression of the *polyph* transcript in the testes can subtly increase the level of phagocytosis then it may become indistinguishable from wild-type levels. Alternatively, it may be that *polyph* expression by the testes does not normally affect phagocytosis, however when there is no hemocyte contribution present, testes expression may be able to compensate.

### 3.3.6 *polyph* flies exhibit increased ROS and decreased bead phagocytosis when exposed to PAMPs

The closest relative to *polyph* is *excitatory amino acid transporter 1* (*eaat1*), a known glutamate transporter in the central nervous system (Takayasu et al., 2009; Stacey et al., 2010). Glutamate transport is known to regulate the cells' GSH and thus ROS levels. Therefore, we measured the ROS levels in the *polyph* mutant flies using the substrate CM-H2CDFDA, which becomes fluorescent when oxidized. When flies were first injected with PBS, followed by the substrate, *polyph* flies showed no significant difference in ROS levels as compared to wildtype. However, when the flies were first exposed to *S.aureus* and then injected with the substrate, *polyph* flies made significantly more ROS than wildtype (Figure 3-13A). To then determine if the increase of ROS in *polyph* flies is occurring in the blood cells, CM-H2CDFDA was added to *ex vivo* blood cells, which were then incubated with *S.aureus*. At 30 minutes there was no difference between wildtype and *polyph* cells indicating that *polyph* flies do not have a significantly higher basal

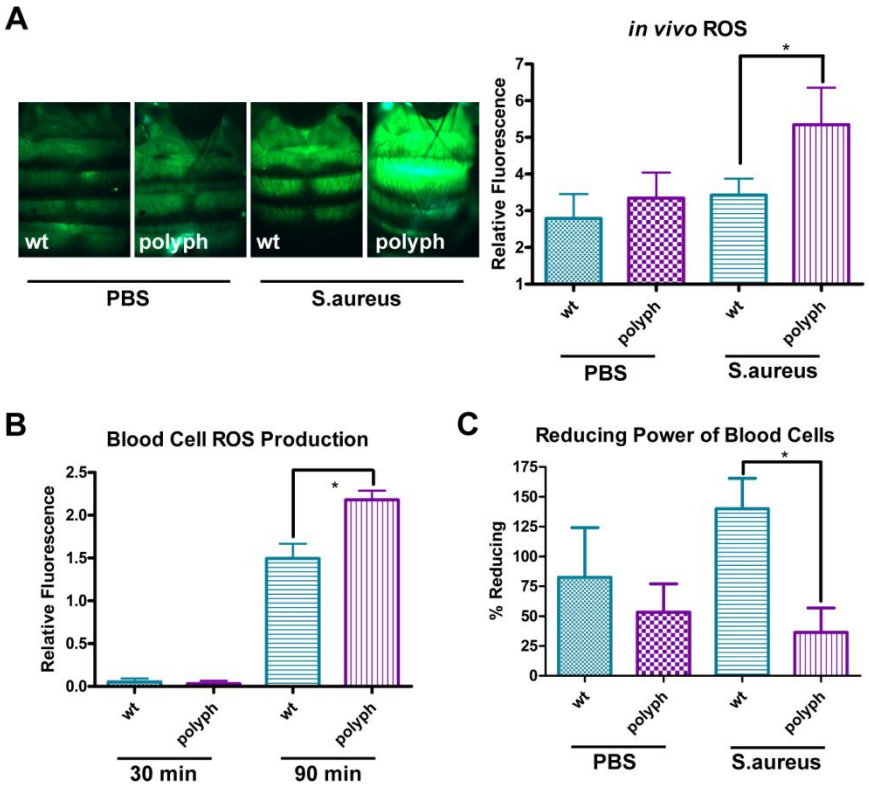


**Figure 3-13: Bacteria induce increased ROS levels in *polyph* flies as compared to wildtype flies.**

(A) Representative pictures of oxidized CM-H2CDFDA-derived fluorescence in wt and *polyph* flies after a 30 minute preinjection of either PBS or overnight culture of *S.aureus*. Quantification follows. n = 12-24 flies. (B) Measurement of oxidized CM-H2CDFDA-derived fluorescence in wt and *polyph* blood cells incubated *ex vivo* with overnight *S.aureus* culture. (C) Blood cells from wt and *polyph* larvae were either immediately incubated with PrestoBlue reagent for 10 minutes or first incubated *ex vivo* with PBS or *S.aureus* for 30 minutes. Fluorescence derived from reduced-Presto Blue was measured. Error bars, ±SE. *$p<0.05$.

level of ROS. However, after 90 minutes of incubation the ROS levels in *polyph* blood cells was significantly higher than those in wildtype cells (Figure 3-13B). The *in vivo* ROS experiment indicated that wildtype flies do not show a significant increase in ROS when exposed to *S.aureus* as compared to PBS (Figure 3-13A). It is possible that wildtype flies are able to control the ROS initiated by the bacteria by increasing the availability of antioxidants, while *polyph* flies cannot. The reducing power of the hemolymph was measured with the reagent PrestoBlue, which turns blue to red as it is reduced. Although *polyph* hemolymph has a similar reducing power as compared to wildtype when exposed to PBS, when the cells are incubated with *S.aureus*, wildtype cells respond by increasing their reducing power, while *polyph* cells cannot (Figure 3-13C). The high levels of ROS and correspondingly low levels of reducing power in *polyph* blood cells are consistent with the hypothesis that *polyph* plays a role in regulating the antioxidant capacity of blood cells via glutamate transport.

The increase in ROS after exposure to *S.aureus* correlates with a decrease in phagocytic capacity. When flies are first injected with PBS and then latex beads, *polyph* flies show a phagocytic capacity equal to wildtype flies. However, when flies were pre-injected with *S.aureus, polyph* flies take up significantly less latex beads than wildtype flies (Figure 3-14). To examine the possibility that *polyph* flies might have a more limited phagocytic capacity that is being overwhelmed by the double injection of bacteria and beads, flies were pre-injected with yellow/green fluorescently labeled latex beads followed by an injection with red fluorescently

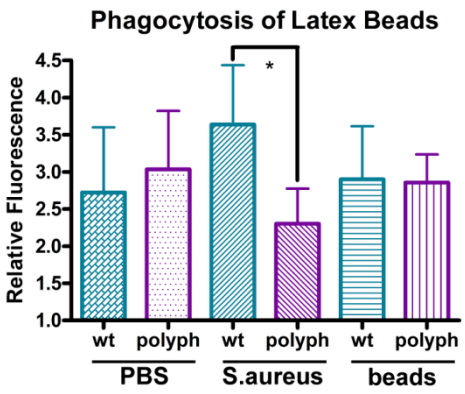


**Figure 3-14: Exposure to bacteria decreases the phagocytic capacity of *polyph* hemocytes.**

Quantification of the phagocytosis of red fluorescently labeled latex beads in wt and *polyph* flies after a 30 minute preinjection of PBS, overnight culture of *S.aureus*, or yellow-green fluorescent latex beads. n = 15-18 flies. Error bars, ±SE. *$p$<0.05

labeled latex beads. After the double bead injection, *polyph* flies were able to uptake the red beads in a manner indistinguishable from wildtype (Figure 3-14). Hence, *polyph* flies do not have a more limited phagocytic capacity but rather become unable to undergo phagocytosis once they have been exposed to *S.aureus*, and this decrease in phagocytosis correlates with an increase of ROS production in blood cells.

### 3.3.7 Phagocytosis is affected when blood cell amino acid transport is either blocked or modulated

As normal glutamate transport in the nervous system is essential for healthy nerve function, multiple drugs have been developed to modulate glutamate transport. One of them, riluzole, acts via an unspecified means to decrease extracellular glutamate and therefore alleviate the corresponding toxic effects (Chang et al., 2010; Mazzone and Nistri, 2011). When *polyph* flies are fed food supplemented with riluzole they are able to survive an *S.aureus* infection better as compared to *polyph* flies fed food supplemented with PBS (Figure 3-15A). The drug had no effect on wildtype fly survival. Hence, the increased susceptibility seen in *polyph* flies is at least partially due to a glutamate imbalance. Given that glutamate deregulation clearly leads to deleterious effects during an infection, it was notable that *polyph* flies did not have shortened lifespans and that male *polyph* flies actually lived significantly longer than wildtype flies (Figure 3-15B). Flies with increased ROS levels have been shown to have longer lifespans than their wildtype counterparts (Albrecht et al., 2011). It could be that a small increase in basal ROS levels is protective against infections from the fly's commensal flora.

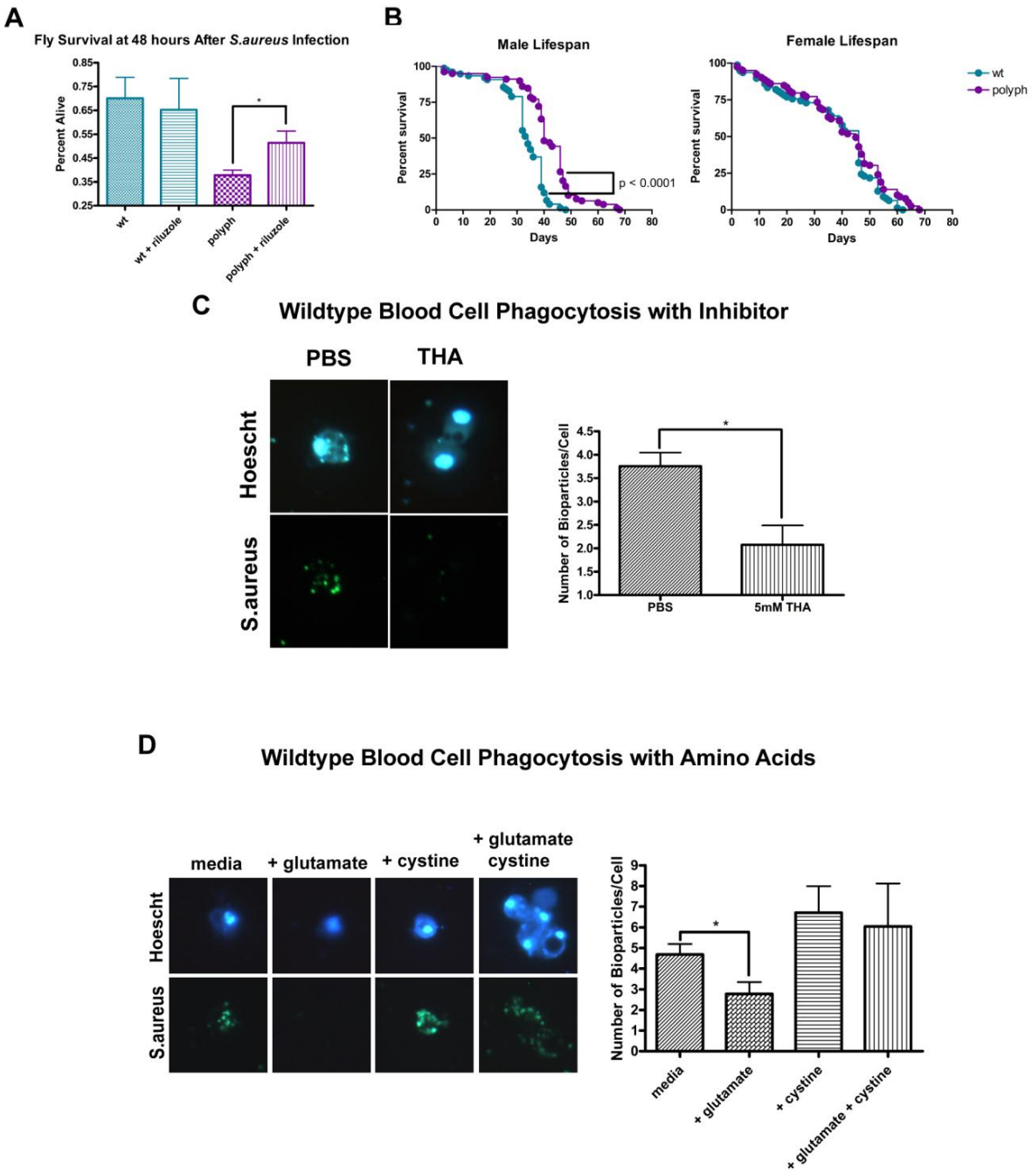
A
Fly Survival at 48 hours After S.aureus Infection
Percent Alive
0.85
0.75
0.65
0.55
0.45
0.35
0.25
*
wt
wt + riluzole
polyph
polyph + riluzole
B
Male Lifespan
Female Lifespan
Percent survival
100
75
50
25
0
p < 0.0001
0 10 20 30 40 50 60 70 80
Days
wt
polyph
C
Wildtype Blood Cell Phagocytosis with Inhibitor
PBS
THA
Hoescht
S.aureus
Number of Bioparticles/Cell
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
*
PBS
5mM THA
D
Wildtype Blood Cell Phagocytosis with Amino Acids
media
+ glutamate
+ cystine
+ glutamate cystine
Hoescht
S.aureus
Number of Bioparticles/Cell
9
8
7
6
5
4
3
2
1
0
*
media
+ glutamate
+ cystine
+ glutamate + cystine

**Figure 3-15: Modification of glutamate transport affects the fly's survival and phagocytic capacity.**

(A) Comparison of the proportion of wt and *polyph* flies that survive 48 hours after an *S.aureus* (OD 0.5) infection while feeding on food supplemented with either PBS or 1mM riluzole. (B) The lifespan of wt and *polyph* male and female flies was recorded. All flies eclosed within 24 hours of each other. n = 80 flies. Blood cells were incubated *ex vivo* with fluorescein *S.aureus* bioparticles in PBS or 5mM THA (C), or media plus nothing, glutamate, cystine, or glutamate and cystine (D). After 30 minutes the number of bioparticles/cell was counted in approximately 20 cells per group in each experiment. All experiments, with the exception of lifespan measurement, were done in triplicate. Error bars, ±SE. *$p<0.05$.

Another modulator of glutamate transport is the aspartate analog, threo-beta-hydroxyaspartate (THA), which acts as a general inhibitor of the eaat transporters (Rimaniol et al., 2001). As *polyph* is most closely related to *eaat1*, it may also be inhibited by THA. Blood cells were collected from wildtype larvae, incubated in either PBS or THA, and assayed for their ability to uptake *S.aureus* bioparticles. Blood cells incubated with 5mM THA showed significantly less phagocytosis of the bioparticles than the blood cells in PBS (Figure 3-15C), indicating that blocking glutamate transport in blood cells inhibits phagocytosis. This was further supported by measuring the phagocytic capacity of cells in minimal media with glutamate, or cystine, or both added to the solution. Cells showed significantly less phagocytosis when incubated with only glutamate and consistently more (albeit not significant $p = 0.061$) phagocytosis when given cystine (Figure 3-15D). The base minimal media contained an extremely small amount of cystine (0.099mM) and no glutamate. When a large amount of excess glutamate was added extracellularly, this mimicked the presumed situation of *polyph* cells. By artificially forcing a high extracellular [glutamate] and a relatively low intracellular [glutamate], this would drive the glutamate/cystine antiporter to pump glutamate into the cell and cystine out, thereby depleting the cells' store of cystine and preventing them from making GSH. Without their ability to counteract bacteria-induced ROS, the cells become unable to function effectively and are unable to phagocytose efficiently. In contrast, when the cells are given high levels of cystine, this drives the antiporter in the canonical direction, allowing the cell to continually make more GSH. These cells seem to have a higher phagocytic capacity than cells in minimal media. The cells in minimal media, while

not actively losing their intracellular cystine stores, are also not able to obtain any more from their environment. Taken together, these data indicate that glutamate transport into blood cells is a tightly regulated process, which is necessary for blood cells to remain effective against an infection.

### 3.3.8 Other amino acid transporters, when expressed in blood cells, appear to play a similar role in the immune response as *polyph*

Because glutamate is an extremely important neurotransmitter, most research on glutamate transport is focused on this aspect of its function. However, it also plays a more complicated role in glutamate toxicity, a phenomenon where neurons become sick from increased intracellular ROS due to excess extracellular glutamate (Miyamoto et al., 1989; Murphy et al., 1989). Cells depend on antioxidants such as GSH to reduce excess oxidized molecules within the cell. The limiting reagent in GSH production is the amino acid cysteine, which is brought into the cell as the oxidized form cystine via a glutamate/cystine antiporter (Rimaniol et al., 2001). The antiporter is concentration dependent, so if *eaat1* is functioning properly, then there is more intracellular glutamate than extracellular, driving glutamate out and cystine in (Bannai, 1986). However, if *eaat1* or the *Drosophila* glutamate/cystine antiporter, *genderblind* (*gb*) is mutated, then cystine is no longer transported in, GSH is limited, and ROS rises. Therefore, we examined if *eaat1* or *gb* were expressed in blood cells and if mutants in these genes would show phenotypes similar to those seen in *polyph*. We found that *gb* (Figure 3-16A) but not *eaat1* (Figure 3-16B) was expressed in blood cells. Consistent with this observation, *gb* mutant flies showed increased

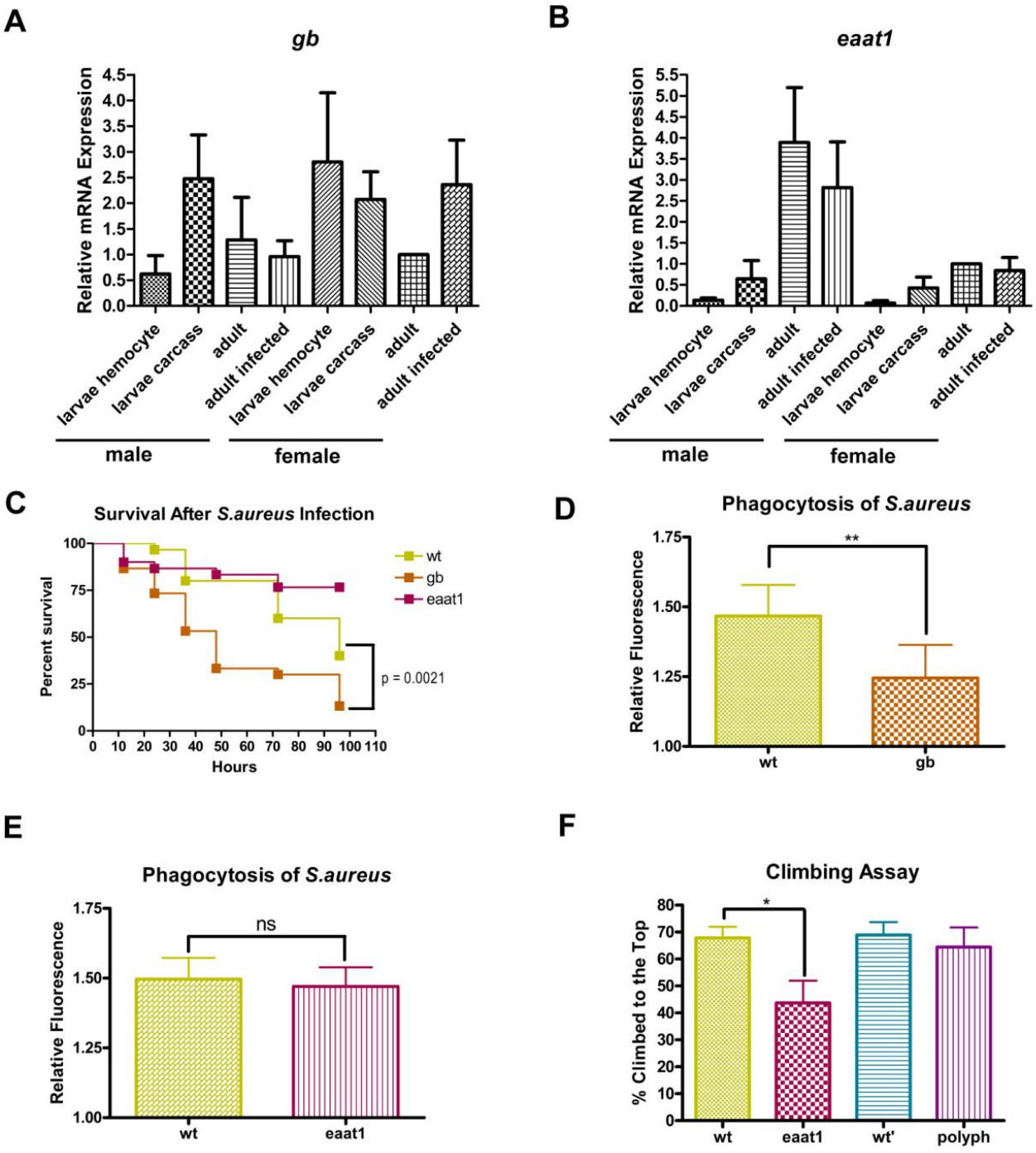


**Figure 3-16: Flies without the cystine/glutamate antiporter, *genderblind*, phenocopy *polyph* flies.**

Using qPCR, the expression of *gb* (A) and *eaat1* (B) was measured over developmental stage, sex, tissue, and infected status. Relative expression was measured using *rp49* as an

endogenous control. Infected flies were injected with overnight culture of *S.aureus* and collected at 6 hours postinfection. (C) Representative survival curve of wt, *gb* and *eaat1* flies after injection of *S.aureus* (OD 0.05). Groups consisted of equal numbers of male and female flies. n = 30 flies. Quantification of the phagocytosis of fluorescein-labeled *S.aureus* bioparticles in wt and *gb* flies (D) and wt and *eaat1* flies (E). n = 15-18 flies. (F) Vials containing 30 wt, *eaat1*, wt', or *polyph* flies were gently tapped on the bench knocking the flies to the bottom of the vial. The number of flies that reached the top inch of the vial within 30 seconds was recorded. Experiments were done in triplicate. Error bars, ±SE. *$p<0.05$, **$p<0.01$, ns = not significantly different.

susceptibility to *S.aureus* infection whereas *eaat1* mutants did not (Figure 3-16C). *gb* flies also showed decreased phagocytosis of *S.aureus* bioparticles (Figure 3-16D) while *eaat1* flies were able to undergo phagocytosis at a rate similar to wildtype (Figure 3-16E). Conversely, it is unlikely that *polyph* is playing a role in the CNS due to its expression pattern. To confirm this, we performed a climbing assay to measure CNS function in *polyph* flies. When knocked down, flies will instinctively climb upward. Because *eaat1* mutants have a motor defect due to inefficient glutamate transport in their CNS, mutant flies climb more slowly than wildtype flies (Stacey et al., 2010). This motor defect was observed in *eaat1* flies but not in *polyph* flies (Figure 3-16F), confirming that *polyph* does not appear to affect the nervous system. Rather, the gene operates primarily in the blood cells where it regulates the macrophages' phagocytic capacity.

If Polyph is functioning in a similar manner to Eaat1 but in a different tissue, then blood cell-specific expression of *eaat1* in a *polyph* mutant background should rescue the phenotypes seen in *polyph* flies. There is indeed a strong rescue of the phagocytosis phenotype (Figure 3-17A) and a small but significant increase in *cecropin* induction over the *polyph* mutant (Figure 3-17B). The discrepancy between the strength of these rescues could either be due to a difference in the sensitivity of the assays or that the production of *cecropin* is more difficult to rescue because it is a complex process that requires many steps. Blood cell-expression of *eaat1* also showed a small decrease in susceptibility to *S.aureus* as compared to the mutant but it was not significant (Figure 3-17C). Taken together, these data indicate that Polyph is

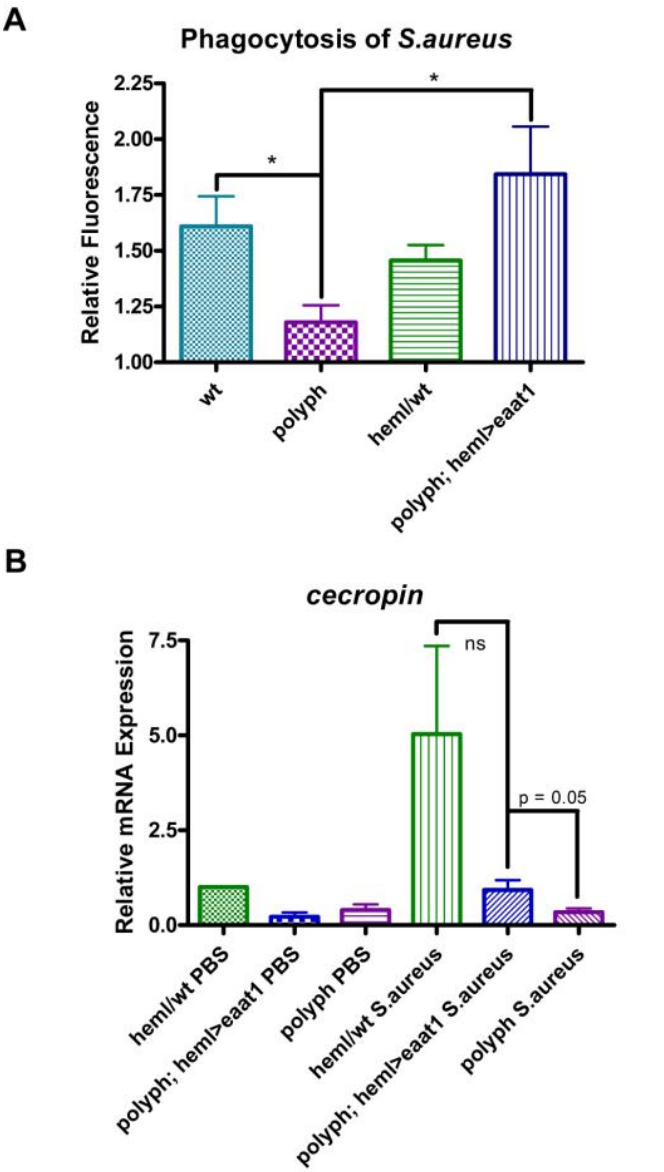
A
Phagocytosis of S.aureus
Relative Fluorescence
2.25
2.00
1.75
1.50
1.25
1.00
*
*
wt
polyph
heml/wt
polyph; heml>eaat1
B
cecropin
Relative mRNA Expression
7.5
5.0
2.5
0.0
ns
p = 0.05
heml/wt PBS
polyph; heml>eaat1 PBS
polyph PBS
heml/wt S.aureus
polyph; heml>eaat1 S.aureus
polyph S.aureus
C
Survival After S.aureus Infection
Percent survival
100
75
50
25
0
0
25
50
75
100
125
150
175
200
Time
wt
polyph
heml/wt
polyph; heml>eaat1

**Figure 3-17: Hemocyte expression of the glutamate transporter *eaat1* can partially rescue *polyph* phenotypes.**

(A) Quantification of the phagocytosis of fluorescein-labeled *S.aureus* bioparticles in wt, *polyph, heml*/wt and *polyph; heml>eaat1* flies. n = 15-18 flies. (B) Using qPCR, the induction of *cecropin* in *heml*/wt, *polyph*, and *polyph; heml>eaat1* flies was measured 6 hours after injection with either PBS or an overnight culture of *S.aureus*. Relative expression was measured using *rp49* as an endogenous control. Pools of 2-10 flies with an equal distribution of females and males were used in each experiment. (C) Representative survival curves of wt, *polyph*, *heml*/wt, and *polyph*; *heml>eaat1* flies after injection of *S.aureus* (OD 0.5). Experiments were performed in triplicate. Error bars, ±SE. *$p<0.05$, ns = not significantly different.

a highly important glutamate transporter expressed on blood cells. It works with the cystine/glutamate antiporter Gb via a complicated and highly regulated process to control the cells' internal ROS in order to maintain macrophage function. Without the tight regulation of glutamate transport across the blood cell membrane, the fly loses its resistance to a pathogenic infection via a decrease in both phagocytic capacity and AMP production.

### 3.3.9 polyph larvae have an increased melanization response

*Drosophila* also produce ROS through the melanization process via specialized hemocytes, crystal cells (Rizki et al., 1985). Crystal cells produce and release prophenoloxidase (PPO) following microbial infection or wounding. These signals initiate a serine protease cascade that ends in prophenoloxidase activating enzyme (PPAE) cleaving PPO to active phenoloxidase (PO) (Lee et al., 1998b; Satoh et al., 1999; Kanost et al., 2004). PO then leads to the production of melanin from tyrosine, a process which releases antimicrobial ROS byproducts into the surrounding hemolymph (Zhao et al., 2007). As melanization is another important component to surviving a septic infection, we investigated whether *polyph* mutants exhibit a compromised melanization response.

Curiously, when *polyph* larvae are wounded, they experience enhanced melanization as compared to wild-type larvae (Figure 3-18A). Hemolymph was collected from *polyph* and wildtype larvae and incubated *ex vivo* with nothing, live bacteria, or dead bacterial bioparticles. Regardless of what was added, *polyph* hemolymph melanized

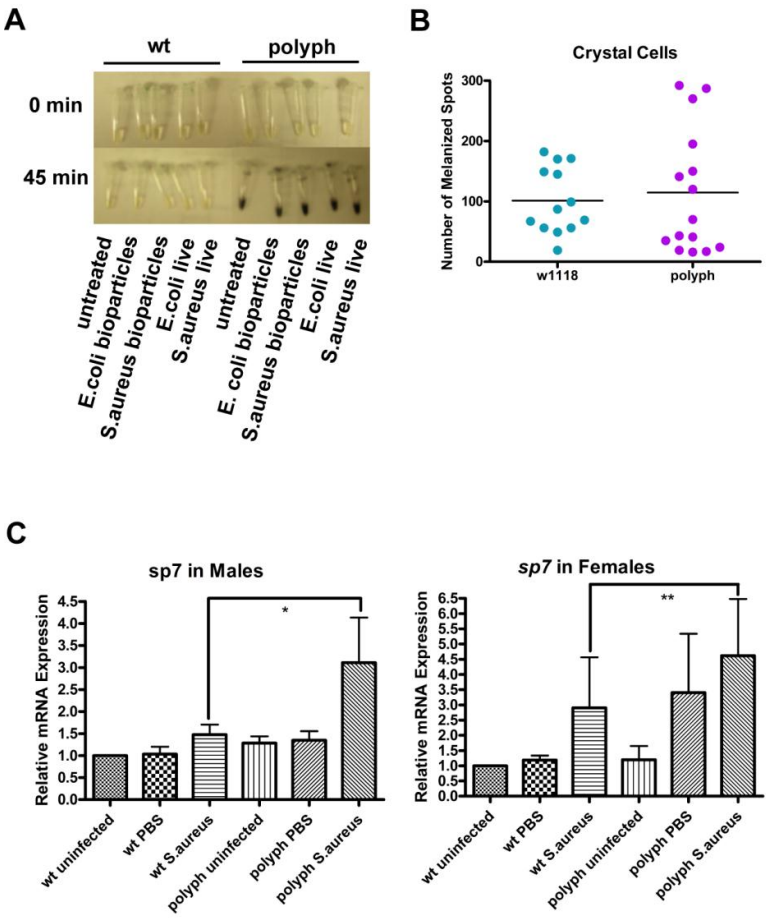


**Figure 3-18: *polyph* flies have a hyperactive melanization response.**

(A) Larvae were bled into Schneider's media and 1µL of sterile PBS, overnight culture of either *S.aureus* or *E.coli*, or 4mg/mL *S.aureus* or *E.coli* fluorescein labeled bioparticles was added to the hemolymph. Picture was taken after 45 min of incubation at room temperature. (B) Larvae were incubated in 1mL of water at 65ºC for 10 min, followed by an hour on ice. The high temperature causes the crystal cells to burst creating localized melanized spots. Each melanized spot was counted as a single crystal cell. n = 13-15 larvae. (C) Using qPCR, the *sp7* transcript was measured in wt and *polyph* flies that were either uninfected or injected with PBS or overnight culture of either *S.aureus* or *E.coli*. Male and female animals were measured separately as they differentially express *sp7*. Relative expression was measured using *rp49* as an endogenous control. Error bars, ±SE. *$p<0.05$, ** $p<0.01$

faster than wildtype hemolymph indicating that the phenomenon was independent of non-commensal bacteria. This increase in melanization in *polyph* mutants was not due to an increase in the number of crystal cells (Figure 3-18B) but was correlated with a higher expression of *serine protease 7* (*sp7*) (Figure 3-18C). Sp7/MP2/PAE1 is one of the serine proteases involved in the cascade which activates PPAE. Overexpression of Sp7 leads to constitutive melanization whereas *sp7* mutants have a decreased melanization response to septic infections (Tang et al., 2006; Ayres and Schneider, 2008).

Hemolymph from double mutant *polyph;sp7* larvae exhibited less melanization than *polyph* larvae but were unable to restore wildtype levels of melanization (data not shown). It is possible that removing *sp7* from *polyph* mutants was unable to completely rescue the increase in melanization because of redundancies in the serine protease signaling cascade. For example, *sp7* mutants still exhibit melanization following wounding and are therefore still able to activate the cascade in a *sp7*-independent manner. Interestingly, *polyph;sp7* double mutants exhibited wildtype induction of *cecropin* (Figure 3-19A) but were unable to phagocytose any better than *polyph* mutants (Figure 3-19B). This discrepancy may be explained by the fact that multiple tissues are capable of *cecropin* expression, while plasmatocytes are the predominant phagocytic cells. *polyph* mutants produce more melanin in a septic infection and therefore more ROS byproducts. Some of these byproducts are permeable to cell membranes and are equally toxic to fly cells as they are to microbes. If the ROS is highly concentrated, it may diffuse throughout the

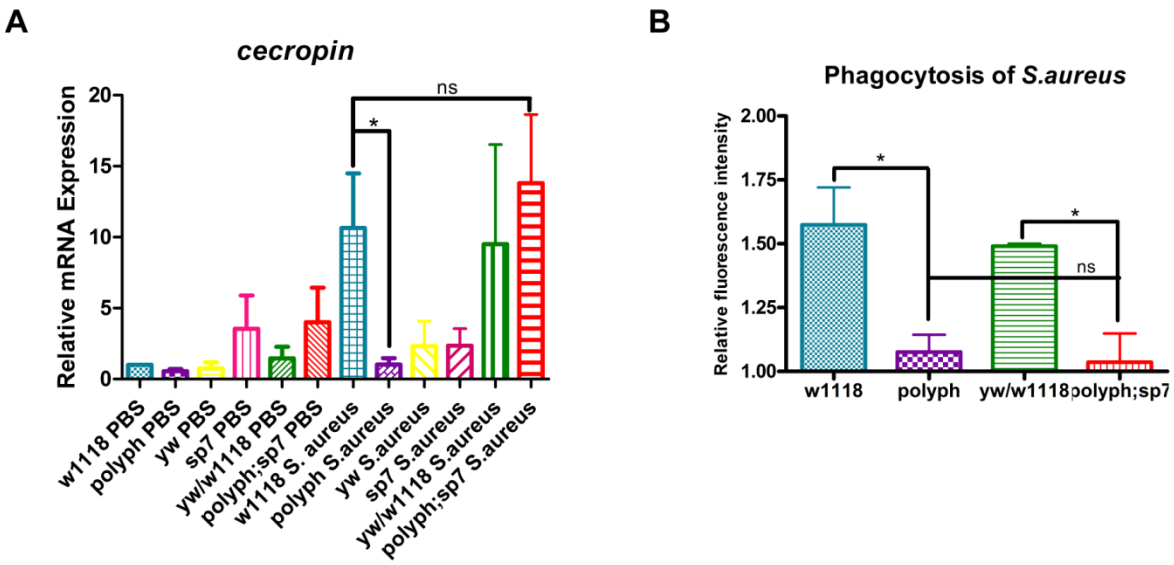


**Figure 3-19: Reducing melanization in *polyph* mutants restores *cecropin* induction.**

(A) Using qPCR, the induction of *cecropin* in $w^{1118}$, *polyph*, *yw*, *sp7*, $w^{1118}$/*yw*, *polyph;sp7* flies was measured 6 hours after injection with either PBS or overnight culture of *S.aureus*. $w^{1118}$ is the background of the *polyph* mutant, while *yw* is the background of the *sp7* mutant. As the two backgrounds often behave differently, they most both be taken into account. Relative expression was measured using *rp49* as an endogenous control. Pools of 2-10 flies with an equal distribution of females and males were used in each experiment. (B) Quantification of the phagocytosis of fluorescein-labeled *S.aureus* bioparticles in $w^{1118}$, *polyph*, *yw*, *sp7*, $w^{1118}$/*yw*, *polyph;sp7* flies. n = 15-17 flies. Error bars, ±SE. *$p<0.05$, ** $p<0.01$, ns = not significantly different.

hemolymph and damage many peripheral tissues including those that produce cecropin such as the fat body and the testes. If the overmelanization is partially reduced in *polyph;sp7* animals, the ROS may be more contained to a smaller area of the hemolymph and only negatively affect the immediately neighboring cells such as the plasmatocytes. In addition, the plasmatocytes when exposed to bacteria already have an increase in ROS and *polyph* cells are unable to respond with an increase in glutathione. Therefore these cells are particularly sensitive to the presence of any additional ROS.

## 3.4 Discussion

The focus to date has been on how lymphocyte-derived glutamate results in neurotoxic effects and causes neurological diseases (Piani et al., 1991; Tian et al., 2008; Yawata et al., 2008). How lymphocyte function is affected by glutamate deregulation has been largely overlooked. Using a well-defined model system, *Drosophila melanogaster*, we find evidence for the importance of glutamate regulation in blood cells in mounting an effective cellular response to infection *in vivo*. We identify a previously uncharacterized putative amino acid transporter, *polyph*, as important in the fly's resistance to infection. *polyph* expression in blood and testicular cells is necessary for the fly's phagocytic response, AMP production, melanization response, and general ability to survive a pathogenic infection. In addition, these flies have increased levels of ROS upon subsequent exposure to bacteria. Polyph is predicted to have an amino acid transporter domain and is most closely related to the well-studied glutamate transporter Eaat1. Taken together, these facts led to the prediction that Polyph may be playing a role in glutamate transport.

Glutamate transporters, specifically those of type $X_{AG-}$ (of which Eaat1 is a member), are expressed and function on human macrophages (Rimaniol et al., 2001). Their function is intimately tied to the $X_{C-}$ cystine/glutamate antiporters in order to regulate cystine transport into the cell (Watanabe and Bannai, 1987). Cysteine is the limiting reagent in the synthesis of the antioxidant GSH (Rimaniol et al., 2001). When glioma cells were subjected to mM amounts of extracellular glutamate, their cystine uptake plummeted and their levels of GSH were depleted. The ubiquitous extracellular glutamate led to cell death within 24 hours. Death could be rescued by either the addition of cystine or the antioxidant Vitamin E indicating that the cell death was dependent upon GSH regulation of internal ROS (Kato et al., 1992).

Polyph appears to function in an Eaat-like manner but in blood and testicular cells instead of neurons. Our model indicates that *polyph* flies have an increased level of extracellular glutamate in their hemolymph due to an inability to effectively transport it into the cell. This would then lead to a decrease in GSH and an increase in internal ROS. The small number of blood cells per larvae made it unfeasible to directly measure blood cell GSH levels. However, a marked increase in ROS was observed when *polyph* flies were exposed to bacteria. GSH levels have been shown to decrease in neutrophils following phagocytosis of *S.aureus* (Roos et al., 1979). Given that *polyph* flies are homozygous viable and do not have fewer blood cells or higher levels of ROS than wildtype flies, they must be able to maintain enough antioxidant to control their resting ROS levels. However, as *polyph* phagocytes begin to take up *S.aureus*, their GSH likely becomes depleted and their ROS levels begin to rise. In

extreme cases, as seen with the glioma cell, it can lead to cell death (Kato et al., 1992). However, this does not occur with *polyph* phagocytes, as there is no loss of blood cells following infection (data not shown). In lymphocytes, an unbalanced GSH redox system leads to decreased microtubule formation (Oliver et al., 1976), a process which is necessary for phagocytosis. This decrease in phagocytosis occurs when cells encounter a broad range of microbes, given that *polyph* flies show decreased phagocytosis of *S.aureus, E.coli* and zymosan. As *polyph* flies are able to uptake latex beads, it appears that the blood cells are functional until presented with a "danger" stimulus present in the microbial-derived particles. This conclusion is consistent with a study that showed increased cystine uptake and GSH synthesis in mouse peritoneal macrophages when exposed to either LPS or zymosan, indicating that the redox system is activated by a variety of danger signals (Sato et al., 1995).

When *polyph* phagocytes are exposed to one of these signals, their internal ROS increases and they are unable to respond with upregulation of redox components as a normal phagocyte would. Thus, the intracellular ROS continues to increase and the phagocyte is no longer able to function normally under oxidized conditions. The excess ROS will likely damage critical proteins needed for cellular function, which will then need to be replaced for the cell to survive. In order to maintain homeostasis, the cell is forced to divert resources away from secondary processes such as phagocytosis. The high concentrations of ROS are only exacerbated by the high levels of melanization in *polyph* flies. It is unclear as to why *polyph* flies exhibit hypermelanization. Hemolymph derived from *polyph* larvae undergoes accelerated

melanization even without the addition of bacteria, which would indicate that the process is solely dependent on wounding. However, in reality the hemolymph most likely becomes contaminated with any bacteria present on either the surface or in the gut of the larvae during the bleeding process. In contrast, when *polyph* adults are subjected to a sterile wound with a needle, they do not exhibit a melanization response at the wound site beyond what is seen in wildtype flies. Therefore, it is possible that, similar to the decrease in plasmatocytes' phagocytic activity, the activation of crystal cells is also dependent upon the introduction of microbial products. In fact, it may be that the two phenomena are interconnected as sick and dying hemocytes are known to initiate melanization (Bidla et al., 2007). *polyph* plasmatocytes become stressed from ROS-induction from a bacterial infection and subsequently initiate the serine protease cascade and crystal cell lysis. This would explain why increased melanization was observed in infected *polyph* larvae but not adults (data not shown). In the larvae, the hemocytes are circulating throughout the animal and therefore the crystal cells would come into contact with the plasmatocytes. In the adult, however, the hemocytes are mainly sessile and therefore rarely contact one another. The crystal cells could be activated by the circulating bacteria, but *S.aureus* is known to not induce a robust melanization response (Ayres and Schneider, 2008). The putative induction of melanization by the *polyph* plasmatocyte, would result in a further increase in ROS production, allowing the destructive molecules to travel throughout the hemolymph harming peripheral tissues, such as the testes.

The role that *polyph* plays in the testes and how it relates to the immune response is less clear. The *Drosophila* testes are involved in localized immune responses as flies are often exposed to microbes through intercourse (Miest and Bloch-Qazi, 2008). The male reproductive system has therefore evolved to independently induce and regulate AMPs and melanization (Junell et al., 2010). In fact *Drosophila* males deposit AMPs as well as sperm into females during intercourse (Lung et al., 2001). Mammalian testes express multiple glutamate receptors (Storto et al., 2001) and transporters (Pines et al., 1992; Storck et al., 1992), although any function they may have in the immune response has not been characterized. Interestingly, the introduction of high concentrations of extracellular glutamate does cause oxidative damage to rat testes (Vinodini et al., 2008), which may indicate that there is a similar glutamate signaling process present in the testes as in neurons. Testes from *polyph* flies may therefore be unable produce a rigorous *cecropin* response to infection as compared to wildtype, which could affect the flies' survival. As *Drosophila* possess an open circulatory system, it appears that glutamate transport in the testes may affect glutamate concentrations in the hemolymph. This would explain how *polyph* expression in the testes could rescue the phagocytosis defect in *polyph* mutants. However, the *polyph* testes must still be partially functional as the mutants are not sterile and do not have any obvious defects in mating.

Increased glutamate levels in the plasma have been associated with many serious diseases including ALS (Iwasaki et al., 1992a), epilepsy (Janjua et al., 1992), stroke-associated headache (Castillo et al., 1995), HIV-associated dementia (HAD)

(Ferrarese et al., 2001), Parkinson's disease (Iwasaki et al., 1992b), breast cancer, colorectal carcinoma, and AIDS (Ollenschläger et al., 1989). To date, most research dissecting the relationship between glutamate and these diseases focuses specifically on how macrophage-derived glutamate causes excitotoxicity in neurons, resulting in neuronal damage or death. For example, patients with HAD develop cognitive impairment resulting from nerve death in the basal ganglia, cerebral cortex, and hippocampus, which is dependent on macrophage mitochondrial glutaminase (Zhao et al., 2004; Erdmann et al., 2007; Tian et al., 2008). This enzyme deaminates glutamine to glutamate and is elevated in HIV-infected macrophages. The elevation in glutamate concentrations results in the macrophages releasing glutamate into the extracellular milieu, leading to glutamate-associated toxicity in the surrounding nerve tissue. Our study indicates that in addition to the effects it has in the CNS, glutamate transport in the blood cells may be playing a more direct role in regulating macrophage function. The role of the Drosophila $X_{c-}$ transporter, *gb*, in glutamate transport and GSH synthesis has already been well established (Augustin et al., 2007). *gb* larvae had significantly less glutamate in their hemolymph as compared to wildtype animals, indicating that *gb* is expressed on a tissue with access and ability to affect the hemolymph amino acid concentrations (Piyankarage et al., 2008). We found that *gb* is expressed on blood cells, and when mutated, *gb* mimics the immune deficiencies found in *polyph* flies. This confirms the importance of glutamate/cystine transport in blood cells on macrophage function. In addition, *ex vivo* experiments using either the glutamate transporter competitive inhibitor THA or the addition of amino acids directly, showed that deregulation of glutamate transport in blood cells results in

impaired phagocytosis. Our data clearly demonstrate that macrophage function is dependent upon a complicated interdependent amino acid transport system, of which both *polyph* and *gb* are a part.

When the GSH redox system becomes unbalanced, there is potential for human disease. For example, people with a genetic disorder with a mutation in the glutathione reductase gene, have impaired neutrophil function. When the antioxidant GSH reduces ROS, it is converted into its oxidized state, GSSG. The enzyme glutathione reductase facilitates the conversion of GSSG back to its reduced state GSH. Without the enzyme, the redox system becomes skewed towards the oxidized state and is less able to reduce any free reactive species. In one study, neutrophils from individuals with the genetic disorder showed between 10-15% of the normal amount of glutathione reductase activity. The neutrophils could maintain normal levels of ROS for a short period of time following incubation with bacteria, but following an extended incubation, the levels of hydrogen peroxide rose significantly higher than in neutrophils from healthy individuals. In addition, the glutathione levels decreased very quickly in the deficient neutrophils (Roos et al., 1979). These findings not only support our blood cell glutamate transport model, but also show that understanding the delicate balance of this system has biological relevance in human disease. Our results demonstrate that a tightly regulated redox system in the blood cells is necessary to maintain immune-cell function and therefore the health of the whole organism.

## 3.5 Materials and Methods

### 3.5.1 Fly Stocks

The following stocks were from Bloomington Drosophila Stock Center: $w^{1118}$; Mi[ET1]CG12943$^{MB02238}$, $y^1$ $w^{67c23}$; P{EPgy2}Eaat1$^{EY20741}$, $y^1$ $w^{1118}$; P{UAS-Eaat1.Exel}3/TM6B, $Tb^1$, $y^1$ $w^{67c23}$; $ry^{506}$ P{SUPor-P}gb$^{KG07905,}$ and $y^1$ $w^{67c23}$; P{SUPor-P}Sp7$^{KG02818}$ $ry^{506}$. $w^{1118}$ flies were used as background control for *polyph* experiments and $y^1w^{67c23}$ flies were used in *eaat1*, *gb,* and *sp7* experiments. The Vienna Drosophila RNAi Center provided the *cecropin A1* RNAi line, $w^{1118}$; P{GD3965}, the *polyph* RNAi line $w^{1118}$; P{GD421} , and the isogenic control line $w^{1118}$ . The fat body driver flies, *yolk*GAL4, the blood cell-specific driver flies, *hem*lΔGAL4, and the testes driver flies, $w^{1118}$; P{GawB}DJ691, were all received from Bloomington.

The *polyph* rescue construct was made by amplifying *polyph-RA* from the Drosophila Genomics Resource Center plasmid IP11938 using a forward primer containing a BglII site: CCGAGATCTATGGAGCCAAAGTCGCAGGATCAGGCT, and a reverse primer containing a XhoI site: CCGCTCGAGTTACATACTGTTGAGCGTCAACTGAAA. The Flag tag, MDYKDDDDK, was added to the C-terminus of *polyph* by PCR using the above forward primer and the following reverse primer, which also contains a XhoI site: CCGCTCGAGTTATTTATCGTCATCGTCTTTGTAATCCATCATACTGTTGAG. Products were cloned into the pCR 2.1 cloning vector (Invitrogen). Plasmids were transformed, following manufacturer's instructions, into TOP10 Chemically Competent *E.coli* Cells (Invitrogen). Constructs were isolated using the QIAprep Spin

Miniprep Kit (Qiagen), according to manufacturer's instructions, sequenced, and then cloned into a pUAST vector. The constructs were transformed as above. The Plasmid Midi Prep Kit (Qiagen) was used according to manufacturer's instructions to isolate and purify the final constructs which were transformed into *Drosophila* embryos (BestGene).

### 3.5.2 Adult Phagocytosis

To assay *S.aureus* phagocytosis, approximately eight flies per genotype per experiment were injected with ~0.2µL of 5 mg/mL fluorescein-labeled heat or chemically killed *S.aureus* bioparticles (Invitrogen) using the Pneumatic Picopump Injector (World Precision Instruments). They were then incubated for 30 minutes at room temperature, injected with Trypan blue, and mounted ventral side down and wings spread to the side onto black electrical tape. Flies were treated as above for the phagocytosis of fluorescein-labeled *E.coli* and zymosan bioparticles (Invitrogen) with the exception that flies were incubated for 10 minutes after the first injection. The 0.2µm Red Fluorescent Carboxylate Modified FluoSpheres (Invitrogen) were injected at a 1:20 dilution. Fluorescently labeled particles were visualized using either a GFP or Texas Red filter on the Discovery.V8 SteREO Microscope (Zeiss). AxioVisionLE software was used to quantify the results. Relative fluorescence calculated as: $[\text{fluorescence}]_{\text{dorsal vein area}}/[\text{fluorescence}]_{\text{adjacent area}}$.

To assay secondary phagocytosis of beads, flies were first injected with approximately 0.04µL of one of the following: PBS, overnight culture of *S.aureus* resuspended in PBS, or 1.0µm Yellow/Green Fluorescent Carboxylate Modified

FluoSpheres diluted 1:20 (Invitrogen). Flies were incubated at room temperature for 30 minutes, then injected with approximately 0.2µL of 1.0µm Red Fluorescent Carboxylate Modified FluoSpheres diluted 1:20 (Invitrogen), incubated at room temperature for 10 minutes, injected with Trypan Blue, and then mounted and visualized as described above.

### 3.5.3 Survival Following Infection

Bacteria, either *S.aureus* or *E.coli*, were grown overnight at 37°C. Bacteria was then subcultured and grown to log phase, OD = 0.8-1.0 for *S.aureus* and OD = 0.5-0.7 for *E.coli*. The culture was spun at 10000 rpm for 10 minutes and cells were resuspended at the appropriate OD in sterile PBS. For survival curves with *E.coli*, OD = 50. For *S.aureus* survival curves with flies that carry the *yolk*GAL4 driver, OD = 0.1. For *S.aureus* survival curves with *gb* and *eaat1* flies, OD = 0.05. All other *S.aureus* survival curves used an OD = 0.5.

Flies of the appropriate genotype were injected with 0.04µL of the appropriate bacterial resuspension. Flies were kept at 25°C (except when specifically indicated otherwise), transferred to new food as appropriate, and death was monitored on a daily basis. To assay the effect of riluzole on survival, flies were placed on food supplemented with 250 µL of either water or 1mM riluzole (Sigma-Aldrich). None of the fly lines used in survival showed significant death after injection with sterile PBS. All survival curves were done, at minimum, in triplicate.

### 3.5.4 Reverse Transcriptase Quantitative PCR

To collect RNA from larval blood cells and carcasses, approximately ten larvae of the appropriate sex were carefully lacerated with tweezers on their anterior end in 100µL nuclease-free water. Carcasses were allowed to rest in the water for an additional minute before being homogenized in 500µL STAT-60 (Tel-Test, Inc.). The diluted hemolymph was added to a new tube containing 500µL STAT-60. To collect RNA from adult flies, 2-10 animals were anesthetized with $CO_2$ and homogenized in 500µL STAT-60. Once samples were in STAT-60, RNA was harvested from all homogenized samples per manufacturer's instructions.

The concentration of RNA was measured using the Nanodrop 1000 (Thermoscientific). Between 100 and 500 ng of RNA was then used to make cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions.

To measure AMPs, flies were injected with 0.04µL of overnight culture of *S.aureus* or *E.coli* as indicated. Flies were collected at 6 hours postinfection to measure all AMPs except *drosomycin*, which was measured from flies infected for 24 hours. AMPs were measured using LUX-based qPCR, where one primer of each pair was tagged with the fluorescent reporter FAM (Invitrogen). The ROX qPCR Mastermix (2X) (Fermentas) was used. Each sample was measured in technical triplicate or quadruplicate. Using the 7300 Real Time PCR System (Applied Biosystems) samples were placed at 50°C for 2 minutes, 95°C for 10 minutes, and then cycled between 95°C for 15 seconds and 60°C for 1 minute 40 times. Relative quantification was

calculated via the $2^{-\Delta\Delta Ct}$ method using RP49 as the endogenous control. Experiments were run at least in triplicate and were analyzed by one-tailed paired t-tests.

To measure *polyph*, *gb*, *eaat1*, and *sp7* expression infected flies were collected 6 hours after injection with 0.04µL of overnight culture of *S.aureus*. Gene expression was measured using SYBR-based qPCR with the Maxima® SYBR Green/ROX qPCR Mastermix (2X) (Fermentas). Each sample was measured in technical triplicate or quadruplicate using the cycling and analysis method described above. The total *polyph* and *polyph-RA* primers were found to have comparable amplification efficiencies ($p = 0.207$) using a standard curve indicating that the transcript levels measured can be compared.

### 3.5.5 Immunostaining

Approximately 5 *heml>polyph-flag* larvae were bled into cold PBS on a polylysine coated coverslip. The carcass was removed after five minutes to allow the blood cells to attach to the coverslip. Excess liquid was removed and cells were fixed in 4% formaldehyde, washed, blocked, and incubated with a 1:500 dilution of a mouse anti-Flag antibody (Sigma) overnight. Cells were then incubated with a 1:200 dilution of the goat α-mouse secondary antibody (Invitrogen) for 2 hours, washed, and mounted in Prolong (Invitrogen). Slides were sealed and incubated at 4°C for at least 24 hours. The LSM 710 confocal microscope (Zeiss) was used for visualizing the cells.

### 3.5.6 Bacterial Load

*S.aureus* was grown overnight in a shaking incubator at 37°C, subcultured to an OD of 0.8-1.0, and resuspended in sterile PBS to an OD of 1.0. Approximately 18 flies per genotype per experiment were injected with 0.04µL of the bacterial suspension. Six flies from each group were then immediately ground in individual tubes with 200µL LB + 1% TritonX-100, serially diluted 1:10 twice in sterile PBS, and plated in triplicate on LB plates. After 24 hours of infection at 25°C, six additional flies from each genotype were assayed as above with the exception that each sample was serially diluted 1:10 five times. Bacterial colonies were counted after the plates were incubated for 24 hours at 37°C.

### 3.5.7 Larval Phagocytosis

To assay the effect of THA on phagocytosis, 30 $w^{1118}$ larvae were bled into 400µL of Schneider's media (Gibco). The carcasses were allowed to rest for one minute before they were removed. In microcentrifuge tubes, 90µL of the diluted hemolymph was then supplemented with sterile PBS or THA (Sigma-Aldrich) at a final concentration of 5mM. Fluorescein-conjugated *S.aureus* bioparticles were added at a final concentration of 16µg/mL. Samples were incubated in the dark for 30 minutes before being pipetted onto polylysine coated coverslips (Fisher) where cells were allowed to attach for one minute. Excess liquid was aspirated, and 100µL of Hoescht (10µg/mL) was added for two minutes. Coverslips were then washed twice with PBS, mounted with PBS, and immediately visualized with a Leica DM RB Microscope. The number of bioparticles per cell was recorded for approximately 20 cells per treatment group per experiment. Experiments were performed in triplicate.

To assay the effect of amino acids on phagocytosis, 10 $w^{1118}$ larvae were bled into 100µL minimal media (Gibco) supplemented with nothing, 20mM glutamate (Sigma-Aldrich), 0.417mM cystine (Sigma-Aldrich), or both. Fluorescein-conjugated *S.aureus* bioparticles were added at a final concentration of 16µg/mL and samples were processed as described above.

### 3.5.8 *In vivo* ROS

Approximately 6 flies per genotype per experiment were injected with 4nL of either sterile PBS or *S.aureus* cultured overnight and incubated at room temperature for 30 minutes. Flies were then injected with 20nL 1mM CM-H2CDFDA (Invitrogen) diluted in DMSO and incubated in the dark at room temperature for 10 minutes. They were mounted ventral side down and wings spread to the side onto black electrical tape and visualized using the GFP filter on the Discovery.V8 SteREO Microscope (Zeiss). AxioVisionLE software was used to measure fluorescence in a polygon drawn in the dorsal vein area of the injected flies, which was then divided by the autofluorescence measured in the same polygon on an uninjected fly. Experiments were done in quadruplicate.

### 3.5.9 *Ex Vivo* ROS

Thirty larvae of the appropriate genotype were bled into 400µL Schneider's media, carcasses were allowed to rest for one minute before being removed. The diluted hemolymph was divided into two wells in a Costar 96 well black clear bottom plate (Fisher Scientific) with 98µL per well. Wells were treated with 2µL PBS or 1µL

*S.aureus* cultured overnight + 1µL 1mM CM-H2CDFDA diluted in DMSO. The SpectraMaxM2 Plate Reader (Molecular Devices) was used with an excitation wavelength of 485 nm and an emission wavelength of 538 nm to measure the fluorescence at 0, 30, 90 minutes. Relative fluorescence was calculated by first subtracting the fluorescence of the PBS-treated cell from the fluorescence measured in the cell treated with *S.aureus*. Then, to normalize for any difference in cell numbers, the number was divided by the fluorescence of the PBS-treated cells at 0 minutes. Experiments were done in triplicate.

### 3.5.10 Measurement of Cells' Reducing Power

Thirty larvae of the appropriate genotype were bled into 400µL sterile PBS, carcasses were allowed to rest for one minute before being removed. The diluted hemolymph was divided into two microcentrifuge tubes, 150µL per tube, that were then treated with 1µL of either PBS or *S.aureus* grown overnight and incubated for 30 minutes at room temperature. 90µL of each sample was then transferred to an individual well in a Costar 96 well black clear bottom plate (Fisher-Scientific). Thirty additional larvae of the appropriate genotype were then bled into 400µL sterile PBS and 90µL of the diluted hemolymph was immediately added to the 96 well plate. 10µL of the PrestoBlue Reagent (Invitrogen) was added to each well and the plate was incubated for 10 minutes at 37°C. The SpectraMaxM2 Plate Reader (Molecular Devices) was used with an excitation wavelength of 570 nm and an emission wavelength of 612 nm to measure the fluorescence. The % reducing power of the blood cells was calculated by first subtracting the fluorescence of PBS + PrestoBlue from all wells, and then dividing the fluorescence of the experimental wells by that of the wells containing the

hemolymph that was immediately added to the plates following bleeding. Experiments were performed in triplicate.

### 3.5.11 Lifespan

For each genotype, 100 flies of each sex were collected within 24 hours after eclosing. The population was distributed into 20 flies/vial and transferred onto new food as appropriate. Flies were kept at 25°C and survival was monitored on a daily basis.

### 3.5.12 Blood Cell Counts

To count total blood cells, single larvae of the appropriate genotype were bled into 20µL PBS. The carcasses were removed after 1 minute and 10µL of the diluted hemolymph was loaded onto a hemocytometer. n = 20. To count crystal cells, larvae were places in Eppendorf tubes with 1mL of PBS and incubated at 65ºC for 10 min. Tubes were then placed on ice for an hour. The high temperatures cause the crystal cells to burst creating localized melanized spots. Each melanized spot was counted as a single crystal cell. n = 13-15 larvae.

### 3.5.13 Fly Weight

Flies were collected from crosses of 10 females and 10 males to avoid overcrowding issues affecting growth. Five groups of 10 flies of each sex from each genotype were weighed on weigh paper. The weight was then adjusted to mg/fly.

### 3.5.14 Climbing Assay

Thirty flies of the appropriate genotype were placed in a clean fly vial. The vial was gently tapped on the bench in order to knock all of the flies to the bottom. The number of flies that reached the top inch of the vial within 30 seconds was recorded. The experiment was done in triplicate.

### 3.5.15 Larval Melanization Assay

Twenty-five larvae were washed in sterile PBS before being bled into 250µL Schneider's media. Carcasses were removed after one minute incubation and 29µL of hemolymph was aliquoted into PCR-grade tubes. Subsequently 1µL of sterile PBS, overnight culture of either *S.aureus* or *E.coli*, or 4mg/mL *S.aureus* or *E.coli* fluorescein labeled bioparticles was added to the hemolymph. Tubes were monitored over the course of several hours to watch melanization occur.

# Chapter 4: Discussion

The innate immune response is a multifaceted system that rapidly provides broad-spectrum defense against infection through germline encoded components. In mammals, it also activates the adaptive immune system to produce antibodies through antigen presentation and the release of costimulatory molecules. One highly conserved component of the innate immune system is the activation of the family of NFκB transcription factors. In mammals, the translocation of NFκB transcription factors into the nucleus can be initiated by TLRs binding to PAMPs (Abbas, 2003). Many of the components of the TLR signaling pathways are highly conserved between species (Ganesan et al., 2011) although there are a few noteworthy exceptions. For example, cows, in particular, are remarkably sensitive to LPS (Berczi et al., 1966), a TLR4 ligand. Several proteins that signal downstream of TLR4, have been found to function differently in bovine cells as compared to human cells (Bannerman et al., 2002c; Bannerman et al., 2004; Szperka et al., 2005; Szperka et al., 2006). Given that LPS-induced septic shock is still a prominent cause of death in surgical patients (Dahmash et al., 1993b; Zanetti et al., 1997b), understanding what makes bovine cells more sensitive to LPS may be helpful in treating human patients.

MyD88 and TIRAP are two TIR-containing adaptor proteins that are recruited to the intracellular region of TLR4 upon LPS stimulation (Medzhitov et al., 1998; Fitzgerald et al., 2001; Horng et al., 2001). Both bovine proteins demonstrate >80% amino acid identity with the human proteins and are predicted to encode the same domains as those found in the human proteins. We demonstrated that both proteins

mediate TLR4-dependent NFκB activation in bovine cells in response to LPS stimulation. This was measured by two independent means: NFκB-luciferase reporter construct and E-selectin expression. These data are consistent with what was predicted from the function of MyD88 and TIRAP in other mammalian species. Additionally, TIRAP's role in modulating LPS-induced apoptosis is also conserved between cows and humans, although our results remain unclear regarding bovine MyD88's role. It may be that bovine MyD88's function is different from the human protein and does not play a role in TLR4-mediated apoptosis. It is also possible that MyD88 is playing a subtle role in the pathway and our assays were not sensitive enough to measure the effect. A third possibility is that bovine cells may have another protein that acts redundantly with MyD88 in this pathway, which is not present in human cells. This would prevent us from measuring a decrease in apoptosis when a MyD88 dominant negative construct was expressed if another protein was able to compensate. Although multiple redundancies are currently known in the human TLR4 pathway, expression of a MyD88 dominant negative construct in human cells does decrease LPS-stimulated apoptosis (Bannerman et al., 2002b). This indicates that there is no human protein that can completely compensate for a lack of MyD88 in this pathway. Preliminary results also indicated that a third member of the TLR4 signaling pathway, TRAF6, functions differently in the cow than what would be predicted from other mammalian studies. Specifically, a dominant negative bovine TRAF6 construct downregulated LPS-induced NFκB induction in human, but not bovine, cells. It would be interesting for future experiments to assay how human TRAF6 functions in bovine cells. Previously, the TLR4 signaling molecule, FADD,

was been shown to act differently in bovine versus human cells (Bannerman et al., 2002c; Bannerman et al., 2004; Szperka et al., 2005; Szperka et al., 2006). While FADD is proapoptotic in both species, overexpression of human FADD, but not bovine FADD, decreases NFκB induction. It may be that similar to FADD, TRAF6 is another bovine protein that deviates from the canonical TLR4 signaling pathways, and contributes to the cow's increased sensitivity to LPS. Although it is currently unclear how bovine TRAF6 is functioning, human TRAF6 stimulates NFκB nuclear translocation following LPS stimulation. If bovine TRAF6 is unable to fulfill this role, the TLR4 signaling pathway may become more skewed from NFκB induction to apoptosis.

While NFκB activation in mammals results in the upregulation of a multitude of proteins including cytokines and adhesion molecules, the nuclear translocation of *Drosophila* NFκB homologs, Dif and Relish, primarily results in the upregulation of antimicrobial peptides (AMPs) (Ganesan et al., 2011). This upregulation of AMPs along with phagocytosis are the fly's primary defense mechanisms against septic infections (Williams, 2007). The amino acid glutamate is highly concentrated in the fly's hemolymph (Piyankarage et al., 2008), and is therefore in direct contact with the fly's immune cells. While glutamate has been carefully studied in the context of the CNS, it is unclear what role, if any, it plays in the immune system. We demonstrated that a putative amino acid transporter, *polyphemus* (*polyph*), is required for the fly to phagocytose, produce a full spectrum of AMPs, control ROS production, and survive a septic infection. We further demonstrate that manipulating the glutamate/cystine

balance affects the blood cells' ability to phagocytose. Increased glutamate in human plasma has been associated with a variety of serious diseases (Dröge et al., 1987; Iwasaki et al., 1992a; Janjua et al., 1992) and monosodium glutamate (MSG) is prevalent in many foods (Yamaguchi and Ninomiya, 2000). Therefore, the scientific field would be remiss to ignore the possible effect of glutamate transport on the immune system. Future experimentation is needed to carefully dissect the relationship between glutamate, antioxidants, and immune function in the mammalian macrophage.

We have demonstrated that both the cow and the fly are interesting organisms in which to study the immune system. The cow, being a mammal, is more closely related to humans. While the immune pathways between the two species are not identical, they are highly similar and the small differences give big insights into how and why the two species respond differently to infectious stimuli. *Drosophila*, being arthropods, share a much older common ancestor with humans. But they are a well-established model system with a well-developed genetic toolbox and a simplified immune system. Together, both organisms have provided and will continue to provide thought-provoking insights into the mechanics of human innate immunity.

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