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The Investigation of Innate Immune Pathways in the Yellow Fever Mosquito, *Aedes aegypti*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

Kanwal Shakeel Alvarez

June 2010

Dissertation Committee

Dr. Alexander S Raikhel, Chairperson

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The Dissertation of Kanwal Shakeel Alvarez is approved:

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University of California, Riverside

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Dedication

To my husband, Ignacio. I love you.

ABSTRACT OF DISSERTATION

The Investigation of Innate Immune Pathways in the Yellow Fever Mosquito, *Aedes aegypti*

by

Kanwal Shakeel Alvarez

Doctor of Philosophy Graduate Program in Cell, Molecular, and Developmental Biology
University of California, Riverside, 2010
Dr Alexander S. Raikhel, Chairperson

Mosquitoes are vectors of many parasitic and viral diseases that continue to burden the global public health system. The innate immune system of insects is the first line of defense against invading pathogens. This system is governed by two major pathways: Toll, and Immune deficiency (IMD). This dissertation involves the investigation of these pathways. Aim 1 involves the exploration of the Toll and PPO activation pathway in response to pathogen invasion. Aim 2 seeks to investigate the signaling components of the IMD pathway and lastly, Aim 3 provides a comparative genomic study of the mosquito immune system. The results of the first aim identified an interleukin receptor associated kinase (IRAK), IRAK4, in the mosquito, *Aedes aegypti*. Furthermore, it was found that IRAK4 serves an important immune function in the Toll immune pathway. Additionally, several cytoplasmic components of the IMD pathway were identified

including PGRPLC, IMD, IKK β , and FADD (Aim 2). Lastly, the study involving the last Aim revealed that the conservation of immune pathways; however, it also revealed species-specific gene expansion, which is probably incorporated to approach particular aspects of immune reactions.

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CHAPTER 1: INTRODUCTION

Vectors are defined as organisms that carry pathogens either physically or biologically, from one host to another; thus, transmitting numerous diseases. The pathogens multiply inside the vector and are passed on when the vector bites to take a blood meal. Arthropods account for over 85 percent of all known animal species, and they are the most important disease vectors in which they continue to affect the public health system. Several genera of arthropods such as lice, fleas, ticks and mosquitoes contribute to human disease, the later being most notable disease vectors.

Mosquitoes are vectors of parasitic and viral diseases that continue to burden the global public health system. Diseases caused by mosquito borne viruses such as Dengue fever, Yellow fever and West Nile virus continue to afflict large populations around the world. Specifically, Dengue fever affects 50 million people per year including several hundred thousand cases of Dengue hemorrhagic fever. Yellow fever afflicts 200,000 people and the West Nile encephalitis virus continue to cause morbidity and mortality despite enormous control efforts (CDC 2002). Mosquitoes also transmit devastating parasitic diseases, most importantly malaria which is caused by the protozoan species *Plasmodium falciparum* and *Plasmodium vivax*, affects 350-500 million cases each year worldwide and kills over three million people each annually, most of them young children in Sub-Saharan Africa (The World Health Report 2002). Lymphatic filariasis, a nematode-based disease transmitted by mosquitoes, affects millions of people in tropical regions of the world.

Control measures for vector-borne diseases are important because most disease are maintained through vertebrate host; therefore are “zoonoses” and are not amenable to

eradication. Therefore, control methods generally focus on targeting the arthropod vector. One control method is the use of protective measures by establishing physical barriers such as house screens and bed nets; wearing appropriate clothing and using insect repellents. Other efforts to control vector-borne diseases focus on the pathogen. For example, there are vaccines available for diseases such as Yellow fever, tick-borne encephalitis, Japanese encephalitis, and plague but despite ongoing efforts, malaria continues to be a tremendous health problem because of the lack of vaccine, and parasite resistance to drugs and mosquitoes' resistance to insecticides. The development of a vaccine is slow and it is difficult to generate one targeting a specific stage in the *Plasmodium* life cycle. For this reason, there is an urgent need for scientists to develop new technologies for controlling the spread of mosquito-borne diseases.

Novel strategies need to encompass a triangular strategy in order to combat diseases including but not limited to protection, prophylaxis/treatment and blocking transmission in mosquitoes. The latter requires mass killing of mosquitoes, blocking parasite development in vector, and or genetic manipulation of the vector. The latter requires investigating the mechanisms involved in parasite development in vector insect in order to develop novel approaches to controlling mosquito-borne diseases based on the interactions between pathogens and the mosquito. These new approaches to control mosquito-borne diseases based on interrupting the transmission cycle in the mosquito require a deeper understanding of the specific interactions between the parasites and their vectors.

The concept that immune responses of arthropod vectors limit the development of parasites and pathogens was proposed over seventy years ago (Huff, CG 1927). Insects utilize multiple defense mechanisms against microbial pathogens and parasites (Hoffmann and Lemaitre, 2007). Recent years have witnessed several breakthroughs including transformation of mosquitoes (*Aedes*, and *Anopheles*), analysis of gene function using RNAi using various mosquito pathogen combinations, genome wide expression profiles using microarrays, and most importantly sequencing of the *Aedes*, *Culex* and *Anopheles* genomes.

These advances have helped to discern some of the mechanisms underlying the dynamic vector-pathogen interactions. Of particular interest is to characterize the restriction of parasite proliferation by vector and the point of parasite evasion. Vector immunity is the major barrier that limits parasite proliferation so the main goal of the vector-pathogen research is to elucidate components and mechanisms of immune pathways of vectors that cause devastating diseases and to utilize our understanding of the immune system to develop novel strategies in order to combat diseases such as malaria.

The innate immune system is the first line of defense against invading pathogens. In insects, it lacks the specificity of the adaptive immune response; however, it can distinguish between self and non-self. Insects have four innate immune mechanisms, which can be sub-grouped under humoral and cellular immunity. Cellular immune response is governed by circulating blood cells (hemocytes), lamellocytes and plasmatocytes that encapsulate or phagocytose invading pathogens,

respectively (Lavine et al., 2002). Humoral immunity comprises the production of antimicrobial peptides (AMPs) by the mosquito fat body (analogous to vertebrates liver) and has been implicated in the antimicrobial response, along with the serine proteolytic melanization and coagulation cascades.

The insect innate immune system relies on two major signaling pathways, Toll and Imd pathway, that result in the activation of specific NF κ -B signaling transcription factors and underlying target immune genes. These pathways are discussed thoroughly in the next few chapters.

The development of genome-based vector control strategies requires detailed knowledge about the organization and function of the mosquito genome. Recent sequencing of the two mosquito species, *Aedes aegypti* and *Anopheles gambiae* provides an opportunity to do comparative genome analysis. Recent sequencing of *Culex* genome is likely to enhance and refine comparative genome analysis. Availability of mosquito genomes and gene annotation has been instrumental for dissecting mosquito biology and distinguishing gene functions related to vector-pathogen interactions. The long term goal of my project is to elucidate the components of the Immune deficiency (IMD) and Toll Immune pathways which attribute to the signal transduction events that lead to the activation of the NF-kappaB transcription factors and effector immune molecules.

My research has three specific aims listed as the following:

Aim 1: Exploration of the Toll and PPO activation pathway in response to pathogen invasion.

Activation of innate immune factors in both mammals and insects share a conserved pathway in which Rel/NF κ B molecules serves as principal regulators. Both the mosquito and *Drosophila* utilize two major immune pathways, Toll and Imd, to overcome foreign pathogens and parasites invasion. In *Drosophila*, the Toll pathway regulates the establishment of the embryonic dorsoventral patterning as well as the innate immune response against fungi or gram-positive (Lys type) microbial infection in adults. In both scenarios, the induction of the Toll pathway needs the participation of a kinase, IRAK, to activate the translocation of NF- κ B and trigger the downstream genes transcription.

Sequencing and annotation of the *Aedes aegypti* genome (Nene et al., 2007 and Waterhouse et al., 2007) revealed two IRAKs, one homologous to mammalian IRAK4, and the other homologous to IRAK2. However, *Drosophila* only has one IRAK, pelle. This genomic information directs our focus to study the distinct function of IRAKs in mosquito, IRAK4 and Pelle using RNA interference and cell culture in vitro assays. Co-Immunoprecipitation (Co-IP) will also give us a clear idea how this new IRAK4 interacts with the other components in the Toll pathway.

Aim 2: Identification of the components of the IMD pathway in *Aedes aegypti*.

Although the major parts of the intracellular components of the IMD pathway are highly conserved between *Drosophila* and mosquito, striking differences do exist between them. Most prominent difference is the absence of Dorsal like factor, Dif. In *Anopheles*, it suggested the possibility of another transcription factor besides Rel1 and Rel2. Furthermore, three isoforms of Rel2 have already been identified and well characterized in *Aedes* (Shin et al 2002). These isoforms also had contrasting function as compared to *Drosophila*. Together this evidence implies important differences in the immune signaling between the mosquito and *Drosophila*. Therefore, a complete investigation of the *Aedes* IMD pathway is necessary. It is important to investigate the pathway that leads to activation of Rel2 since overexpression of Rel2 confers anti-plasmodium immune response while inhibition of Rel2 shows opposite results (Antonova et al. 2009, and Miester et al 2005). We expect differences between immune factors in mosquitoes while Rel2 exhibits unique characteristics. Due to the significant differences in *Drosophila* immune response, we have decided to investigate and map the IMD pathway in *Aedes aegypti*, specifically examining the multiple branches of the IMD pathway and asking if REL2 gain of function has an affect on Plasmodium development.

Aim 3: Comparative genomic study of the mosquito immune system.

Studies of *Drosophila* innate immunity had a chief impact on insect immunity leading to discoveries answering fundamental questions on how insects fight general pathogens. Ten years ago, the completion of genome sequencing of *Drosophila* provided the foundation for further functional studies. Also, a comparative genome analysis of *Anopheles* and *Drosophila* immune related gene families revealed an extensive diversification and expanded insect innate immunity which dates back 250 million years (citation). The availability of the *Aedes* and *Culex* genome will enhance our understanding of mosquito immunity and elucidate important features of vector biology. In this aim, a multiple comparison of the insect innate immune system will be made to specifically analyze common and species specific immune molecules, which are located in the Toll and Imd pathway. We will use the tools in the computation biology to study this puzzle, such as multiple sequence alignment, phylogenetic analysis, gene annotation, and expression data mining.

CHAPTER 2: LITERATURE REVIEW

2.1 Innate Immunity: An overview

Innate and adaptive immunity comprise of the two mechanism utilized by most organisms to defend themselves. Innate immunity is defined as the existence of pathogen recognition receptors that are encoded in the germline. On the other hand, adaptive immune response is characterized by the presence of a specific mechanism of gene arrangement and recombination that leads to the creation of molecules that interact with a particular antigen. However, it can effectively distinguish between self and non-self. In general, the innate immune response is initiated upon recognition of pathogens by the pathogen recognition receptors (PRR). This leads to activation of the cytoplasmic signaling cascades which culminate in the nuclear translocation of NF- κ B transcription factors. These factors bind to the promoters of activate genes encoding immune defense molecules.

2.2 Local and Systemic immunity of insects

The insect immune system can be divided into two branches: local and systemic immunity. Local immunity includes synthesis of antimicrobial immune factors in the barrier epithelia. Systemic immune response involves the immune functions of hemocytes and fat body (Lemaitre and Hoffmann, 2007). Systemic immunity is further divided into three branches: cellular immunity that includes the immune functions of hemocytes; humoral immunity that depends on immune reactions of the fat body; and

melanization reaction which is a combination of both humoral and cellular immune response.

Local immunity consists of immune reactions in the epithelia. The barrier epithelia have developed specific defense immune responses because they are in constant contact with surrounding environment. The insect epithelium is programmed to synthesize its own antimicrobial peptides (AMPs) and lysozymes. As it has been shown in *Drosophila*, the insect gut epithelium can also produce reactive oxygen species (Lemaitre and Hoffmann, 2007). These immune molecules can be constitutive or inducible.

2.3 Cellular Immunity and Humoral Immunity

Insects have four innate immune mechanisms which can be subgrouped under humoral and cellular immunity. Insect defense includes melanization of the invading microorganism, cellular responses such as phagocytosis or encapsulation and humoral systemic responses. The cellular immunity in *Drosophila* involves three processes carried out by circulating blood cells called hemocytes: phagocytosis (plasmatocytes), encapsulation (lamellocytes) and melanization (crystal cells). Phagocytosis relies on recognition between self and non-self. Plasmatocytes comprise 90-95% of all mature larval hemocytes and their main function is phagocytosis of dead cells and microbial pathogens. Lamellocytes are large flat cells that encapsulate and neutralize large object or pathogens that are too large to be phagocytosed. Lastly, crystal cells are non-phagocytic

cells involved in melanization. Mature crystal cells possess prophenoloxidasases (PPOs), and when disrupted, they release these PPOs that eventually leads to the deposition of melanin.

In mosquitoes, cellular immunity is directed by four types of hemocytes: granulocytes, oenocytoids, adipohemocytes, and thrombocytoids (see more recent classification by M Strand, *Insect Biochem Molec Biol*?). Granulocytes and oenocytoids are circulating hemocytes while adipohemocytes and thrombocytoids are sessile and attached to tissue. Oenocytoids were shown to contain PPO (Hillyer et al., 2003a, b). Mosquito granulocytes are major phagocytic cells (Castillo and Kouri, 2004). The same study showed that mosquito granulocytes express PPO in response to bacterial invasion and after phagocytosis of bacteria. Specifically, these granulocytes are capable to phagocytize Gram-positive and Gram-negative bacteria as well as *Plasmodium* sporozoites (Hillyer et al., 2003a; Moita et al., 2005).

Series of humoral reactions are also activated in parallel to cellular reactions after infection (Gillespie et al., 1997). Humoral immunity comprises the production of antimicrobial peptides (AMPs) by the mosquito fat body (analogous to vertebrate liver) and has been implicated in the antimicrobial response, along with the serine proteolytic cascade. The insect humoral innate immune system specifically relies on two signaling pathways, Toll and Imd pathway, that result in the activation of NF κ -B signaling transcription factors. These pathways are discussed below.

Melanization is an immediate immune response in Arthropods, including insects, can be visualized at the site of cuticular injury and on the parasitic surface. The reaction can be characterized by a blackening of the target caused by deposition of melanin. The reaction requires the activation of PO, an enzyme that catalyzes the oxidation of mono- and diphenols to orthoquinones, which polymerize to form melanin. The inactive PPO is cleaved by a serine protease (SP) called prophenoloxidase activating enzyme (PPAE). PPAE is also a zymogen which is activated through a series of reactions involving a SP cascade. Several studies indicate that melanization is initiated upon injury or recognition of pathogens by pathogen recognition receptors (PRRs) (Lee et al., 2003). In mosquitoes, melanization serves as an anti-Plasmodium immune response which will be discussed later in detail. The PPO reaction is therefore one example of a non-self recognition system in invertebrates.

2.4 Initiation of Immune Response by Pattern Recognition Receptors (PRR)

The innate immune system recognizes invading microbes through receptors that are heritable through the germline. Charles Janeway (1996) describes pattern recognition molecules as innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs) which stimulate an immune response. The surfaces of microorganisms bear repeating patterns of molecules such as peptidoglycan, β -1,3 glucans, and lipopolysaccharides (LPS) referred to as pathogen associated molecular patterns (PAMPs). The innate immune system recognizes pathogens with the use of receptors or pattern

recognition receptors (PRR) that bind to these repeated PAMPs. While some PRRs are constitutively expressed and secreted surveillance sensors; others are synthesized in response to pathogen invasion. Recognition of pathogens by pattern recognition molecules culminates into activation of phagocytic events and activation of immune pathways such as the IMD and Toll Pathway which lead to the production of antimicrobial peptides. These receptors include blood circulating receptors as well cell membrane bound receptors of host cells.

PGRPs were first identified in *Bombyx mori* in 1996 and since then homologues in various species have been identified subsequently (Yoshida et al., 1996). Evidence based on sequence homology also suggests that PGRPs are likely involved in innate immunity and are conserved from insects to humans (Kang et al., 1998). They have now been identified in mollusks, echinoderms, and several vertebrates like fish, amphibians, and birds (Charroux et al., 2009). Four PGRP have been identified in *Homo sapiens*, 13 in *Drosophila melanogaster* and 7 in *Anopheles gambiae*, 8 in *Aedes aegypti* and 10 in *Culex quinquefasciatus*. All PGRPs share a conserved C-terminus PGRP domain of approximately 165 amino acids (Werner et al., 2000), and can be subdivided into long and short classes, where long forms are intracellular or transmembrane and short forms are located extra-cellularly. In *Drosophila*, three of the seven genes encode for short PGRP-S (S1, S2, S3); they have a signal secretory peptide, a single PGRP domain and no transmembrane domain. On the other hand, long PGRPs (PGR-L) can contain up to 2 PGRP domains in the C-terminus. There are 6 or 7 PGRP-Ls (LA1, LA2, LB, LC1, LC2, LC3, LD) some being splice variants. Insect PGRP-S and most other short PGRPs are

expressed in the hemolymph and cuticle. These are either constitutively expressed or are induced upon immune signal by the fat body.

Several classes of PRR have been identified in *Anopheles gambiae* including peptidoglycan recognition proteins (PGRPs), gram-negative binding proteins (GNBPs), thioester containing proteins (TEPs), scavenger receptors (SCRs), C-type lectins (CTLs), galactoside binding lectins (GALE), and fibrinogen-like domain immunoprotections (FBNs) (Christophides et al., 2000; Christophides et al., 2004; Zdobnov et al., 2002). The *Drosophila* immune system is able to differentiate pathogens based on the composition of their outer membrane. In order to distinguish pathogens, *Drosophila* differentiates between a lysine residue in the third position of the peptidoglycan stem peptide in Gram-positive bacteria. In Gram-negative bacteria, the lysine is replaced by mesodiaminopimelic acid (m-DAP). In order to activate the IMD pathway, PGRP-LC and -LE recognized DAP peptidoglycan while PGRP-SA and -SD recognize and bind to LYS type peptidoglycan to activate the Toll pathway (Leulier et al., 2003). PGRP-LC mutants fail to activate the IMD pathway-dependent genes after immune challenge and are susceptible to Gram-positive bacteria infection (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002b). PGRP-SA mutants respond to fungal challenge normally; however, they are susceptible to Gram-positive bacteria, such as *Micrococcus luteus* (Michel et al., 2001). PGRP-SA and PGRP-SD cooperate with GGBP1 in the detection of Gram-negative bacteria, such as *Staphylococcus aureus*, and can be found in a complex together (Wang et al., 2009). Recent studies implicate Gram-negative binding protein, GGBP-3, as a potential fungal receptor upstream of the Toll pathway (citation). The role of PGRPs

and other PRRs in the detection of pathogens has been under intense scrutiny in recent studies. It is clear now that PGN recognition by PGRPs is central to immune signaling in insects. Several lines of evidences show that PGRPs are expressed based on pathogen invasion. These studies have shown that the activation of the Toll and Imd pathways results from the pathogen recognition by PGRPs. This recognition is specific; in general, Gram-negative bacteria are recognized by PGRP-LC family and Gram-positive by the PGRP-SA and Gram-negative binding protein (GNBP1). Together, these studies suggest a discriminatory and specificity of response to pathogens in innate immunity; thus, expanding the recognition and response repertoire in innate immunity. Further dissection of the role of PRRs in insect immunity including the study of which proteins have been conserved in the course of evolution will be interesting.

PGRPs have also been implicated in the down regulation of immune pathways. Studies suggest that some PGRPs with amidase activity could act *in vivo* as scavenger receptors and be involved in down regulation of immune activity. Flies depleted with PGRP-SC1 and PGRP-LB by RNAi have shown an over-activation of the IMD pathway after bacterial challenge (Bischoff et al., 2006; Zaidman-Remy et al., 2006). Additionally, PGRP-LF depletion leads to activation of IMD and JNK pathways in the absence of infection; perhaps suggesting that the function of PGRP-LF function is to control constitutive activation of this pathway (Maillet et al., 2008).

These PGRPs are upregulated upon exposure to bacteria or purified PGN (Christophides et al., 2001; Dimopoulos et al., 2002; Kang et al., 1998; Werner et al., 2000). There is evidence that suggests differential expression and induction of these

PGRPs where both are influenced by immune elicitors. In some insects, PGRPS are involved in activation of the Toll and Imd immune pathways through differential activation of each pathway (Choe et al., 2002; Michel et al., 2001; Ramet et al., 2002b).

Investigations in the pattern recognition molecules in insects also lead to the discovery of C-type lectins which bind bacteria lipopolysacchrides thereby activating antibacterial immune response in hemocytes or activating the PO pathway (Jomori and Natori, 1991, 1992; Yu et al., 2002). Thioester containing proteins or TEPs may function as immune surveillance factors against pathogen invasion. *Anopheles* TEP1 binds to Gram-positive and Gram-negative bacteria through a covalent bond and bacteria are subsequently cleared by phagocytosis (Levashina et al., 2001). In addition, TEP1 is implicated in anti-*Plasmodium* activity. RNAi depletion of TEP1 in *A. gambiae* susceptible strain causes a 5-fold increase in *Plasmodium* oocysts number. Additionally, melanization of oocysts was not seen in these mosquitoes. These data suggest that TEP1 may function in the defense against this pathogen invasion.

The vertebrate immune system is able to discriminate between a large pathogen spectrum. Invertebrates do not have similar antidotes but first evidence of Ig immunoglobulin involvement in insects comes with *Drosophila* Down Syndrome cell adhesion molecule, Dscam followed by evidence in *Anopheles* (Dong et al., 2006; Watson et al., 2005). *Drosophila* study showed that immune competent cells have the potential to express more than 18,000 isoforms of Dscam and is expressed in hemolymph and loss of Dscam inhibited the uptake of phagocytotic bacteria (Watson et al., 2005). Additionally, the *Anopheles* showed that an alternatively spliced hypervariable

immunoglobulin domain encoding gene, Dscam, generates broad range of PRRs implicated in immune defense in the malaria vector *Anopheles gambiae*. AgDscam, has 101 exons and can produce over 31,000 potential alternative splice forms. AgDscam produces pathogen challenge specific splice form repertoires in response pathogens. This study suggests that though invertebrates do not have a memory antibody system, they are capable of immune specific responses (Dong et al., 2006).

2.5 *Drosophila* Innate Immunity: IMD and TOLL immune pathways

Toll-like receptors (TLR) are primordial receptors in mammals and are responsible for mediating host immune response to distinct microbial patterns such as lipopolysachrides (LPS) in bacterial cell wall by TLR4 and viral dsRNA by TLR3. *Drosophila* Toll receptor is shown to encode a transmembrane receptor that also includes an intracytoplasmic domain similar to the interleukin-1 receptor (IL-1R) thus referred to as the TIR (Toll/IL-1R Resistance) domain. The ectodomain of the Toll receptor is characterized by leucine rich motifs rather than immunoglobulin-like motifs in IL-1R. *Drosophila* harbors nine Toll receptors including Toll, 18-Wheeler, Toll-3, and Toll-9. The Toll (Toll1) receptor was characterized genetically as a key component in dorso-ventral patterning in embryogenesis (Anderson et al., 1985) and later shown also to signal for antifungal and anti-Gram-positive bacterial immune responses (Lemaitre et al., 1996). A complex signalosome assembles around the TIR domain of the Toll receptor.

TLRs are differentially activated by a variety of PAMPs such as bacterial DNA, LPS, peptidoglycan, teichoic acids, flagellin, pilin, viral dsRNA and fungi zymosan. Specifically, TLR2 recognizes soluble peptidoglycan, lipoteichoic acid and whole Gram-positive bacteria while TLR4 responds to the Gram-negative component lipopolysaccharide (LPS). Activated TLRs differentially trigger the expression of cytokines such as the interferons; the interleukins; IL-2, IL-6, IL-8, IL-12, IL-16, and TNF-alpha. Both *Drosophila* Toll and mammalian TLRs are activated upon microbial challenge. However, TLRs have been shown to be directly induced by microbial challenge whereas *Drosophila* Toll requires a cytokine, Spatzle (Spz) for its activation. (Hu et al., 2004; Weber et al., 2003).

In *Drosophila*, the Toll family of receptors comprises nine members, Toll and Toll-2 to Toll-9. With the exception of Toll-9, phylogenetic tree analysis shows that *Drosophila* Tolls are most closely related to each other than to mammalian TLRs. (Du et al., 2000). These data suggests that these two receptor families evolved independently (I do think that this is correct). A dual role of Toll pathway in immunity and development is quite apparent; however, the exact functions of Toll-2 to -9 are still under investigation. Toll-5 and -9 have been shown to activate the Drosomycin promoter in tissue culture cells; however, the other Toll receptors do not activate transcription of the genes encoding antimicrobial peptides or interact with DmMyd88 (Tauszig-Delamasure et al., 2002). Most Tolls are highly expressed during embryogenesis and metamorphosis (Tauszig et al., 2000).

Four genes from *An. gambiae*, TOLL 1A, 1B, 5A, and 5B, form an orthologue group with the fruit fly Toll and Toll-5. Transient expression analysis in *Drosophila* cell culture revealed that both Toll1A and Toll5A activated the expression of a firefly luciferase gene under the control of Drosomycin promoter (Luna et al., 2006; Luna et al., 2002). In *Aedes aegypti*, three homologous genes of *Drosophila* Toll (Toll-1) have been identified and named as AeToll1A, AeToll1B, and AeToll5 (Waterhouse et al., 2007). In *A. aegypti*, Toll5A was shown to be a major regulator of the *Aedes* immune Toll pathway (Shin et al., 2006).

The activation of the Toll pathway occurs after the binding of Spätzle to the Toll receptor (Weber et al., 2003). The cleavage and thus activation of Spätzle is induced by a proteolytic cascade that is activated by circulating PRR and culminates in the release of the cytokine that binds to Toll receptor. The proteolytic cascade consists of a series of serine proteases that undergo zymogen activation by serine proteases; mostly CLIP domain proteins. Two clip-domain serine proteases, Snake and Easter, are involved in embryos. However, Persephone is a cascade member in adult insects that activates the Toll pathway in anti-fungal response. The cascade is positively and negatively regulated by C-type lectins (CTLs), serine protease inhibitors (SRPNs), and clip domain serine proteases (CLIPAs). As it has been observed in *Drosophila*, the activation of the Toll receptor involves multimerization of the receptor(s). Following cleavage of Spätzle, the cytokine binds to the N-terminal extracellular domain of Toll which triggers the conformational changes in the receptor that leads to signaling (Weber et al., 2007; Weber et al., 2005).

Activation of the Toll receptor leads the assembly of a signalosome that consists of MyD88 (Myeloid differentiation primary-response gene 88), Tube and Pelle. Tube and Pelle are homologous to IRAK family of serine-threonine kinases of mammals. MyD88, Pelle, and Tube that interact through their death domains which are recruited to the receptor via their Toll/Interleukin-1 receptor (TIR) domains (Horng and Medzhitov, 2001; Medzhitov et al., 1998; Sun et al., 2002; Tauszig-Delamasure et al., 2002). This results in the full activation of the cytoplasmic signaling cascade that triggers rapid phosphorylation and degradation of Cactus, a homologue of the mammalian NF- κ B inhibitor I κ B (Nicolas et al., 1998). The latter reaction results in the release of the NF- κ B transcription factor, Dorsal or Dif and induction of developmental or immune responsive genes, respectively. Signaling through the Toll receptor also results in phosphorylation of Dif and Dorsal by unknown kinase, a step which is important for their functional activity (Han and Ip, 1999; Meng et al., 1999; Rutschmann et al., 2000; Stein et al., 1998).

2.6 Imd pathway

The second major immune pathway, Immuno-Deficiency or Imd, is triggered in response to Gram-negative bacteria and requires activation by a third NF- κ B transcription factor, Relish that activates Diptericin, Defensin, Attacin, Cecropin and others AMPs (De Gregorio et al., 2002b). This pathway is named after *Drosophila* mutant that has a mutation in the *imd* gene, resulting in a strong susceptibility to Gram-negative bacteria (Lemaitre et al., 1995). Signaling by this pathway mimics signaling by

TNFR (tumor necrosis factor receptor) signaling in mammals. Figure 2 illustrates the current knowledge of the Imd pathway in *Drosophila*.

In *Drosophila*, the Imd pathway commences with the recognition of DAP-type PGN of Gram-negative by PGRP-LC which serves as the receptor for this immune pathway (Lemaitre and Hoffmann, 2007). Association of the PGRP-LC receptor with IMD, an adaptor protein-similar to mammalian RIP (receptor interacting protein) in the next step in the pathway. This occurs through the association of the RIP domain in the N-terminus of the receptor. IMD is a 25kD protein that contains the death domain (DD) that is similar to mammalian RIP (Georgel et al., 2001).

Immunoprecipitation and yeast-two hybrid assay revealed that the activation of the pathway, leads to the formation of cytoplasmic complex consisting of IMD, dFADD, and Dredd, a caspase (Hu and Aksoy, 2006; Naitza et al., 2002). This results in the activation of TAK1 (a MAP3K) by ubiquitination, which leads to phosphorylation and cleavage by IKK and Dredd, a caspase 8 homolog respectively. IKK complex consists of two subunits-IRD5, which is mammalian orthologue of IKK β and Kenny, similar to mammalian IKK γ . The exact mechanism of TAK1 and IKK activation is unknown however, the involvement of a ubiquitin conjugation has been proposed (Zhou et al., 2005). The involvement of ubiquitin has been implicated in activation of TAK1 and IKK complex since this modification is important mechanism of activation during signaling through TNF (Imd) pathway. In mammals, TRAF5 and TRAF2 (ubiquitin ligases) along with UE1A and Ubc12 (Ubiquitin conjugating enzyme) have been implicated in the mechanism of activation. In *Drosophila*, Ubc13 homolog, Bendless, and UEV1a were

reported to be components of a complex with protein-ubiquitin complex. Moreover, it was that Bendless is required for resistance to Gram-negative bacteria (Zhou et al., 2005). However, no TRAF was shown to participate in the *Drosophila* Imd pathway. Nonetheless, an inhibitor of apoptosis (DIAP2) with a RING domain was discovered and is a likely candidate as a ubiquitin E3 ligase activating TAK1.

Several studies reveal that the Imd pathway bifurcates at the level of TAK-TAB2 complex where TAK1-TAB2 activates JNKK hemipterous (Hep) (Park et al., 2004; Silverman et al., 2003). Therefore, in addition to activation of Relish, IMD pathway also participates in activation of JNK pathway. This pathway is implicated in wound healing and apoptosis (Bosch et al., 2005; Galko and Krasnow, 2004; Ramet et al., 2002a). Microarray analysis of *Drosophila* S2 cells shows that TAK1 activates JNK pathway in response to bacteria via the JNK kinase basket (Boutros et al., 2002). Furthermore, JNK signaling was proposed in AMP gene expression by the fat body (Delaney et al., 2006; Kallio et al., 2005).

Cleavage of Relish results in release of its Rel domain containing part from the I κ B domain. Epistatic analysis also implicate the involvement of dFADD and Dredd upstream in the pathway; however, this hypothesis is a currently under much debate (Zhou et al., 2005). *Drosophila* FADD is mammalian homologue of FADD and contain a death domain that interacts with IMD (Naitza et al., 2002). Additionally, FADD contains the death effector domain (DED) domain. After immune activation, IMD recruits FADD which then interacts with DREDD those results in the cleavage of Relish.

Immunoprecipitation experiments revealed the association of DREDD and Relish; however, direct cleavage of Relish by DREDD is yet to be shown (Stoven et al., 2003).

A screen of *Drosophila* mutants led to the identification of the immune repressor of Imd pathway, Caspar. The mutation of Caspar caused an increase in resistance to infection by Gram-negative bacteria. Caspar mutation also caused a modest increase in Diptericin expression whereas the overexpression of Caspar led to suppression of this AMP gene expression and increase susceptibility to bacterial infection. Additionally, Caspar suppressed the nuclear localization of Relish (Kim et al., 2006). In addition, it was found that Caspar controls resistance to *P. falciparum* in *Anopheles* (Garver et al., 2009).

Immune regulation and apoptosis

Regulation of apoptosis is crucial to maintain homeostasis and failure to do so is linked to several pathogenic diseases. Inhibitors of apoptosis (IAPs) are negative regulators of apoptosis by directly acting on caspases. Caspases are involved in apoptosis and in non-apoptotic processes, such as regulation of actin dynamics, innate immunity, cell proliferation, differentiation and survival. These processes are activated by caspases without killing the cell.

Although, an E3 ligase remains elusive in the Imd pathway, dIAP2 was recently identified as a likely candidate (Gesellchen et al., 2005). dIAP2 was recognized as an essential component in the IMD pathway (Gesellchen et al., 2005; Huh et al., 2007;

Leulier et al., 2006; Valanne et al., 2007). dIAP2 contains a RING finger motif which is necessary for Imd signaling. DREDD, an apical caspase, is also involved in signaling between IMD and TAK1.

Specificity in the innate immune response has been implicated where some aspects of immune response are tailored to immune challenge. For example, the fungal immune response leads to production of Drosomycin. On the other hand, production of Defensin and Cecropin is induced by gram-negative bacteria. Selective activation of immune pathways reflects some immune specificity.

Synergism of Toll and Imd pathway

Though there is general agreement that Toll pathway is activated upon Gram-positive and fungal infection while Imd pathway responds to Gram-negative bacteria, there is evidence of cross talk between these two pathways. For example, one study showed that Dorsal/Dif and Relish are needed for maximum expression of AMPs Defensin and Drosomycin (Tanji et al., 2007). The same study showed that Drosomycin expression is induced not only by Relish homodimer but also by its heterodimer with Dif. Further evidence showed that the presence of Spätzle and peptidoglycan of Gram-negative bacteria together causes a synergistic activation of representative target genes of the two pathways, including Drosomycin, Diptericin, and Attacin A (Tanji et al., 2007). Additionally, *in vivo* studies showed that Relish mutants were able to express Cecropin A2, Attacins A and C after Gram-positive bacteria, *Staphylococcus aureus*, and

Micrococcus luteus; suggesting an alternative pathway, perhaps Toll pathway, responsible for the induction of these immune genes (Hedengren-Olcott et al., 2004). AMP induction ceased in Toll/Relish double mutants. Recent studies have also shown that the promoters of many AMPs are dually responsible for Doral/Dif and Relish mediated induction (Hedengren-Olcott et al., 2004; Imler and Hoffmann, 2000).

Synergism of Toll and Imd pathway was also seen in mosquitoes. The knockdown of REL1 and REL2 individually did not result in anti-Plasmodium gene expression as dramatically as with double knockdown. Double knockdown of REL/REL2 resulted in an increase in *Plasmodium* number (Frolet et al., 2006). These results suggest that Toll and Imd pathway in the mosquito cooperate as in *Drosophila*.

2.7 NF-kappaB transcription factors

NF-κBs were first discovered in B-lymphocytes as nuclear factors that are required for immunoglobulin kappa (κ) light chain transcription (Sen and Baltimore, 1986). NF-κBs are required for most vital responses such as immune and stress response, inflammation, cell proliferation among others. In mammals, binding sites for NF-κB transcriptional factors (κB) sites are found in genes that regulate factors involved in immune responses including cytokines, acute phase proteins, and cell adhesion molecules.

An accepted model regarding the mechanism of NFκB processing is that an inactive NF-κB factor is constitutively repressed in the cytoplasm by its inhibitor, IκB.

Upon immune activation, I κ B is removed, permitting activation of NF κ B and its translocation to the nucleus, where it binds to the promoters of immune responsive genes causing their transcriptional activation. The constitutive expression of NF κ Bs ensures a rapid immune response (Libert et al., 2006).

The NF κ B family can be divided into two subfamilies: simple Rel-type proteins and composite NF- κ B proteins. The latter contain an additional inhibitory domain. Humans have three simple Rel-type factors, cREL1, RelA, and Rel1B and two NF- κ Bs, p100 and p105. Composite NF- κ B proteins consist of a Rel homology domain (RHD) followed by a transactivation domain and a nuclear translocation sequence (NLS). The RHD domain is approximately 300 nucleotides and contains DNA binding, dimerization and the NLS motifs. The NLS signal of the NF- κ B is disguised by the I κ B in the inactive state in the cytoplasm. Human composite NF- κ B proteins, p100 and p105 have an inhibitor I κ B domain in their C-terminus and require proteolytic cleavage of the inhibitor for activation. The p50 and p52 are generated by proteolytic cleavage of p105 and p100 precursors, respectively. Proteolysis of cleaved an inhibitor I κ B domain includes ubiquitination and partial degradation by the 26S proteasome. It is likely that the mechanism of partial degradation requires that the C-terminus of the precursor protein enters the proteasome and is degraded methodically (Karin and Ben-Neriah, 2000; Takeuchi et al., 2007).

I κ B contains several ankyrin repeats, each one consisting of 33 amino acids. The number of ankyrin repeats contributes to the specificity of I κ B binding to NF κ B (Ghosh et al., 1998),(Gilmore, 2006; Gilmore and Herscovitch, 2006). Additionally, I κ Bs also

have a C-terminus acidic region which is important the cytoplasmic retention of the I κ B/NF κ B complex. The C-terminus also contains a PEST sequence which is responsible for the rapid degradation of the protein (Beauparlant et al., 1996). In addition, the C-terminus is I κ Bs includes the 90 amino acids long Death domain, which is highly conserved in proteins involved in apoptosis, inflammation, immune response, and cell differentiation.

Upon activation and translocation to the nucleus, NF- κ Bs bind to variable DNA 9-10 bp long (κ B sites): 5'GGGR (A/G)N(W)A/T(Y)C/T)Y(C/T)CC-3' where R is A or G, N is any nucleotide, W being A or T and Y is C or T. The NF- κ B responsive sequence is found in the 5'end of the AMP genes of many insects (Reichhart et al 2002). The diversity of the κ B sites allows for the regulation of different but related sets of genes. Besides κ B sites, several other factors affect NF- κ B binding including affinity of NF- κ B dimers to DNA, and the interaction of NF- κ B and coactivators on promoters of immune responsive genes (Gilmore, 2006). Combination of NF- κ Bs can also affect activity of responsive genes where some can act as inhibitors, such p50/p50 and p52/p52, while others act as activators (Ghosh et al., 1998; Miyamoto and Verma, 1995; Plaksin D, 1993).

Dorsal was the first non-vertebrate NF κ B factor to be discovered in *Drosophila*. Genetic studies have shown that Dorsal is involved in the dorsal-ventral patterning of *Drosophila* embryo (Stein et al., 1998). Another NF κ B factor named Dif (Dorsal related immune factor) was found to mediate the Toll pathway in immune responses against fungi and Gram-positive bacteria. Both Dif and Dorsal are members of the Rel-type

subfamily of the NFκB proteins and are homologous to c-Rel, RelB, and RelA (Stewart 1987; Ip et al., 1993); (Ghosh et al., 1998). *Drosophila* also contains an IκB proteins, Cactus. Characteristic to IκB protein, Cactus contains ankyrin repeats and PEST sequence. In the cytoplasm, Cactus is associated with Dorsal and Dif and upon immune stimulation, it is phosphorylated and rapidly degraded (Belvin et al., 1995).

The third *Drosophila* NFκB protein, Relish, is a composite NFκB protein homologous to mammalian p100 and p105. Similar to these NFκB factors, *Drosophila* Relish has a DNA binding Rel homology domain (RHD) and a C-terminal inhibitory ankyrin domain (IκB) (Dushay et al., 1996; Hedengren et al., 1999). The RHD domain is involved in DNA binding and activates gene expression, while the IκB domain that has several ankyrin repeats that block nuclear localization. As a compound protein that encodes its own inhibitory protein, it requires proteolytic cleavage of the inhibitor for activation and translocation. Upon microbial invasion the Imd pathway causes, phosphorylation of Relish and its processing via endoproteolytic cleavage (Stoven et al., 2000). Cleavage of Relish results in translocation of its RHD to the nucleus where it participates in AMP gene expression.

Genomes of *Aedes* and *Anopheles* contain NF-κB proteins orthologous to *Drosophila* counterparts (Waterhouse et al., 2007; Christopheles et al, 2002; (Holt et al., 2002). However, distinct differences exist between mosquitoes and the fly. First, there is no Dif in mosquitoes and therefore, mosquitoes rely on *Drosophila* Dorsal orthologue, REL1. Initially, a Rel family member, Gambif1, was identified in *An. gambiae*, and found to be most similar to Dorsal and Dif, it was later renamed as REL1 (Barillas-Mury

et al., 1996). *Anopheles* has one REL1 while *Aedes* has two: REL1A, and REL1B. Both mosquito species contains one orthologue to *Drosophila*, Cactus. Secondly, mosquito homologue of *Drosophila* Relish, REL2, has three isoforms in *Aedes* and two in *Anopheles* (Garver et al., 2009; Meister et al., 2005; Shin et al., 2002). The primary form (3.9 kb) contains a RHD and an I κ B domain (full length Rel2). This form is similar to mammalian p100 and p105 in that it has the death domain. The second form is the RHD N-terminal form that consists only of a RHD domain and lacks an I κ B coding region. The third form has only the I κ B domain and thus is referred to as C-terminal transcription repressor form. It has been shown that only the RHD containing transcript binds to *Drosophila* Cecropin A1 and *Aedes* Defensin κ -B motif (Shin et al, 2002).

In mosquitoes, REL1 is implicated in the regulation of Spatzle and Serpin2 expression in anti-fungal immune response (Bian et al., 2005; Shin et al., 2005). In *Anopheles*, REL1 has been shown to be involved in the regulation of TEP1 as well as phagocytosis and anti-*Plasmodium* immunity (Blandin and Levashina, 2007; Frolet et al., 2006). *Aedes* REL2 is involved in responses to Gram-negative bacteria infection ((Bian et al., 2005; Blandin and Levashina, 2007; Frolet et al., 2006; Shin et al., 2002) while in *Anopheles*, REL2 regulates the AMPs Cecropin, and Gambicin. REL2 also controls the expression of the anti-*Plasmodium* factor, LRIMI (Blandin and Levashina, 2007; Frolet et al., 2006; Meister et al., 2005). Lastly, reports of crosstalk of both the IMD and Toll pathway has been reported in regulating antimicrobial peptide response and antiparasitic response (Frolet et al., 2006; Hergannan and Rechhart, 1997).

In addition to the κ B sites, the regulatory regions of many immune genes also contain a GATA motif which is located in a close proximity to the κ B sites. GATA sequences or GATA boxes are commonly found in the 5' region of insect genes. The consensus sequence-A/T(GATA)A/G-was first identified in a promoter of *Bombyx mori* (Engstrom 1993-JMB). The consensus sequence has been identified in cis-regulatory region of many genes in vertebrates as well as invertebrates. In *Drosophila*, an intact GATA box is needed for the induction of immune genes by Rel1 (Kadalayil et al., 1997). Serpent, a GATA binding transcriptional factor, was found to induce systematic expression of immune genes in cooperation with other genes (Petersen et al., 1999); specifically, this study showed that the GATA motif is necessary for the expression of Cecropin A1 gene. Additionally, the regulatory regions of Dipterocin and Metchikowin harbor GATA sites that are required for expression in the midgut (Senger et al., 2006). In mosquitoes, it was shown that *Aedes* Lipophorin gene (Lp) and lipophorin receptor (LpRfb), in which LpRfb contains binding sites for GATA are upregulated after Gram-positive bacteria challenge and *Plasmodium gallinaceum* infection (Cheon et al., 2006).

2.8 Induced expression of Immune proteins

Antimicrobial peptides (AMPs) were the first immune effector molecules to be identified and characterized. Isolated from the hemolymph of the giant silk moth, *Hyalophora cecropia*, Cecropin was the first AMP to be identified in 1981 (citation).

Most AMPs were identified using functional tests and have been shown to be active against bacteria and fungi. These molecules are often used as markers to monitor activation of Toll and IMD pathway. Since then, over 170 of AMPs have been identified from different insect species (Bulet et al., 1999). Produced mostly by the fat body and hemocytes, the first AMPs to be identified were Diptericin (mostly active against Gram-negative bacteria), Drosomycin (antifungal immune peptide) and Defensin (active against Gram-positive bacteria). Eight classes of AMPs have been identified in *Drosophila*; however transcriptional profile analysis suggests more (De Gregorio et al., 2001; Hultmark, 1993). Others include Cecropins and Gambicins possess both antibacterial and anti-parasitic immune response (Vodovar et al., 2004).

Most AMPs have a low molecular weight (less than 5kD) and contain amphiphilic α -helices, hairpin like sheets, and multiple disulfide bonds. In insects, AMPs are produced: by the fat body (analogous to the liver) before they are secreted in the hemolymph (body fluid), by blood cells or by some epithelia. AMP expression is not only induced in the Fatbody but some evidence suggests that barrier epithelia can express at least one AMP (Tzou et al., 2000). The expression of most AMPs is regulated by Toll and Imd pathway. The Toll pathway controls the expression of Drosomycin while the production of cecropins, attacins, and defensins is controlled by both pathways. The *Drosophila* JAK/STAT pathway has also been implicated in the regulation of genes involved in the humoral opsonization factors (Boutros et al., 2002).

2.9 Melanization reaction in insects

Melanization reaction is a key component of arthropods immune defense and can be found in insects, including flies and mosquitoes. It is an acute immune response in which melanin deposition occurs in injury sites, damaged basal epithelial, or on the surface of invading microorganisms. Melanization is essential for cuticle maturation, wound healing, phagocytosis, encapsulation, and pathogen killing (Lemaitre and Hoffmann, 2007). Phenoloxidase (PO) is the central enzyme of the melanization reaction that catalyzes formation of quinines from phenolic compounds. These quinines non-enzymatically polymerize in to melanin, cross-links with proteins surrounding the injury site and thereby encapsulating the injury and or invader (Barillas-Mury, 2007; Jiang et al., 1998). The encapsulation of invader seals off oxygen and thus, suffocating the pathogen. Additionally, quinones and other reaction intermediates produce toxic substances such as reactive oxygen species that are harmful to pathogens.

Phenoloxidase exists as a zymogen called prophenoloxidase (PPO) and is stored in crystal cells in *Drosophila* and oenocytoids in mosquitoes (Castillo and Kouri, 2004; Hillyer et al., 2003a, b). PPO is activated in the hemolymph upon microbial challenge and cleaved by Clip-domain serine protease cascade which culminates in the synthesis of melanin (Saton et al 1999; (Ross J, 2003)). The serine protease cascade is negatively regulated by serpins (serine protease inhibitors) such as *Drosophila* Spn27A, and *Anopheles* Serpin-2 and Serpin-6 ((De Gregorio et al., 2002a) (Ligoxygakis et al., 2002) (Abraham et al., 2005b; Zou et al.).

Drosophila contains three PPO genes: PPO-A1, -A3 and PPO. RUNX are RUNT related transcription factors that contain the conserved RUNT domain. These RUNX factors are implicated in several developmental process from hematopoiesis to carcinogenesis. Recent reports have shown that a *Drosophila* RUNX factor, Lozenge (Lz) and GATA transcription factor- Serpent- are necessary for hemocyte differentiation and proliferation. It was shown that expression of PPO in crystal cells is dependent on Lz (Ferjoux et al., 2007).

Since the discovery of a refractory *Anopheles gambiae* strain against *Plasmodium cynomolgi*, several studies have focused on the mosquito melanization reaction against the malaria parasite. The characteristics of this strain led to melanization of ookinetes, which prevented development into the oocysts stage. Genome analyses revealed a gene expansion in the PPO genes in mosquitoes that consist of 9 PPO genes in *An. gambiae* and 10 in *Ae. aegypti* (Waterhouse et al., 2007). The role of these PPO genes as well as their regulation is both interesting and elusive.

2.10 JAK/STAT pathway and antiviral immunity

The JAK/STAT pathway constitutes another pathway involved in insect immunity (Lemaitre and Hoffmann, 2007). This pathway was first identified in mammals and was found to include several cytokines as well as growth factors. The JAK-STAT pathway is triggered by the binding of the unpaired ligand, Upd3, which leads to phagocytosis and the antiviral immune response. The other main components of this pathway are the

receptor domeless (DOM), the JAK (Hopscotch Hop) and the STAT. The binding of Upd3 to the transmembrane receptor Domeless (Dome), a homolog of vertebrate type cytokine receptors (Brown et al., 2001), leads to receptor dimerization and subsequent juxtaposition of two JAK molecules which phosphorylate each other. The activated Hop then phosphorylates Dome resulting in the formation of docking sites for the cytoplasmic STATs. The recruitment of STATs leads to their phosphorylation and activation, culminating in the translocation to the nucleus where they participate in the induction of specific genes. The comparative genome analysis of *Drosophila*, *Aedes*, and *Anopheles* led to the discovery of orthologs of Dome, Hop and STAT in all three of the species (Waterhouse et al., 2007). These analyses also identified orthologs of the negative regulators of JAK/STAT pathway, PIAS and SOCS (citations).

After its initial discovery in mammals, the JAK/STAT pathway has been implicated in antiviral immunity, including defense against the Dengue Virus ((Dostert et al., 2005; Dupuis et al., 2003); (Gimeno et al., 2005). In *Drosophila*, JAK/STAT immunity was found to control *Drosophila* C virus (DCV); specifically, JAK/STAT signaling is required but not sufficient for antiviral response (Dostert et al., 2005). In this study, expression of DCV-induced genes that required JAK kinase; however, these genes were not induced in flies constitutively expressing Tumorous lethal-1 (Tum-1) allele hop, suggesting that activation of JAK/STAT pathway is necessary but not sufficient to trigger an antiviral response. Flies deficient of hop had a higher DCV concentration and were more susceptible to infection compared to wild type.

Early evidence of STAT involvement in *Anopheles* defense responses involved work which revealed that AgSTAT protein translocates to nuclei of the fat body cells after bacterial challenge (Barillas-Mury et al., 1996). Furthermore, bacterial challenge results in the induction of genes with STAT responsive sites. Recent evidence shows that JAK/STAT immunity may be involved in anti-Dengue viral defense in *Aedes aegypti* (Souza-Neto et al., 2009). This study names two putative JAK/STAT pathway regulated dengue virus restriction factors, DVRF1 and -2.

Surprisingly, the Imd pathway, was recently implicated in the anti-viral immune response in *Drosophila*. Evidence suggested that though AMPs regulated by Imd pathway were not induced after CrPV infection, it does imply that hemocyte mediated immunity in immunity against CrPV (Costa et al., 2009). In this study, flies with mutations in PGRPLC, Tak1, ird5, key and rel were susceptible to CrPV challenge. However, flies deficient in dFADD did not exhibit similar phenotype; suggesting either that antiviral immunity is specifically regulated by one branch of the Imd pathway or that mutation of major Imd pathway genes abolishes general immunity which may act as a primary signal for antiviral response.

Lastly, new evidence suggests that STAT pathway may mediate anti-*Plasmodium* immunity (Gupta, 2008). The duplication of the STAT gene in *Anopheles*, AgSTAT-A and -B established investigation for the role of these STATs and it was discovered that STATA participates in transcriptional activation of the nitric oxide synthase (NOS) response to bacterial and *Plasmodium* infection. AgSTAT-A silencing enhances

Plasmodium infection by increasing overall oocysts survival and silencing of the suppressor, SOCS, reduces parasite infection by increasing NOS expression.

2.11 *Plasmodium* Life Cycle

In order for *Plasmodium* transmission to take place, the parasite must undergo several developmental states which are outlined in Figure 3. The *Plasmodium* life cycle initiates with the mosquito ingesting an infectious blood meal consisting of microgametocytes (male and female). Male gametocytes give rise to eight haploid gametes through three rounds of mitosis. Fertilization takes place within 1 hour resulting in zygotes which gradually differentiate into motile ookinetes through a series of developmental events. The ookinetes then traverse the peritrophic matrix (PM), a chitin containing extracellular matrix that surrounds the blood bolus, and then penetrate the midgut epithelium, maturing into oocysts approximately 24 hours post blood meal. This cycle can vary for different *Plasmodium* species and hosts systems. In the case of *Anopheles* and *Plasmodium berghei*, several mitotic divisions in the oocysts give rise to sporozoites. Approximately nine days post infection, oocysts rupture releasing the sporozoites into the hemolymph (mosquito open circulatory system), where they migrate to the salivary glands (figure 3; (Moreira et al., 2004). The cycle continues in the vertebrate host (human, rat or chicken) when the infected mosquito bites to infect by injecting the sporozoites in the host. A number of significant interactions between parasite and host tissue take place that are vital for the development and proliferation of the malaria parasite. Important tissues to

investigate include organs such as fat body, midgut and salivary glands where significant interaction between parasite and host take place. These organs produce a number of immune peptides, releasing them into the hemolymph (see Appendix A for *Aedes/Plasmodium gallinaceum* cycle).

2.12 Mosquito transgenesis and anti-*Plasmodium* immunity

It is essential to understand the mechanisms of the innate immune response in mosquitoes given its vector status in hopes to develop novel strategies against *Plasmodium*, and thus limit malarial infections. As mentioned in the previous section, *Plasmodium* undergoes a complex life cycle that consists of oscillating asexual and sexual phases. The mosquito has ample opportunity to fight the parasite before transmission while the parasite traverses two epithelial barriers in the mosquito and circulates in the hemolymph. The genetic modification of mosquitoes including introducing foreign genes in to these vectors is a strategy under intense study as a means to combat malaria. Currently, methods include introducing foreign genes under the control of tissue specific promoters which enables the expression of these genes in the tissues of interest at the appropriate time points. This enables the characterization of effector molecules capable of interfering with parasite development. These points of interactions have been the focus of recent studies.

Insect vector control can be subdivided into two broad categories. One is genetic modification of mosquitoes which includes releasing transgenic mosquitoes carry anti-plasmodium genes. Another approach is population suppression using sterile insect technique (SIT). Advances in genetic transformation using transposable elements is due to identification of transposable elements, use of marker genes, tissue/state specific markers, and identification of effector molecules including antibodies against parasite or mosquito midgut proteins, and synthetic microbial peptides. Several success in genetic transformation and inhibition of parasite are discussed below.

The use of transposition assays led to researchers to pinpoint a feasible strategy for genetic transformation (Coates et al., 1997; O'Brochta et al., 2003). This method measures the transposition of an antibiotic gene using a helper plasmid containing a heat shock promoter. However, the use of eye markers such GFP for positive germline transformation proved to be far superior and successful than antibiotic gene resistance. Additionally, GFP can be detected in early larval stages. Much has been learned from these studies including the efficiency of these transposable elements. For example, the piggy-Bac element has a high transformation rate and seems to integrate precisely (Grossman et al., 2001; Grossman et al., 2000; Kokoza et al., 2001).

Several lines of evidence suggest that the interruption of the *Plasmodium* life cycle at various points may lead to refractoriness. The addition of Nitric oxide synthase showed a reduction in the prevalence of *Plasmodium* in *Anopheles* (Luckhart et al., 1998). However, depletion of Defensin A in *Anopheles* did not affect parasite numbers in contrast to the anti-Plasmodium affect of exogenous defensin (Blandin et al., 2002;

Shahabuddin and Pimenta, 1998). On the other hand, transgenic alterations such as expression of a synthetic peptide, SM1, resulted in decreased numbers of *Plasmodium berghei* oocysts in *Anopheles* (Ito et al., 2002). CEC 1 transgene expression in the midgut of transgenic *Anopheles gambiae* significantly reduced *P. berghei* numbers (Kim et al., 2004). Several lines of evidence have also shown that systemic production of AMPs can adversely affect parasite development and consequently block parasite transmission (Vizioli et al 2000; (Kokoza and Raikhel, 2009). Along these lines, it has also been shown that addition of immune peptides to *Plasmodium berghei* gametocyte cultures inhibits the development of ookinetes. Furthermore, feeding peptide treated gametocytes to *Anopheles albimanus* *check spelling* inhibits parasite development in the midgut (Nirmala and James, 2003). Moreover, oocysts development was blocked by 96% compared to control mosquitoes when Shiva1 was expressed as a recombinant protein was fed to mosquitoes (Yoshida et al., 2001). Several studies have also shown that inhibition of functional activity of Rel2 and deletion of Rel-1A results in susceptibility to gram-negative bacteria and fungi, respectively (Bian et al., 2005; Shin et al., 2003). Similarly, Rel2 gene silencing nearly doubles the number of parasites in the midgut as compared to control mosquitoes (Meister et al., 2005) while over-expressing REL2 led to a 94% reduction in oocysts number (Antonova et al., 2009). Similar evidence was shown with the expression of phospholipase A2 (PLA2) in midgut epithelia reduced oocysts formation by 80% in these transgenic mosquitoes (Ag-Aper1-PLA2) (Abraham et al., 2005a).

One major issue remains is that how to introduce the transgene(s) into wild type mosquito populations. Several theories have been discussed, one being the use of transposable elements like the P-element which spread in *Drosophila* populations. However, elements for this purpose remain unidentified and have several drawbacks i.e inability to cross species and internal mutations. Another idea is to use *Wolbachia*, intracellular maternally transmitted bacteria, which can be used as a driving mechanism to replace natural insect population since it has the ability to spread rapidly through population by cytoplasmic incompatibility. However, ethical and political issues regarding the release of genetically modified mosquitoes need to be addressed. While the prospects of this application remain at large, the identification of effector genes should continue since the parasite is constantly evolving and the effectiveness of single effector gene will be short-lived.

The idea that that by introducing genes in mosquito populations and hence rendering them refractory, could hinder and or block transmission of parasite was introduced three decades ago. Though considerable research since then reveals promising results, it is essential to understand the relative fitness and population ecology of genetically modified mosquitoes. Fitness of a mosquito depends on several factors. One is the location and type of protein being expressed since ubiquitously expressed proteins are more detrimental to fitness. Additionally, proteins being expressed from a strong and ubiquitous promoter causes a larger fitness load. Furthermore, the design of the construct should be so that it has minimal effect on non-targeted populations. Secondly, the disruption of native genes may also reduce fitness. However, disruption of genes is

unlikely to be homozygous. Most insertions have little or no effect on fitness either because they integrate into uncoded regions or do not disrupt native gene function (Marrelli et al., 2006). Several lessons can be derived from recent published studies. First, inbreeding strongly influences fitness; specifically, heterozygotes are not affected by inbreeding, and mutations by insertions are recessive. Second, chose a stage and tissue specific promoter and lastly, consider the position of the insertion (position effect).

2.13 References

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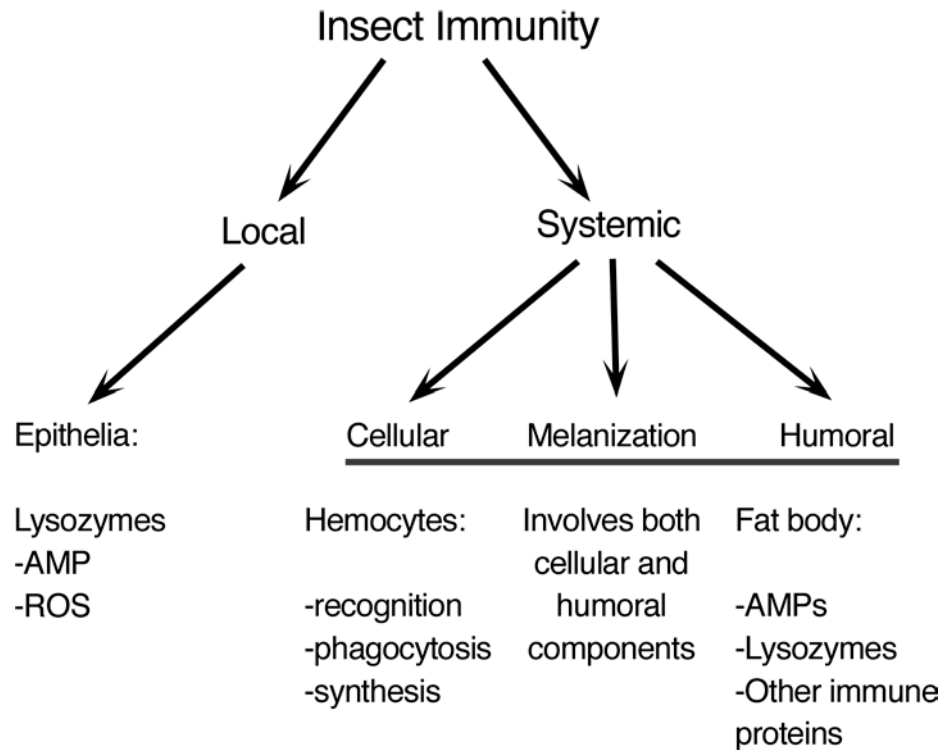


Fig. 2.1. Overview of Insect innate immunity. Insect immunity consists of both local and systemic immune responses. Local immunity comprises of responses dictated by the epithelia. Humoral responses. Systemic immunity includes humoral responses, melanization and systemic immunity. All these dictate antipathogen immunity in insects.

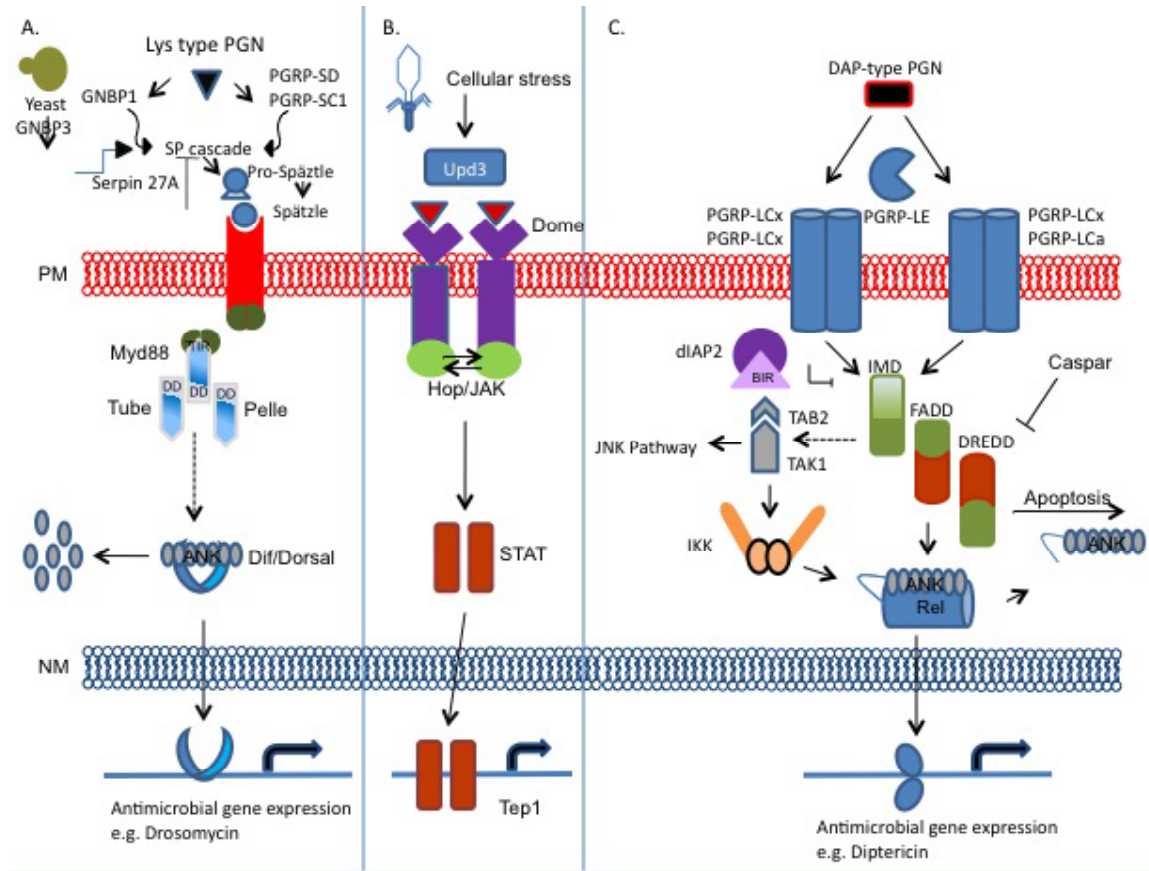


Fig. 2.2. Immune pathways in insects. A) Toll pathway. B) JAK/STAT pathway. C) IMD pathway. Toll pathway responds to Lys-type or Gram-positive bacteria and fungi while the JAK/STAT pathway is for antiviral immunity and cellular stress. IMD pathway responds primarily to DAP-type bacteria or Gram-negative bacteria invasion. All three pathways culminate in the activation of NF- κ B transcription factors which results in the production of anti-pathogen peptides.

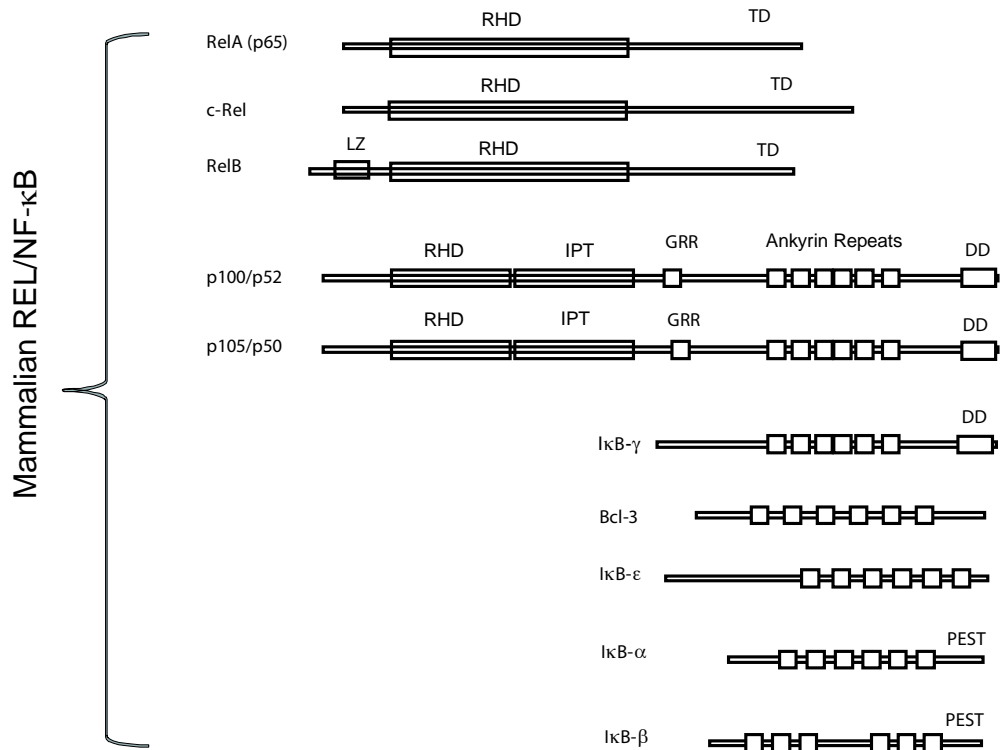


Fig. 2.3. The Rel/NFκappaB/IkappaB family of proteins. The number of amino acids in each protein is shown on the right. *RHD*, *rel* homology domain; TD, transactivation domain; LZ, leucine zipper domain of rel-B; GRR, glycine-rich region; SRR, serine-rich region in Relish. Figure adapted from: Sankar Ghosh, Michael J. May, and Elizabeth B. Kopp. (1998) NF-κB AND REL PROTEINS: Evolutionarily Conserved Mediators of Immune Responses. *Annual Review of Immunology*. 16: 225-260.

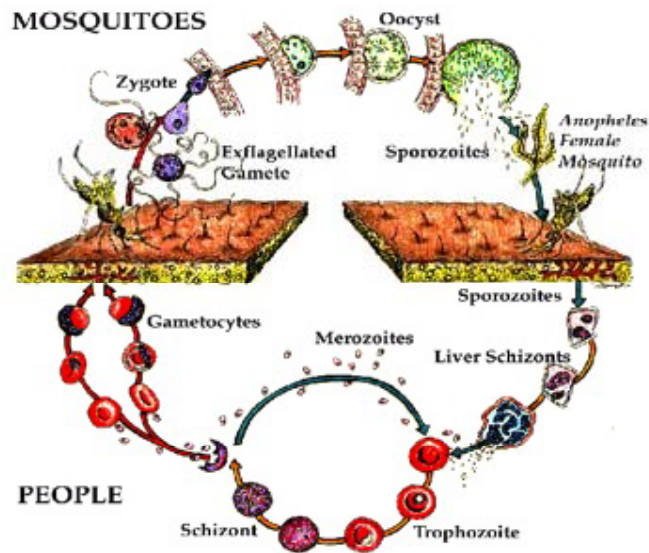


Fig. 2.4. *Plasmodium* sexual life cycle. The microgametocytes give rise to eight microgametes soon after blood meal ingestion. Next, the fertilization of macrogametes formulate the zygote. The zygote then develops into motile ookinetes which invade the midgut epithelium approximately 24 hours post infection (hpi). Ookinetes then develop in the non-motile oocysts in the basal lamina. Several mitotic divisions in the oocysts stage give rise to thousands of sporozoites which are then released in to the hemolymph nine days post infection (dpi). The sporozoites migrate and reside in the salivary glands of the mosquito until they are inoculated into the vertebrate host during feeding. (Image courtesy of www.malariatest.com).

**CHAPTER 3: THE REGULATION OF THE TOLL PATHWAY IN THE
MOSQUITO, *Aedes aegypti*.**

ABSTRACT

Interleukin receptor associated kinases (Fitzgerald et al.) mediate major immune responses in both insects and mammals. Sequencing and annotation of the *Aedes aegypti* genome revealed two IRAKs, one homologous to mammalian IRAK4, and the other homologous to *Drosophila pelle*. Furthermore, phylogenetic analysis revealed that the arthropod IRAK4 forms an evolutionary pathway that is independent of *Drosophila Pelle*. This unique genomic information directs our focus to study the distinct function between mosquito IRAK4 and Pelle. Expression profiles examining the immune response revealed that IRAK4, but not Pelle, is dramatically induced after microbial infection. The expression of IRAK4 was also up-regulated in REL-1 gain-of-function transgenic mosquitoes. Additionally, ectopic expression of IRAK4, and Pelle showed that IRAK4 induced stronger Drosomycin gene expression versus Pelle in *Drosophila* S2 cell line. Additionally, IRAK4 expression was upregulated in REL1 gain-of-function transgenic mosquitoes. RNAi mediated depletion of IRAK4 compromised the immune activation of CLIPB29 and Serpin-2 in the mosquito, *Aedes aegypti*, which mimicked Rel1 or Toll5A depletion. Moreover, mosquitoes with IRAK4 depletion were significantly more susceptible to fungal infection. Lastly, the concurrent depletion of IRAK4 with Cactus compromised the refractoriness of Cactus-depleted mosquitoes against the avian malaria parasite, *Plasmodium gallinaceum*. Altogether, these results indicate that IRAK4 is a key component of the mosquito Toll immune pathway and is involved in the antifungal and antiparasitic defense response.

3.1 Introduction

Hematophagous arthropods, such as mosquitoes and ticks, utilize vertebrate blood as a nutritional source for their egg production; in turn pathogens of numerous devastating diseases exploit insect-vertebrate interaction for their life cycles. Essential life stages of pathogen development occur in the vertebrate and the vector insect host, and both hosts rely on their immune defenses to modulate and control development of pathogens. Thus, control of pathogens can be achieved not only by targeting pathogens via drug therapy or immunization of humans but also interrupting pathogen transmission in vector insects. Deciphering immune defenses of vector insects against pathogens is essential for developing new strategies for managing emerging and reemerging diseases.

Innate immunity is evolutionarily conserved from lower metazoans to vertebrates. They share conserved pathways in which Rel/NF- κ B molecules serve as principal transcription activators of innate immune factors. In *Drosophila melanogaster*, the Toll pathway owns dual functions of regulating the establishment of the embryonic dorso-ventral patterning as well as the innate immune response against fungi and Gram-positive (Lys-type peptidoglycan) microbial infection (Galindo et al., 1995; Grosshans et al., 1994; Leulier et al., 2003; Towb et al., 1998). In both scenarios, the induction of the pathway involves signaling of Toll receptors through adaptor molecules, Myd88 and Tube, and an IRAK (Interleukin associated kinase), Pelle, which culminates in the activation of NF- κ B transcription factors (Shen and Manley, 2002; Sun et al., 2002). Pelle is implicated to initiate a MAPK kinase signaling cascade, which results in the phosphorylation and

subsequent degradation of Cactus, the I κ B-like inhibitor. The dissociation of Cactus from NF- κ B factors, Dorsal and Dif, allows for their nuclear translocation and binding to corresponding cis-elements (κ B motif) of antimicrobial gene promoters by which the transcription of antimicrobial genes are activated (Matova and Anderson, 2006; Shin et al., 2005).

Drosophila Toll pathway has been implicated to be analogous to the vertebrate Toll-like receptor pathway (TLRs/IL-R) (Yamamoto et al., 2004). Similar to *Drosophila*, the activation of the TLR pathway in vertebrates leads to transmembrane receptor dimerization and downstream culminating in NF- κ B activation through MyD88 and IKK complex. Thus, it resulted in the activation of immune molecules. Similar to *Drosophila* pelle, vertebrate IRAKs mediates cytoplasmic signaling of TLR pathway. To date, four IRAKs have been identified in the human genome: IRAK1 (Cao et al., 1996), IRAK2 (Muzio et al., 1997), IRAK-M (Wesche et al., 1999) and IRAK4 (13). IRAK family members are composed of an amino terminal death domain and a serine-threonine kinase domain. Recent studies implicated IRAK4 as an essential factor in the TLR/IL-1R mediated responses (Koziczak-Holbro et al., 2007; Li et al., 2002; Li, 2008; Lye et al., 2008; Suzuki et al., 2002; Suzuki et al., 2003). Specifically, IRAK4 activity is indispensable in cytokine production such as IRAK4 deficiency in humans leads to impaired TLR responses (Suzuki et al., 2002). Additionally, mutants expressing kinase-deficient IRAK4 failed to induce NF- κ B activation (Suzuki and Saito, 2006). Recently, it was also shown that MyD88 is another essential adaptor for recruiting IRAK4 to the IL-R complex and for bringing IRAK1 and IRAK4 in close proximity to each other. Recent research clearly demonstrates

distinct downstream targets of each IRAK and therefore their contributions to specific activation of distinct cellular response upon specific TLR ligand challenge (Qin et al., 2004).

Several studies implicated IRAK-M as a negative regulator of IRAK4-mediated immune reaction (Kobayashi et al., 2002). Contrary to IRAK4, cell lacking IRAK-M showed an increase in cytokine production in response to bacteria and various TLR ligands (Kobayashi et al., 2002). On the hand, IRAK1 knock-out mice are still able to produce cytokines after TLR or IL-1R stimulation; suggesting a redundant role of immune response mediated by IRAKs (Gottipati et al., 2008). Additionally, IRAK2 is implicated in apoptosis in which it associates with Mal/TIRAP, a TLR intracellular adaptor molecule (Nagpal et al., 2009).

The involvement of the Toll pathway in immune response against fungi was previously reported in the mosquito *Ae. aegypti*, where the receptor, Toll5A, homolog of *Drosophila* Toll receptor, is specifically induced after fungal challenge. (Shin et al., 2006). Additionally, the same study revealed that Spätzle 1C (Spz1C) also functions as a cytokine specific to Toll receptor mediated immune response following fungal infection in the mosquito (Shin et al., 2006). Furthermore, it was also shown that Rel1, a homologue of *Drosophila* Dorsal, regulates Toll mediated antifungal immune pathway in the mosquito, *Aedes aegypti* (Shin et al., 2005).

Besides antifungal immune response, several studies suggest the involvement of the Toll immune pathway in the anti-*Plasmodium* as well as antiviral immune response. It was shown that depletion of Cactus results in resistance to *Plasmodium* as shown by low

oocysts number in Cactus-depleted mosquitoes (Garver et al., 2009; Zou et al., 2008). In both studies, the Cactus-depleted mosquitoes had a low oocysts number, suggesting that the Toll pathway regulated by REL1 also exerts an anti-*P. falciparum* effect (Garver et al., 2009). However, the latter study showed that Cactus depletion also leads to an increase in melanized oocysts. Furthermore, the same study showed that the depletion of RUNX4 abolished anti-parasitic causes by Cactus depletion. This information suggests that RUNX4 cooperates with REL1 in the antiparasitic immune response. Toll pathway has also been implicated in the antiviral immune response using Sindbis and Dengue Virus (Sanders et al., 2005; Xi et al., 2008).

Here, we show that IRAK4 is a key player in the *Aedes* Toll immune pathway. Sequencing and annotation of the *Aedes aegypti* genome (Waterhouse et al., 2007) revealed two IRAKs, one homologous to mammalian IRAK4, and the other homologous to *Drosophila* pelle. This genomic information directs our focus to study the distinct function of IRAKs- IRAK4, and Pelle in the mosquito, *Aedes aegypti*. The immune challenge expression profiles revealed that IRAK4 but not Pelle was induced specifically after microbial infection. Additionally, ectopic expression of IRAK4 induced a stronger Drosomycin gene expression in *Drosophila* S2 cells than Pelle. Additionally, IRAK4 transcript expression was upregulated in the REL1-gain-of function transgenic mosquitoes. Furthermore, the RNAi mediated depletion of IRAK4 and Pelle showed that only the knockdown of IRAK4 results in the compromised induction of depletion of Serpin-2 and CLIPB29 after fungal infection, both which are established marker genes of the mosquito Toll pathway (Zou et al., 2008). Survival rate analysis revealed that mosquitoes with

RNAi-mediated depletion of IRAK4, not Pelle, are susceptible to fungal infection. The double knockdown of IRAK4 and Cactus rescued the *Plasmodium* oocysts numbers as compared to Cactus depletion alone. These results lead us to believe that IRAK4 may have been retained evolutionarily as a central regulator of Toll innate immune response in mosquitoes.

3.2. Materials and Methods

Experimental animals — The wild type *Aedes aegypti* mosquito strain, UGAL/Rockefeller, was maintained in the laboratory culture with 27 °C, 80 % humidity. Adult mosquitoes were fed on water and 10% sucrose (Hays, 1990). Naïve adult female mosquitoes were collected at 24 hours post eclosion for RNA injection experiments. All dissections were performed in *Aedes* physiological solution (APS).

Computational analysis — Known kinase domain of *Drosophila*, *Aedes* IRAK4 and Pelle as well as other insect IRAKs were used as queries to perform TBLASTN searches in NCBI, Vectorbase, Ensembl, and Genboree Database. Retrieved sequences from different metazoan species were analyzed by PROSITE (Hulo et al., 2006) SMART (Schultz et al., 1998) and CDRT at NCBI (Marchler-Bauer Aron et al., 2007) to confirm conserved domain structures required for the specific function. Alignments were done using CLUSTALW and manually adjusted using GENEDOC. Secondary structures labeled in the death domain and kinase domains of IRAK4 were identified from PDB (Protein Data

Bank). Exon-Intron boundary sequences were obtained from Ensembl or predicted using TBLASTX. Sequences used for alignment and phylogenetic analysis are summarized in Table 1. Sequences were aligned using ClustlX2.0 (Blosum 30 matrix, a gap penalty of 10, and an extension gap of penalty of 0.1). Phylogenetic trees were constructed using the neighbor joining method and displayed using Treeview.

RT-PCR and Northern analysis — RNA was extracted from eight body mosquitoes' whole by using Trizol method (Invitrogen) according to manufacturer's protocol. 5 µg of total RNA from each sample was separated on a formaldehyde gel, blotted and hybridized with the corresponding ³²P-labeled DNA probe. Probes were generated using PCR and followed by labeling using the High Prime (Roche) protocol. Actin was used as a loading control. cDNAs were synthesized from 2 µg total RNA using Omniscript Reverse Transcriptase kit (Qiagen). PCR was performed by using Platinum High Fidelity Supermix (Invitrogen). Naïve, 5h post infection with *Enterobacter cloacae*, and 2 day post fungal, *Beauveria bassiana*, infection mosquitoes were used to construct the immune profile of IRAK4 and Pelle. Toll5A was used as a control. To investigate the expression of presence of IRAK4 and Pelle during mosquito development, ovaries were collected from adult female mosquitoes one day and three days post blood meal. Additionally, eggs of one day post oviposition were also collected for RNA. PCR was performed under the following conditions: 35 cycles for IRAK4 and Pelle, 30 cycles for Toll5A, Toll1b and Actin. See Table 3, Supplemental data for primer sequences.

Gene expression knockdown — For gene silencing, double-stranded RNA (dsRNA) was synthesized with T7 RNA polymerase. A T7-phage promoter sequence was incorporated into both sense and antisense sequences of target genes to generate template cDNAs containing the T7 tag. RT-PCR was performed using the Titan one-step RT-PCR kit (Roche) with samples of 0.2 µg total RNA as templates to generate a 400-600 bp gene-specific cDNA fragment. Amplification conditions involving synthesis of dsRNA was accomplished by simultaneous transcription of both strands of template DNA using the MEGAscript kit (Ambion). The luciferase gene was used to generate control iLuc dsRNA. After RNA synthesis, the samples were treated by means of phenol/chloroform extraction and then ethanol precipitation. The dsRNA was then suspended in RNASE Free water with a final concentration of 5µg/µl. The concentration, size, and formation of dsRNA were confirmed by running 0.2µl of the reaction on a 1% agarose gel in TBE. A Picospritzer II (General Valve, Fairfield, NJ) was used to introduce corresponding 3-5 µg dsRNA into the thorax of CO₂-anesthetized adult *Aedes aegypti* mosquito females, at one or two days post eclosion.

Septic injury and RNA extraction — Septic injuries were performed by pricking female adult mosquitoes in the rear part of the abdomen with an acupuncture needle (0.20 x 25 mm) dipped into either *Enterobacter cloacae* bacterial culture (stationary phase of bacteria in LB broth; OD₆₀₀ ≈ 2.0) or a fungal spore suspension (~5 × 10⁷ viable spores/ml) of *Beauveria bassiana* strain GHA. RNA was extracted from infected mosquitoes using

the Triazol method (Invitrogen) as described above.

Survival rate experiments — To test the anti-pathogen response, 24h eclosed mosquitoes were subjected to treatment with various dsRNAs (REL1, Pelle, IRAK4, TOLL5A and IMD. Mosquitoes were challenged with fungal spore suspension $\sim 5 \times 10^7$ viable spores/ml) of *Beauveria bassiana* strain GHA four days post RNAi injections. The number of dead mosquitoes was calculated every 12 hours; survival rate was recorded and plotted using Microsoft Excel. Each experiment was performed minimum three times.

Transfection assay in Drosophila S2 cells — Full length cDNA sequences of IRAK4 and Pelle were obtained from Zdobnov Computational evolutionary genomics group (<http://cegg.unige.ch/Insecta/immunodb>) and confirmed by subcloning the cDNA into TOPO Vector (Invitrogen) followed by sequencing. Primers were designed for full length transcripts including unique restriction sites and cloned into pac5.1/HisA/V5 plasmid. See Table 3, Supplemental data for primer sequences. Coding region sequences of *Ae. aegypti* IRAK4, Pelle, REL1, and IMD were amplified by means of PCR, inserted into pAC5.1/V5/HisA vector (Invitrogen), and used to transfect *Drosophila* S2 cells. 2×10^6 cells per ml were distributed on 35-mm plates in *Drosophila* Schneider's medium (GIBCO) supplemented with 5% fetal bovine serum (GIBCO) and 1X Antibiotic-Antimycotic (GIBCO). A 1:4 DNA to Lipid ratio was used for all transfections. The cells were transfected with 1 μ g of each plasmid construct and incubated for 6h in serum-free

Schneider's medium, which was then removed and replaced with complete medium. The cells were then incubated for 24 h with 20-hydroxyecdysone, and then incubated for 5 hours with heat inactivated *E. cloacae*. Transfection with empty plasmid was used as a control for both analyses. Total RNA was isolated from harvested cells using the Trizol method (Invitrogen), and samples were then subjected to Northern analysis. Blots were hybridized to *Drosophila* Drosomycin and Dipteracin probes. The Drosomycin blot was stripped and reprobbed with Actin for loading control. For protein expression analysis, cells were transfected with the same plasmids as discussed above and protein extraction was done using a Cell lysis buffer followed by SDS-PAGE gel electrophoresis and Western blot.

Plasmodium analysis – To test the anti-*Plasmodium* response, gene expression of IRAK4, Pelle, REL1, and Cactus was knocked down in mosquitoes 24 hours post eclosion using the RNAi method as discussed above. Then these mosquitoes blood fed on White Leghorn chickens infected with *Plasmodium gallinaceum* 4 days post RNAi injections. The oocysts number was observed and recorded 7 days post infectious blood meal. Statistical analysis was done using Graphpad and relevance was tested using the Man-Whitney t-test. To test the knockdown of Cactus with REL1, Pelle, and IRAK4, equal proportions of dsRNA concentrations were mixed and then injected into the mosquito thorax. Three independent experiments were performed.

3.3. Results and Discussion

3.3.1. *The mammalian IRAK4 orthologue mediates Toll immune pathway in the mosquito, Aedes aegypti*

The presence of IRAK4 with a death domain in the mosquito- In mammals, IRAKs play a significant role in Toll-like receptor (TLR) immune pathways Myd88 serves as an adaptor protein associates with both IL-1 receptor and IRAK, through its TIR and death domain. Mammalian IRAK4 takes the central position in the IL-1R pathway and is able to activate the downstream NF- κ B and MAPK pathways. It functions upstream of IRAK1 and independent of IRAK2.

Drosophila IRAK orthologue, Pelle, mediates Toll signaling in *Drosophila*. In *Drosophila*, the cytoplasmic signaling pathway downstream of Toll involves the adaptor protein, Tube, which transmits signals to Pelle. Pelle, in turn, signals to NF- κ B transcription factor, Dorsal-Cactus complex. We figured out that arthropods with Tube genes, which are orthologues of the *Drosophila* Tube, have an N-terminal death domain that is largely consistent with the mosquito death domain (Fig. 3.2D). These genes are referred to as “Tube like genes.” However, since mosquitoes and other insect Tube-like genes also contain a C-terminal kinase domain, which is orthologous to mammalian IRAK4 kinase domain, we have renamed these Tube-like genes as “IRAK4”. Although the evolutionary origin of Tubes and IRAK4s is predicted to be conserved in arthropods, we will distinguish each with separate names of Tube.

In case of *Drosophila*, we will refer to these genes as Tube or Tube-like genes while in the case of other arthropods, we will refer to Tube like genes as IRAK4 since the kinase domain defines the function of these IRAK proteins.

IRAK4 is present in all the vertebrate genomes from frog to human. Sequencing and annotation of the mosquito genomes revealed that mosquitoes harbor two IRAKs rather than one in *Drosophila* (Fig. 3.1, Table 3-1). One is an orthologue of mammalian IRAK4, and the other, Pelle family, also an IRAK. However, the *Drosophila* Toll pathway has only one IRAK kinase, Pelle (Fig. 3.2). We searched all available arthropod genomes by using *Ae. aegypti* and human IRAK4 as the query sequence and found that IRAK4 is present in louse, tick, silkworm, moth, and pea aphid, but is absent in the fly, honeybee, and wasp (Fig. 3.1 and Table 1). After examining the protein domain structure of these IRAKs using PROSITE and manual alignment, all of these genes contain a highly conserved death domain and a kinase domain (Fig. 3.1).

It was recently observed that the structure of Pelle is well conserved among various Arthropods whereas the structure of IRAK4 differs greatly (Towb et al., 2009). The linkage sequence between kinase and death domain in Pelle group is stable and also similar in length. However, the linkage sequence length in IRAK4 is much more variable than Pelle (Fig. 3.1). According to the ClustalW alignment, the variability of the amino acid sequence could be due to the high variability in the length and content of the amino acid sequence between the death and kinase domains of IRAK4 (linker region). These data also suggest the linkage of the death and kinase domains in Pelle was fixed in the very early stage of the metazoan evolution. Based on the amino acid length and

variability of the linker region (see Fig. 3.1), we suggest that the IRAK4 gene is unstable, supporting that the linkage of two domains could be broken; therefore, dissolving the structure of the gene and leading to the loss of the kinase domain of Tube. This phenomenon shows that IRAK4 with a death domain is extensively present in the arthropod; the reason why some insects lost the kinase domain remains to be elucidated..

Phylogeny analysis of the metazoan IRAK- IRAKs are characterized by a ~100-amino acid N-terminus Death domain, and an approximate ~300-amino acid C-terminus kinase domain (Fig. 3.1). In order to investigate the evolutionary origin of the *Aedes IRAK4* protein, we set out to identify IRAK4 orthologues in other species by classifying the IRAK domain. The Death domain serves as an imperative regulatory domain present in IRAKs and Tubes. The results of the phylogenetic analysis of both domains are largely consistent and illustrated in Figure 3.2B (IRAK domain) and Figure 3.2C (death domain).

According to the phylogenetic analysis of death domains, insect Pelle and IRAK4 form two distinct clusters (Fig. 3.2C). Moreover, *Aedes Pelle*, forms a subcluster with other mosquito Pelles, and *Bombyx Pelle* which does include *Drosophila Pelle*. Mammalian IRAK4 death domain also formed a diverse clade separated from them.

The mosquito IRAK4 grouped with the vertebrate IRAK4s as well as the other IRAK4 from *Tribolium castaneum* (beetle), *Bombyx mori* (silkworm), *Acyrtosiphon pisum* (pea aphid), *Pediculus humanus* (louse), *Ixodes scapularis* (tick) and others. The death domains of three mosquito IRAK4s form an orthologous sub-cluster (Fig. 3.2C). Additionally, Coleoptera (Beetles) and Lepidoptera (Moths) IRAK4 death domain is

deeper rooted. The death domain of Hymenoptera (aphid) Tube evolved earlier and quicker than the other subclade. This suggests that some insect Tube lost their kinase domain after they diverged from vertebrates since we did not see an IRAK domain in these insects.

We noted that two very similar Pelle sequence in both *N.vitripennis*, and in *A.mellifera* so we excluded these and concluded that they may be alleles of the same gene or products of unfinished sequencing not excluding possible misassembled contigs. *Aedes* IRAKs were named Pelle and IRAK4 based on the phylogenetic relationship of kinase domain with *Drosophila* and Human IRAKs respectively (Fig. 3.2).

While studying the kinase domain, we noticed that the three mosquito species (*A. aegypti*, *A. gambiae*, and *C. pipens*) form a sub-clade in both phylogenetic trees of death and kinase domains suggesting a similar evolution of IRAK4 in all three species (Fig.3.2B and 3.2C). IRAK4s of other invertebrate species are deep rooted, which is consistent with the information obtained from the death domain tree. While the mammalian IRAK4 forms a completely different cluster in the Death domain tree (Fig. 3.2C), they form a sub-clade in the IRAK4 domain tree (Fig. 3.2B); suggesting a shared origin and conservation in the insect and mammalian IRAK domain.

We also examined the exon-intron structure of mosquito IRAKs, human IRAK-1, 2, M, 4 and fly Pelle and found a similar distinct pattern of splice site in the kinase domain between human IRAK1, 2, and M (data not shown). Furthermore, vertebrate IRAK1, 2, and 3 forms a different clade than IRAK4, suggesting that they arose from a gene duplication and share the same origin.

Multiple sequence alignment shows the similarity and distinct differences between the Pelle and IRAK4 kinase. Analysis of the kinase domains of fly Pelle, Human, and mosquito IRAK4s revealed that the C-terminal proline residues are also conserved in these organisms (Fig. 3.2A). Three important motifs are recognized as essential for kinase activity: VAIK (Val-Ala-Ile-Lys), HRD (His-Arg-Asp), and DFG (Asp-Phe-Gly). The VAIK motif is present in Pelle but not in IRAK4; however, this motif is not present in the ATP binding domain of most IRAK kinase and therefore its integrity may not be essential for kinase activity. We also found that the DFG (Asp-Phe-Gly) motif in the activation segment is also well conserved in both Pelle and IRAK4. Both motifs are important for kinase activity during biological processes.

Several differences were also noted when examining the kinase domain structure of IRAK4 and Pelle. First, the comparison of primary structure revealed that the unique tyrosine gatekeeper residue is conserved from invertebrate to vertebrate IRAK4, but is absent in the all Pelles. Second, the ATP binding site is starkly different between Pelle and IRAK4 specifically the VAIK motif as discussed above. All IRAK4s were characterized with HRD (His-Arg-Asp) in the start site of the catalytic loop. The conserved aspartate separated by five residues is the metal binding asparagines in the catalytic loop are conserved in the IRAKs. The catalytic loop also contains several serine and threonine residues which are phosphorylated and in some kinases leads to conformational change leading to full activation of the enzyme. However, Pelle was observed to contain HGD. The HGD motif is conserved in most eukaryotic kinases and

eukaryotic like kinases. It serves as a scaffold which binds to aspartate in the DFG motif. This suggests that Pelle does not need to be phosphorylated and activated, versus IRAK4 which may need activation based on this observation; suggesting a differential function for both type of kinases.

Molecular Characterization of Aedes IRAK4- Genomic and Phylogenic analysis show considerable difference between these two IRAKs, which led us to focus on the distinct function of these two IRAKs. We first analyzed induction of these IRAKs in the female adult mosquitoes after bacterial and fungal infections (Fig. 3.3A). The gene expression profile of the IRAKs was quite distinct from each other. The expression of IRAK4 was specifically induced, but not Pelle, after microbial infections.

Next, the tissue specific expression profiles of IRAK4 and Pelle were investigated and compared (Fig 3.3B). Of the two IRAKs, the transcripts of IRAK4 were expressed more after fungal challenge in the mosquito fat body, strongly suggesting its participation in mosquito immunity. IRAK4 expression was not detected in the midgut. Pelle expression was weakly induced in the fat body; however, its expression was strongly present in ovary; suggesting a possible role in ovary development. The induction of IRAK4 after immune challenge in the fat body is similar to that of Toll5A which has been shown previously to be involved in the *Ae. aegypti* Toll Immune pathway (Shin et al., 2006).

Additionally, to test the role of IRAK4 in mosquito development, we tested the expression of IRAK4 and Pelle in the mosquito ovaries of adult females at 1 and 3 day

post blood meal (PBM) and 1 day old laid eggs (Fig. 3.3C). RT-PCR analyses revealed that Pelle is expressed during late stages of ovary development specifically in ovarian tissue 3 days PBM. Both IRAK4 and Pelle were expressed in ovaries after 1D and 3D PBM, suggesting that IRAK4 and Pelle may be involved in *Aedes* ovarian developmental. Interestingly, we did not detect IRAK4 or Pelle expression in 1D old eggs; thus ruling out the involvement of IRAK4 in mosquito development.

Lastly, the regulation of IRAK4 and Pelle was dissected in the Toll pathway using transgenic mosquitoes and the RNA interference technique in the mosquitoes (Fig. 3.4B and 3.4C). To test if IRAK4 is regulated by REL1, the NF- κ B factor in Toll pathway, we looked for IRAK4, Pelle and Toll5A expression in REL1 gain of function transgenics (Fig. 3.4B). IRAK4 was significantly upregulated in REL1 transgenics 24 hours post blood meal; however a slight induction of Pelle was also observed. This suggests that REL1 chiefly regulates IRAK4 whereas Pelle may be involved in a branched pathway.

Functional characterization of the Aedes IRAK4 in mosquitoes- To investigate the *in vivo* role of IRAK4, mosquito REL1, Pelle, IRAK4, and IMD (adaptor molecule of IMD pathway) was isolated and cloned into pAC5.1 expression vector. Like REL1, *Aedes* IRAK4 strongly induced Drosomycin gene expression in *Drosophila* S2 cells, when compared to *Aedes* Pelle and IMD (Fig. 3.4A). IMD was able to strongly induce Dipterucin gene expression. When we co-transfected Pelle and IRAK4 in S2 cells, we found that co-transfection did not induce a stronger activation of Drosomycin gene as compared to IRAK4 alone (data not shown). Drosomycin and Dipterucin are

representative marker genes regulated by *Drosophila* Toll and IMD pathway, respectively. This result indicates that mosquito REL1 and IRAK4 more specifically activate *Drosophila* Toll immune pathway in S2 cells.

To understand the role of *Aedes* IRAKs in Toll immune signaling, we tested the expression of immune genes two days post fungi challenged mosquitoes that were previously depleted with REL1, IMD, Toll5A, MyD88, IRAK4, and Pelle genes. The mRNA levels of Serpin-2 and CLIPB29 declined only in IRAK4 and Myd88-depleted mosquitoes as well as REL1 and TOLL5A that were used as controls and are consistent with previous findings. Serpin 2 and CLIPB29 were used to measure Toll pathway output (Zou et al.). Furthermore, Defensin gene expression was not abolished in the IRAK4-depleted mosquitoes. However, depletion of IMD had no effect on the expression level of CLIPB29 and Serpin 2 whereas Defensin expression was significantly compromised (Fig. 3.4D). These results suggest that IRAK4 and Myd88 along with REL1, and TOLL5A are functional components of the *Aedes* Toll Immune pathway.

Genetic analyses have shown that mammalian TLR pathway mutants are sensitive to immune challenge. To address the role of *Aedes* IRAK4 in mosquito immune response, the susceptibility of IRAK4- and Pelle-depleted mosquitoes was compared after bacterial and fungal challenge. After a 4-5 day recovery following dsRNA injections, the mosquitoes were challenged with fungal spores of *B. bassiana*. The IRAK4-depleted mosquitoes have much higher mortality 72 hours post infection compared to naïve mosquitoes and Pelle-depleted mosquitoes (Fig. 3.5). REL1 and Toll5A-depleted mosquitoes have a similar mortality rate to IRAK4, which means IRAK4 phenotype is

compatible with the genes in the Toll pathway. Still, Pelle-depleted mosquitoes have an increased mortality although weaker than IRAK4-depleted mosquitoes, indicating Pelle may also be involved in the Toll pathway albeit somewhat weaker role; however, further evidence is needed to confirm this hypothesis.

We also investigated the role of IRAK4 in the anti-parasitic response. Previous reports have shown that the malaria parasite development is affected in mosquitoes whose immune system has been activated before parasitic infection (Frolet et al., 2006). We observed that the boosting of mosquito immunity by Cactus depletion resulted in depletion of parasitic numbers (Fig. 3.6 and Supplemental Data S2 for results of single knockdown of iCACTUS, iREL1, iIRAK4). Indeed the depletion of IRAK4 abolished immune activation as observed with Cactus depletion. Interestingly, the double knockdown of Cactus/IRAK4 rescued the killing of parasite oocysts by Cactus depletion. This is similar to the Cactus/REL1 knockdown as shown by Zou et al (Zou et al., 2008). Pelle/Cactus knockdown also comprised an antiparasitic response albeit less than IRAK4. With this comprehensive information, we propose that IRAK4 regulates defense against the malaria parasite and reaffirms the involvement of Toll pathway in the anti-plasmodium response.

3.3.2. Regulation of the Toll Pathway by RUNX factors

RUNX are RUNT-related transcription factors containing a highly conserved RUNT domain. They are key regulators in multiple developmental processes, ranging from hematopoiesis to carcinogenesis. Recent reports have shown that a *Drosophila* RUNX [Lozenge (Lz)] and a GATA transcription factor (Serpent) are both necessary for hemocyte differentiation and proliferation. The crystal cell-specific expression of *Drosophila* PPOs is dependent on Lz (citation); however, the effect of Lz on insect immunity has yet to be elucidated. Below is the partial description of the findings as published in PNAS by Zou et al (2008).

In *A. aegypti*, three key evidences suggest the involvement of an immune inducible killing mechanism of a parasite: the sequence analysis of mosquito PPOs, their gene expression profiles, and comparison between Cactus and Serpin-2 knockdown phenotypes against the avian malaria parasite. We have shown that four out of the ten PPO genes are expressed in response to microbial infection and that their transcription is regulated by a RUNT-related transcription (RUNX) factor and the Toll pathway. Furthermore, the RUNX4-mediated immune activation under regulation of the Toll pathway has a potential role that largely restricts the parasite development.

Among four mosquito RUNX genes, RUNX2 and RUNX4 were inducible by bacterial and fungal challenge, but transcripts of RUNX2 were most abundant in 1-day-old eggs. This suggests a role for RUNX2 in embryonic development. The transcripts of RUNX4 were detected at the highest level in female mosquitoes 2 days after fungal

infection. The expression levels of RUNX1 and RUNX3 transcripts were not elevated by microbial infection (data not shown). Moreover, we identified RUNX4 as a key transcriptional factor that induces PPO genes. In addition, mosquito RUNX4 strongly activated the expression of both *Drosophila* PPO-A1 and PPO3 genes in *Drosophila* S2 cell line (Fig. 3.3C), proving that RUNX4 binds to RUNT motifs and directly activates PPO genes.

In this study, the role of RUNX4 in the anti-parasitic response was also investigated. Previous reports showed that development of malaria parasites was significantly hampered in mosquitoes in which immune systems had been activated before parasite infection (15, 21). Parasitic response in REL1+ transgenic mosquitoes with gain of function indicated the involvement of an alternate immune transcriptional factor in antiparasitic response. Indeed, the depletion of RUNX4 abolished immune effects caused by Cactus depletion. Cactus/RUNX4 double knockdown significantly compromised both the refractoriness of the mosquitoes against the parasite and ookinete melanization, albeit slightly less than with Cactus/REL1 double knockdown. With this comprehensive information, we propose that RUNX4 cooperates with REL1 in the anti-parasitic response.

3.4. Concluding Remarks

In mammals, proteins in the IRAK family are involved in signaling mediated by a family of TLR as well as the IL-1R, which finally activates NF- κ B transcription factor. Comparably, in *Drosophila* and other insects, Tube is a scaffolding protein which contains an N-terminal Death domain as well as a C-terminus which mediates signaling to Dorsal. However, contrary to Pelle, it lacks a kinase domain in its C-terminus. Therefore, the *Drosophila* Toll pathway also relies on Tube which functions as an adaptor protein with Pelle. Both Pelle and Tube are required to transduce signal to NF- κ B factor, Dorsal.

Many insect immune genes were identified based on their homology with the model organism, *Drosophila melanogaster*. Our curiosity about two IRAKs in many arthropod genomes has given us a chance to study its function and origin. Our analysis of insect IRAK4 has led to the conclusion that mosquito IRAK4 has significant immune function. Whether *Drosophila* Tube is an orthologue of mammalian IRAK4 or not is questionable. The first reason is the similarity of death domain between DmTube and HsIRAK4 is very low. Second, a kinase domain is present in mammalian IRAK4 but not in DmTube. Currently, we do not know the reason IRAK4 was lost in the some species. However, mosquito IRAK4 as a good model for kinase domain function was confirmed.

In *Drosophila*, MyD88 contains a TIR domain that interacts with the TIR domain of the Toll receptors, whereas Tube interacts with Myd88 and the protein kinase, Pelle with association of their death domains. Therefore, Tube is an adaptor protein that is

involved in a sophisticated death domain complex structure formed by three protein, MyD88, Tube, and Pelle. However, in mammals, there are exceptions where Myd88 independent signaling exists. Additionally, *Drosophila* Toll receptor does not directly bind to pathogens or pathogen derived compounds instead it is activated by the cytokine Spätzle, a product of a proteolytic cascade.

Up to date, the point at which the *Drosophila* Toll Pathway bifurcates is still a mystery. We have shown that the kinase domain of IRAK4 serves a crucial role in mosquito immunity. Our data shows that *Aedes* IRAK4 has a distinct and major role in immunity and Pelle may provide a residual basal immune function.

Foreign immune signal requires different kinase activity. Pelle and IRAK4 have similar but distinct structure and activity (RD-type and non RD-type). IRAK4 was separated from other IRAKs prior to the split between the vertebrate and invertebrate animals. There is no direct proof to support the hypothesis that they share one common ancestor. However, Pelle structure was kept stable and has regulatory function in both embryonic development and basal epithelial immunity. Conversely, IRAK4 structure was not very stable during the evolution in the arthropod lineage. In *Drosophila* and some other insects, it lost its kinase domain. In mosquito, IRAK4 is involved in the major Toll immune pathway, where its function is more specific than Pelle. The further question will be how they were organized, sequentially or cooperatively. We also could not exclude the possibility of the existence of a branching of a kinase pathway. The origin and dynamic co-evolution of the death domain and the kinase domain deserve imperative investigation as an interesting issue in the insect Toll immune pathway.

The results of the study presented here indicate that the Toll pathway may consist of a branch point at the Toll receptor where one branch is dependent on signaling by IRAK4 and the other branch is dependent on Pelle whose role remains to be elucidated. However, this is inconsistent with *Drosophila* Toll pathway, which consists of a linear pathway, serving both immune and developmental role. The activation of the Toll pathway in *Drosophila* involves receptor dimerization followed by induction of cytoplasmic components, Pelle, Tube, and Myd88. This activation, as previously described, occurs linearly and results in the full activation of the cytoplasmic signaling cascade which results in the release of the NF- κ B transcription factor, Dorsal or Dif and induction of developmental or immune responsive genes, respectively. The role of mosquito IRAK4 is clear in that it serves a prominent role in immunity and signals to REL1; however, the role of Pelle is less clear and needs to be investigated.

Our study on RUNX factors revealed that the expression of the four PPO genes is induced by microbial infection in the adult female *Ae. aegypti* mosquito. We also found that a transcription factor, RUNX4, is directly involved in the transcriptional activation of the PPO genes. Furthermore, RUNX4 might have a crucial role in the defense against the development of the avian malaria parasite *P. gallinaceum* in *A. aegypti*. This newly discovered immune mechanism contributes to our understanding of the immune interaction between the malaria parasite and its mosquito host at the molecular level.

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Fig. 3.1. Phylogenetic analysis of IRAK death domain and kinase domains in the fly, mosquito, and mammals. The amino acid sequence of various invertebrates (A,D) The amino acid sequence of the death domain is conserved across all three divisions (D). The death domain is well conserved in mosquitoes (helix 1 and 3). The kinase domain is only conserved in mosquitoes (2A, helix 1, 5, and 6). Alignment was done using ClustalW and was manually adjusted using GENEDOC. Helix structure was identified using Protein Data Bank (PDB). Phylogenetic relationships of IRAK kinase and death domain respectively (B,C). The amino acid sequence from three mosquito species, *Drosophila*, mammals, worm, and other insect IRAKs are examined. C, The death domain tree, Clade I (blue background) contains *Drosophila* Tube as well as mosquito IRAK4. However, analysis using the only the kinase domain reveals that mosquito IRAK4 form its own subcluster (B). Grouping and edge length suggest evolution from common ancestor around the same time. Alignments were made using ClustalW. Proteins sequences were taken from published data or predicted using BLAST sequence alignments. Red, green and black nodes represent bootstrap values great than 50%. Dm, *Drosophila melanogaster*, Ae, *Aedes aegypti*, Ag, *Anopheles gambiae*, Cp, *Culex pipens*, Am, *Apis mellifera*, Ph, *Pediculus humanus*, Tc, *Tribolium castaneum*, Ce, *Caenorhabditis elegans*, Mm, *Mus musculus*, Hs, *Homo sapiens*, Xt, *Xenopus tropicalis*, Nv, *Nasonia vitripennis*, Ci, *Ciona intestinalis*, Sp, *Strongylocentrotus purpuratus*, Bm, *Bombyx mori*, Ic, *Ixodes scapularis*, Ac, *Acythosiphon pisum*.

A.

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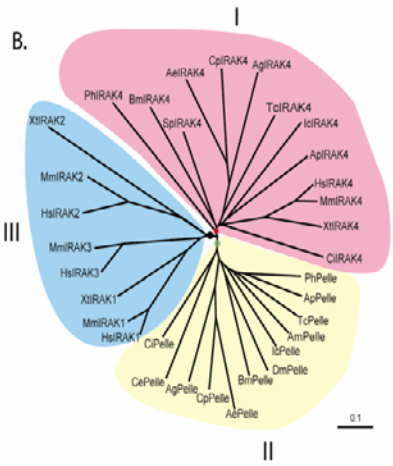
DmPelle : IVQRAAESLLEI . . . . . DYAELENATDGWSPDNR . GGGFQGVYRGKWKQLD . A . KVN . NYRSPNI . DQKML . LQGSYNEL
AeI RAK4 : TNVATVEPLI NFFSFDLTTATQNF . DJ . KRFSN . CNLPQD . RVL . GI . GGF . CEVFLALNLTPTYP . SA . VKR . F . FT . NYKY . E . K . D . . . . . RER
Agi RAK4 : T . . . . . DG . NVAPYTN . NLAAPN . GRSL . GGGF . GAVFLALN . LAPS . P . AA . V . KR . DPD . KYKFR . K . H . . . . . LEL
Cpi RAK4 : TDSATAEPVH . FSYQN . QVATAN . NADPF . NN . CDLRKPN . HFL . GGGF . GCVLAVN . LTSN . AAAA . KR . LPTNENY . ROK . D . . . . . RER
Mmi RAK4 : T . . . . . RFHSFSFHELKSI . TNN . DEQPASAG . . . . . GNRWGGGGF . GVV . YKGCVNNTI . VA . KK . GA . VEI . STEEL . K . C . D . . . . . CEI
Hsi RAK4 : T . . . . . RFHSFSFHELKNTVNN . DERPI . SVS . . . . . GNRWGGGGF . GVV . YKGCVNNTI . VA . KK . AA . VDI . TTEEL . K . C . D . . . . . CEI
      Helix 1                                ATP Binding Site                                Helix 2

DmPelle : KYLNSI . R . DNI . LALY . GYSI . KGGK . PCLVY . QLMK . GSSLEAR . RAHKAQN . LPA . LTV . QQ . RFSI . SL . CT . ARG . YFL . TARGT . PLI . H . GDI . KP
AeI RAK4 : EILSKY . N . HSN . I . VEL . I . GFS . . . . . ENVT . CLVY . EYL . P . GIL . GAA . DKS . RK . G . Q . . . . . LH . TVTR . RI . SYL . FGI . AAA . EYL . HSPL . VQV . VHR . DVTL
Agi RAK4 : DILSKHS . H . TNV . VS . L . L . GSS . SDGP . Q . CLVY . EYL . KOG . N . EAA . SLV . RA . G . Q . . . . . R . MGAT . V . RL . GYL . KDI . ANA . V . A . FL . ER . . . . . AKI . I . HRD . V . KS
Cpi RAK4 : QILSQCH . H . PNI . VKL . FG . Y . SENDQ . CLVY . EYL . P . GIL . ERA . L . KAN . REG . . . . . QTL . PPTS . RLS . YLL . GI . ASA . I . EYL . HALP . DPV . I . HRD . V . TP
Mmi RAK4 : KIMAT . C . CHEN . L . VEL . L . GFS . SDSD . M . CLVY . A . MP . NGS . L . LDR . S . CL . D . ET . . . . . PPL . SWHT . RCK . VAQ . CTANGI . RFL . EN . . . . . HH . HRD . I . KS
Hsi RAK4 : KIMAK . C . CHEN . L . VEL . L . GFS . SDGDD . CLVY . A . MP . NGS . L . LDR . S . CL . D . ET . . . . . PPL . SWHM . RCKI . AQC . A . NGI . NFL . EN . . . . . HH . HRD . I . KS
      Helix 2                                Helix 3                                Helix 4                                Catalytic Loop

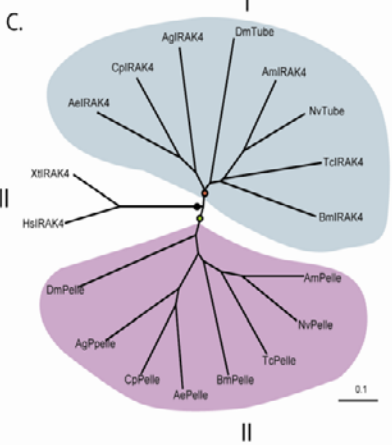
DmPelle : ANI . LLD . QCL . QPKI . GDF . GLV . REG . PKSL . DA . I . VE . VNK . V . FGT . KI . YLP . P . E . FR . NR . FQL . ST . G . DV . V . SF . GI . VLL . EV . F . TR . QV . TDR . V . PEN . ET . KKN
AeI RAK4 : ANI . L . LH . G . DV . AKL . D . F . GL . V . KR . . . . . I . DS . I . TAT . EV . SGT . GPYI . S . P . E . AMRG . TV . T . PAL . DV . YS . Y . GVV . I . A . EV . I . E . EV . L . CE . VLP . . . . . EG
Agi RAK4 : ANI . L . LDG . PV . AKL . D . F . GL . I . KR . . . . . TSS . RTTI . SI . I . OT . NAY . NA . E . AV . R . G . DV . SPALDI . FAF . GI . VVA . ET . V . T . G . P . V . LAEK . ERS . E . I . DIA
Cpi RAK4 : ANI . L . LR . G . TT . AKL . A . DF . GL . V . KR . . . . . VESTTAT . VV . AGTAA . YLP . VEAL . RG . I . VTPAL . DI . YSF . GVV . I . A . EV . S . G . E . DA . I . G . G . V . S . G . SAN . VD
Mmi RAK4 : ANI . L . LD . K . DFT . AKI . SD . F . GLA . R . ASAR . LAQ . V . MTS . R . I . V . GTTAY . NA . E . AL . RG . EI . TP . KS . DI . YSF . GVV . I . L . E . I . T . G . LA . AV . D . EN . REP . QLL . LD
Hsi RAK4 : ANI . L . LD . EAFT . AKI . SD . F . GLA . R . ASEK . FAQ . V . MTS . R . I . V . GTTAY . NA . E . AL . RG . EI . TP . KS . DI . YSF . GVV . I . L . E . I . T . G . LA . V . D . EN . REP . QLL . LD
      Activation Loop                                Helix 5                                Helix 6

DmPelle : LLDY . I . KQ . W . R . QN . RM . ELLE . KHL . A . A . PMG . . . . . K . EL . D . M . C . M . CA . . . . . EAGLH . CTAL . DP . QD . R . P . S . MNA . L . KRF . E . P . F . . . . . VTD . . . . .
AeI RAK4 : VGDL . I . ERV . TARGA . Q . LES . M . D . R . KV . SQ . . . . . HELSE . V . LTY . . . . . GKE . L . KLS . N . YC . RE . F . K . RPS . AAA . V . REC . . . . . VQ . KMK . QGI . C
Agi RAK4 : GYVCR . RAE . . . . . GR . L . GLL . VDRRA . AV . GRDG . . . . . BAR . WCE . AGR . KLE . . . . . AI . K . . . . . O . GE . W . S . RP . NSG . AL . ADA . V . AGI . QL . V . PL . . . . .
Cpi RAK4 : QDDL . ERV . SARDA . Q . FI . GL . V . D . S . R . V . DG . . . . . ERW . EGG . RRLLE . SRR . . . . . C . RD . TK . R . PG . AKE . V . RLI . ED . . . . . VANL . . . . .
Mmi RAK4 : I . KEEI . EDEE . KTI . E . DY . T . K . M . S . D . ADP . . . . . ASV . EA . . . . . MYS . A . SQ . . . . . C . LHE . K . N . R . R . DOI . AKV . Q . LL . Q . EMISA . . . . .
Hsi RAK4 : I . KEEI . EDEE . KTI . E . DY . I . DK . KM . S . D . ADS . . . . . TSV . EA . . . . . MYS . A . SQ . . . . . C . LHE . K . N . R . R . DOI . KKV . Q . LL . Q . EMTAS . . . . .
      Helix 7                                Helix 8
  
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B.



C.



D.

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DmPelle : MSGVQTAEAEQAQNGANGNRTSRSH . DNTMAI . RL . PLP . VRAQL . CAHL . DALDVWQQLATAVKLYPDQV
AeI RAK4 : MT . . . . . PY . TRS . TEVR . KV . . . . . FEL . DELASV . ED . . . . . DMRT . LFYLI . P . R . KI . . . . . AD . VER . P . AGS . YPI . RY . NGT . H . ESE
Agi RAK4 : MH . . . . . RNM . . . . . EIR . H . MA . FH . ET . LAHI . LDTSE . G . V . R . DFLYLI . P . RNL . . . . . DDL . L . KED . P . YPL . KYT . GL . NESI
Cpi RAK4 : MT . . . . . RYQ . RTTE . IRK . V . T . P . D . LES . L . TEI . ED . . . . . DMRT . LFYLI . P . R . KI . . . . . GVD . Q . P . P . GAY . LTY . NGN . QERD
Mmi RAK4 : MN . . . . . KPL . TPSTY . I . R . N . V . GI . L . RK . S . DFI . DP . QEG . Y . K . LAVAL . K . I . P . S . . . . . GDD . . . . . RY . NQF . HI . RR
Hsi RAK4 : MN . . . . . KPI . TPSTY . I . R . N . V . GI . L . RK . S . DFI . DP . QEG . Y . K . LAVAL . K . I . P . S . . . . . GDD . . . . . RY . NQF . HI . RR
      Helix 1                                Helix 2                                Helix 3

DmPelle : EQI . SS . . . . . CKQRGRSASNEF . I . NI . W . G . . . . . GQYN . . . . . HTV . QTL . F . ALF . KKL . K . L . HN . . . . . AMRL . I . K . DYV . . . . .
AeI RAK4 : LDDYGR . I . QN . . . . . TARARI . L . LEE . W . GTS . GRKGERPTL . GHLYTL . VDKGGM . I . R . . . . . AVRH . I . EK . L . . . . .
Agi RAK4 : LETES . . . . . Q . KRN . LSPSKLL . LEE . W . I . SG . . . . . QVRPT . VDHL . K . L . V . RANQI . R . . . . . AA . EY . L . TT . L . . . . .
Cpi RAK4 : LDKHASQERRCA . LQLLEE . . . . . W . GSS . GKL . QDRPTLEHL . L . K . L . V . KGNM . FRAA . DEV . AVN . . . . . L . . . . .
Mmi RAK4 : FEALL . . . . . CTG . . . . . KSPT . CEL . L . F . DW . G . T . TN . . . . . CTV . G . DL . V . D . LL . V . QI . EL . F . . . . . APATL . . . . . LP . DAV
Hsi RAK4 : FEALL . . . . . CTG . . . . . KSPT . SEL . L . F . DW . G . T . TN . . . . . CTV . G . DL . V . D . LL . I . QNEFF . . . . . APASL . . . . . LP . D . . . . .
      Helix 4                                Helix 5                                Helix 6
  
```

Table 3-1: IRAK4, Pelle and Tube in insects. Following sequences were used for alignments and phylogenetic analysis. Table highlights presence and absence of IRAK4s as well as kinase domain for each is indicated with + for presence or – for absence for each genome examined. Gene IDs were obtained from specific genome websites dedicated for each species or NCBI Genebank.

Common name	Species	Abridged Name	Gene ID	Kinase domain	Length (aa)
IRAK4					
Yellow fever mosquito	<i>A. aegypti</i>	Aaeg	AAEL007642	+	672
African malaria mosquito	<i>A. gambiae</i>	Agam	AGAP003062	+	672
House mosquito	<i>C. pipiens</i>	Cpip	CPIJ013746	+	678
Flour Beetle	<i>T. castaneum</i>	Tcas	GLEAN_11895	+	673
Silk moth	<i>B. mori</i>	Bmor	BGIBMGA0024 94	+	598
Deer tick	<i>I. scapularis</i>	Isca	VBpre_P00033 8	+	454
Head louse	<i>P. humanus</i>	Phum	XP_002425995	+	758
Human	<i>H. sapiens</i>	Hsap	AF445802	+	460
Field mouse	<i>M. musculus</i>	Mmus	AAH51676	+	459
Frog	<i>X. tropicalis</i>	Xtro	AAI60587	+	457
Horse	<i>E. caballus</i>	Ecab	XP001488489	+	460
Aphid	<i>A. pisum</i>	Api	XP_001950616	+	438
Water flea	<i>D. pulex</i>	<i>Dpul</i>	Dpp222758	+	489
PELLE					
Fruit fly	<i>D. melanogaster</i>	Dmel	CG5974	+	501
African malaria mosquito	<i>A. gambiae</i>	Agam	AGAP002966	+	516
Yellow fever mosquito	<i>A. aegypti</i>	Aaeg	AAEL006571	+	486
House mosquito	<i>C. pipiens</i>	Cpip	CPIJ015474	+	487
Head louse	<i>P. humanus</i>	Phum	XP_002431275	+	744
Honey bee	<i>A. mellifera</i>	Amel	XP_624002.2 XP001599847.	+	413
Wasp	<i>N. vitripennis</i>	Nvit	1	+	480
Flour beetle	<i>T. castaneum</i>	Tcas	Glean_15365 BGIBMGA0000	+	443
Silk moth	<i>B. mori</i>	Bmor	63 VBpre_P00800	+	316
Deer tick	<i>I. scapularis</i>	Isca	0	+	519
Water flea	<i>D. pulex</i>	<i>Dpul</i>	Dpp64441	+	415
Aphid	<i>A. pisum</i>	<i>Api</i>	XM001942995	+	610
TUBE					
Fruit fly	<i>D. melanogaster</i>	Dmel	CG10520	-	462
Honey bee	<i>A. mellifera</i>	Amel	GB15684	-	181
Wasp	<i>N. vitripennis</i>	Nvit	XP_001601124	-	380

Table 3-1: IRAK4, Pelle and Tube in insects

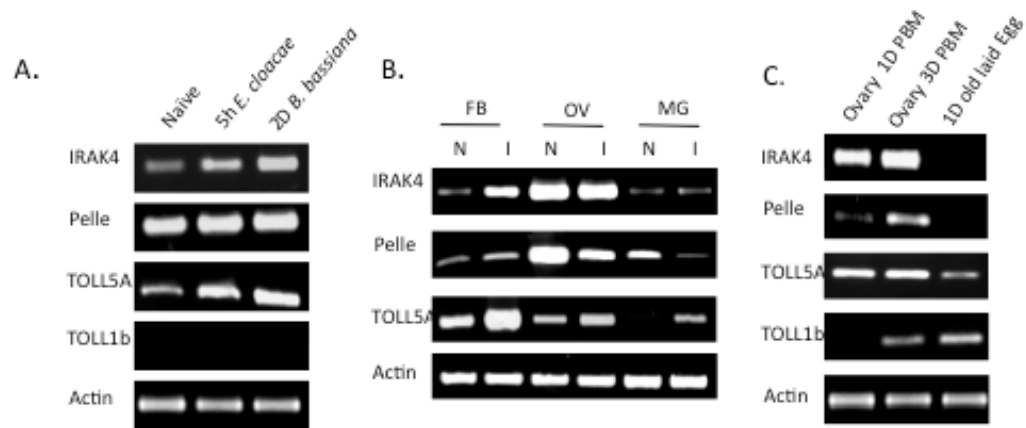


Fig. 3.2. Molecular characterization of *Aedes* IRAK4. Pathogen and tissue specific gene expression profile of IRAK4 and Pelle (A,B). Pathogen specific profile and tissue specific profile shown using RT-PCR. A, The induction profiles by septic injuries with different types of pathogens Gram (-) bacteria, and fungi. B, Tissue specific expression profile of IRAK4, and Pelle in *Aedes*. IRAK4 and Pelle expression profile 48 hours after septic injury with *B. bassiana* spores. IRAK4 expression is Fatbody specific. C, The expression profiles of IRAK4 and Pelle during ovary and egg (embryo) development. Pelle expression is ovary specific. The expression profiles of ovaries and laid eggs shown by RT-PCR analysis. Tissues were collected for RNA extraction and RT-PCR was performed using gene specific primers. FB, fatbody, OV, ovaries, MG, midgut, N. Naïve, and I, infected with *B. bassiana*.

Fig. 3.3. Functional characterization of *Aedes* IRAK4. *A*, Ecotopic expression of *Aedes* IRAK4 and Pelle in *Drosophila* S2 cells. AaIRAK4 activates Drosomycin gene expression in *Drosophila* S2 cells. *Drosophila* S2 cells were transfected to express AeIRAK4, AePelle, AaRel1, AeToll5A, and AeIMD (control). Cells were harvested 48 hours post transfection or 5 hours after heat inactivated *E. cloacae* infection for RNA extraction. Ribosomal protein (rp49) was used as the RNA loading control. (-), uninfected cells, (+) cells infected. *B*, IRAK4 is regulated by REL1. Expression of IRAK4, Pelle, TOLL5A was assessed in REL1-gain of function transgenic mosquitoes. *C*, Role of IRAK4 in *Aedes* Toll immune pathway. Northern blot analysis revealed that RNAi mediated knockdown of *Aedes* IRAK4 compromised the immune activation of CLIPB29 and Spn2 in the mosquito, *Aedes aegypti*. *D*, IRAK4 does not affect Defensin D expression which was used to measure IMD pathway output. For RNAi experiments, 3µg-5µg of dsRNA was injected in to the thorax of the adult *Aedes aegypti* mosquito. Septic injuries were performed by pricking mosquitoes in the rear part of the abdomen with *Beauveria bassiana* strain GHA.

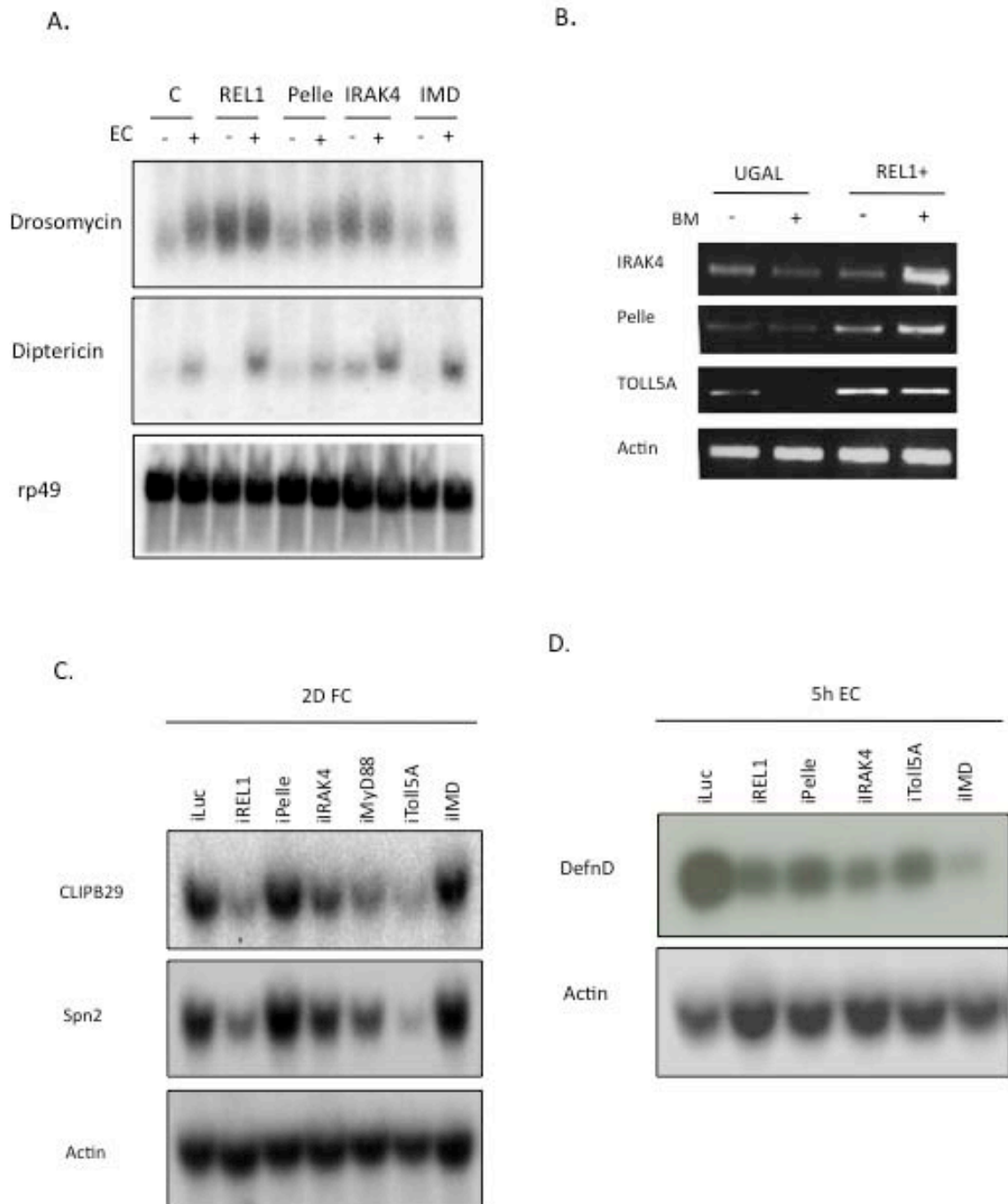


Fig. 3.3. Functional characterization of *Aedes* IRAK4.

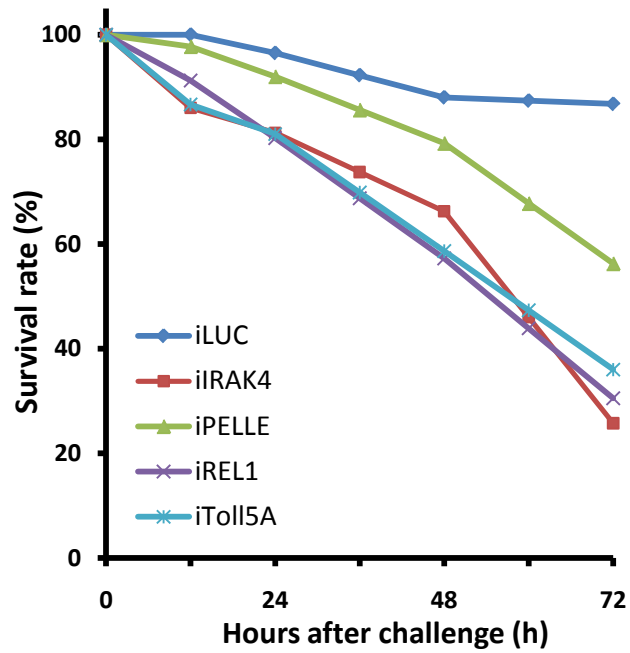


Fig. 3.4. Susceptibility of IRAK4 dsRNA treated mosquitoes to fungal infection. The iIRAK4 mosquitoes were significantly more sensitive to fungal infection than luciferase dsRNA treated mosquitoes. Mosquitoes treated with iLUC., iIRAK4, iPELLE, iREL and iTOLL5A indicate Luciferase, IRAK4, Pelle, REL1 and TOLL5A dsRNA treated mosquitoes, respectively. 3 μ g-5 μ g of dsRNA was injected in to the thorax of the adult *A. aegypti* mosquito. Septic injuries were performed by pricking mosquitoes in the rear part of the abdomen with an acupuncture needle (0.20 x 25 mm) dipped into a fungal spore suspension of *Beauveria bassiana* strain GHA. Number of dead mosquitoes were calculated after 12, 24, 48 and 72 hours post infection. Results represent three independent experiments.

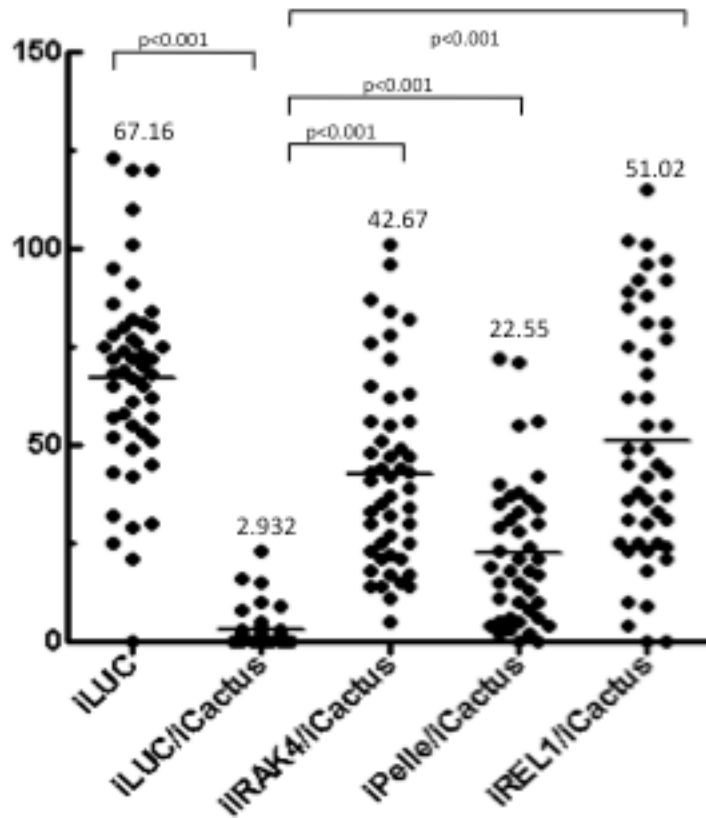


Fig. 3.5. Effect of AeIRAK4 depletion on *P. gallinaceum* expression. Cactus depletion restricts parasite development which is compromised in Cactus/IRAK4 (iCactus/iIRAK4) depleted mosquitoes. Mosquito midguts from each treated sample were dissected and the number of mature oocysts was calculated 7 days post infectious blood meal. Data from oocysts were collected from three independent experiments with dsRNA-treated mosquitoes and pooled. The dots represent the infection level per midgut and the mean numbers from three independent experiments is shown on top of each column. P-values were calculated using the Mann-Whitney Test.

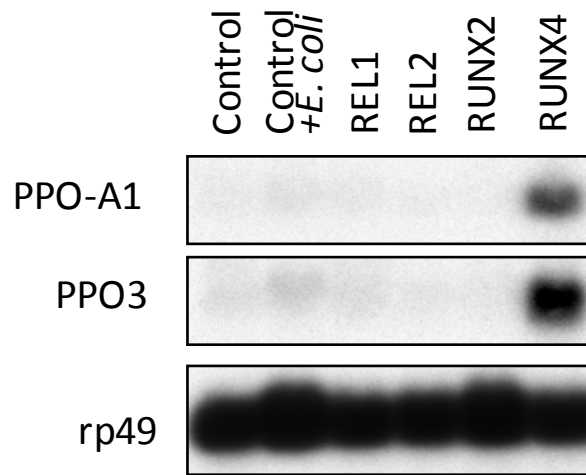


Fig. 3.6. Direct activation of four immune-inducible PPO genes by RUNX4. *B.* Drosophila PPO-A1 (bc) and PPO3 genes were activated by Aedes RUNX4 in Drosophila S2 cells. The activation of Drosophila PPO-A3 was not detected using this Northern analysis.

	AelRAK4	AgIRAK4	CpIRAK4	DmPelle	BmIRAK4	TcIRAK4	HsIRAK4
AelRAK4	100	47	55	23	29	32	29
AgIRAK4	65	100	45	45	26	39	29
CpIRAK4	55	58	100	24	30	34	29
DmPelle	28	59	37	100	28	28	28
BmIRAK4	46	46	49	43	100	29	30
TcIRAK4	51	52	49	44	51	100	35
HsIRAK4	47	46	46	40	50	50	100

Table 3-2 Amino acid identify and similarity of IRAKs in mosquito, fly and human. Percent similarities and identifies are shown in bold and unbolded numbers, respectively. Values were calculated by aligning the sequences using GenDoc.

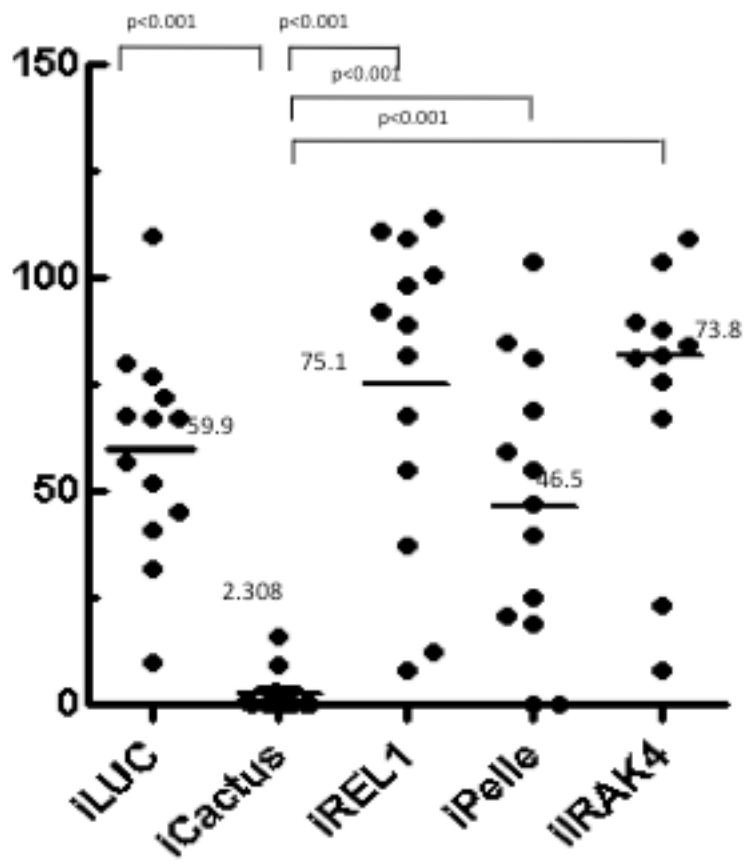


Fig. 3.7. The effect of Plasmodium development after single knockdown of IRAK4 and Pelle. Cactus depletion restricts parasite development which is compromised in IRAK4 depleted mosquitoes. Mosquitoes midguts from each treated sample were dissected and the number of mature oocysts were calculated 7 days post infection. The dots represent the infection level per midgut, and the mean numbers from three independent experiments is shown on top of each column. P-values were calculated using the Mann-Whitney Test.

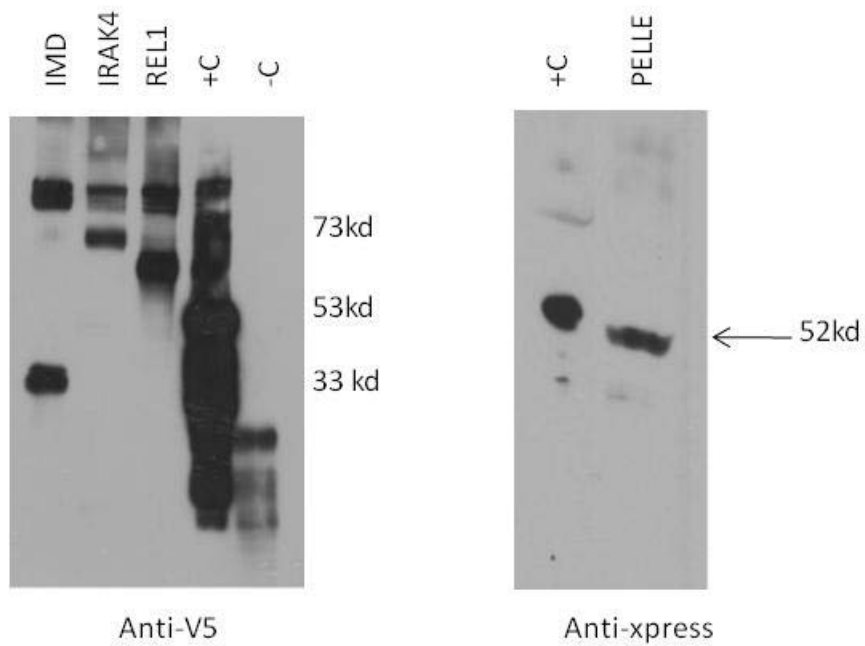


Fig. 3.8. Transient expression of IRAK4, REL1, and IMD in Drosophila S2 cells. Drosophila S2 cells were transfected to express AeIRAK4, AePelle, AeREL1 and AeIMD. Cells were harvested 5 hours post infection for protein extraction. SDS-PAGE electrophoresis was performed followed by Western blot. A, Anti-V5 antibody was used to detect AeIRAK4 (73kd) and IMD (33kd). Anti-xpress was used to detect AePelle (52kd). Positope (Invitrogen) was used as positive control for anti-V5 and anti-xpress antibodies

CHAPTER 4: INVESTIGATION OF THE IMD PATHWAY IN *Aedes aegypti*

ABSTRACT

The Imd pathway responds to gram-negative bacterial infection and is also implicated in antiparasitic response. The cytoplasmic signaling components of the *Drosophila* IMD pathway have been well characterized and well studied. The role of the pattern recognition receptor, a signaling cascade which consists of an adaptor protein, Imd, and a MAP3K, TAK1 have been investigated. However, these components remain unexplored in the mosquito, *Aedes aegypti*. Here, we report the characterization of the *Aedes* IMD pathway; specifically the identification of the PRR, PGRP-LC, and the adaptor gene, IMD. We demonstrate that transient expression of IMD and PGRP-LC induces Diptercin gene expression in S2 cells while the RNAi mediated knockdown of IMD and PGRP-LC impairs Defensin A expression as well as causes severe susceptibility to gram-negative bacterial infection in the mosquito, *Aedes aegypti*. We also tested the role of FADD, Dredd, and components of the IKK complex. The results of the characterization of the Rel-2 gain of function transgenic mosquitoes' response to *Plasmodium* infection (previously reported in (Antonova et al., 2009) revealed that these transgenics have low or no infection. These findings contribute to the current knowledge of IMD pathway and the role of NF- κ B factor, REL2 in mosquito immunity.

4.1 Introduction

Mosquitoes continue to impact the public health system by spreading numerous human diseases. Malaria is particularly devastating, taking a heavy toll on the human population by affecting over 300 million people and killing at least 2 million (WHO, 2002). Vector borne diseases are reaching disastrous levels especially those caused by viruses and transmitted by mosquitoes. Dengue fever continues to threaten roughly 2.5 billion people around the world particularly Africa and South Asia while West Nile Virus persists to spread in the U.S.

Several reasons including lack of effective vaccines against Malaria and other vector borne diseases as well as the development of insecticide resistance by vectors and pathogens respectively are the cause of this tragic situation. Therefore, there is an urgent need to explore novel options in order to tackle these mosquito-borne menacing diseases. Several lines of evidence suggest that the interruption of the *Plasmodium* life cycle at various points may lead to refractoriness

Pattern recognition receptors (PRRs) recognize pathogens and signal the initiation of immune signal transduction pathways. The Imd pathway is activated with the recognition of the meso-diaminopimelic acid (DAP) type peptidoglycan by the pattern recognition receptors, PGRP-LC and -LE (Kaneko et al., 2006). Downstream of the PGRP-LC, signal transduction involves TAK1 in order to activate the IKK complex. The mechanism of this activation is unknown; however, recent data from RNAi studies

showed that ubiquitination is possible. A likely candidate is inhibitor of apoptosis protein 2 (dIAP2) which was recently identified as an essential component of the IMD pathway (Kleino et al., 2005; Leulier et al., 2006; Valanne et al., 2007). Dredd, a caspase, thought to cleave Relish is involved in signaling between IMD and TAK1 (Stoven et al., 2003). Activation of TAK1 culminates in the activation of NF- κ B/Relish activation or JNK (Silverman et al., 2003).

This progress can be attributed to the development and use of genetically manipulated *Drosophila*. The recent use of reverse transgenic techniques such as RNAi and mosquito transgenesis, has led to major advances in mosquito research. RNAi allows the study of gene of interests in *Plasmodium* research by temporarily silencing transcript levels in live mosquitoes. However, RNAi has certain limitations such that it does not allow the study of the affects of overexpression of a particular gene. Additionally, RNAi varies between individual organisms such that the gene expression in one mosquito may vary greatly from that of another mosquito. Use of transgenesis overcomes these limitations by allowing the stable, heritable genetic transformation, allowing for both overexpression and dominant negative phenotypes.

Here, we report the characterization of the IMD pathway; specifically the identification of the PRR, PGRP-LC, and the adaptor gene, IMD. We demonstrate that transient expression of IMD and PGRP-LC induces Diptercin gene expression in S2 cells while the RNAi mediated knockdown of IMD and PGRP-LC impairs Defensin A expression as well as causes severe susceptibility to gram-negative bacterial infection in the mosquito, *Aedes aegypti*. We also tested the role of FADD, Dredd, and components

of the IKK complex. The results of the characterization of the Rel-2 gain of function transgenic mosquitoes' response to *Plasmodium* infection (previously reported in (Antonova et al., 2009) revealed that these transgenics have low or no infection. These findings contribute to the current knowledge of IMD pathway and the role of NF- κ B factor, REL2 in mosquito immunity.

4.2 Materials and Methods

Experimental animals — The wild type *Aedes aegypti* mosquito strain, UGAL/Rockefeller, was maintained in the laboratory culture with 27 °C, 80 % humidity. Adult mosquitoes were fed on water and 10% sucrose (HaysAR and Raikhel AS., 1990). All dissections were performed in *Aedes* physiological solution (APS).

Computational analysis — *Aedes* PGRP-LC (1.1200) was identified using *Drosophila* PGRP-LC sequence using a Blast search using Preassembled *Aedes* genome. A phylogenetic tree was constructed using ClustalX.

Reverse transcription and PCR — Two micrograms of total RNA were used as a template in a cDNA synthesis reaction using the Omniscript Reverse Transcriptase Kit (Qiagen). First, RNA was treated with DNase (DNase Amplification grade, Invitrogen) and then cDNAs were synthesized using the above mentioned process using Oligodt primers. The resultant cDNA were then used for gene specific amplification by PCR using Platinum High Fidelity Supermix (Invitrogen).

Gene expression knockdown — Gene silencing was accomplished by synthesizing double-stranded RNA (dsRNA) with T7 RNA polymerase. T7-phage promoter sequence was incorporated into both sense and antisense sequences of target genes to generate template cDNAs containing T7 tag. RT-PCR was performed using the Titan one-step RT-PCR kit (Roche) with samples of 0.2 µg total RNA as templates to generate a 400-600 bp gene-specific cDNA fragment. Amplification conditions involving synthesis of dsRNA was accomplished by simultaneous transcription of both strands of template DNA using the MEGAscript kit (Ambion). The luciferase gene was used to generate control iLuc dsRNA. After dsRNA synthesis, the samples were treated with phenol/chloroform extraction and then ethanol precipitation. The dsRNA was then suspended in RNASE Free water with a final concentration of 5µg/µl. The concentration, size, and formation of dsRNA were confirmed by running 0.2µl of the reaction on a 1% agarose gel in TBE. A Picospritzer II (General Valve, Fairfield, NJ) was used to introduce corresponding dsRNA into the thorax of CO₂-anesthetized mosquito females, at one or two days post eclosion. 3µg-5µg of dsRNA was injected in to the thorax of a single adult *Aedes aegypti* mosquito.

Septic injury and Survival rate — Septic injuries were performed by pricking female adult mosquitoes in the rear part of the abdomen with an acupuncture needle (0.20 x 25 mm) dipped into either *Enterobacter cloacae* bacterial culture (stationary phase of bacteria in LB broth; OD₆₀₀ ≈ 2.0) or a fungal spore suspension (~5 x 10⁷ viable spores/ml) of *Beauveria bassiana* strain GHA. Each experiment was performed at least three times.

RNA isolation —Total RNA was isolated from whole bodies or specified organs of the mosquito. Tissues of interest were dissected in *Aedes* Physiological saline (Hayes, 1953). Tissues were homogenized using a disposable plastic pestle in TRIzol® (Invitrogen, Carlsbad CA) reagent according to manufacturer's protocol and purified using phenol/chloroform extraction. RNA samples were quantified using the Nanodrop spectrophotometer.

Northern analysis — RNA was extracted from 8 whole body mosquitoes by using the Trizol method (Invitrogen) according to manufacturer's protocol. 5 µg of total RNA from each sample was separated on a formaldehyde gel, blotted and hybridized with the corresponding ³²P-labeled DNA probe. Probes were generated using PCR and then following the High Prime (Roche) protocol. Actin was used as a loading control.

Quantitative Real-Time PCR Analysis -- Total RNA of the mosquito fat bodies was prepared by using the TRIzol technique (Invitrogen). Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Invitrogen). Reverse transcription was carried out by using an Omniscript reverse transcriptase kit (Qiagen) in a 20-µl reaction mixture, containing Oligo-dT primer and 2 µg of total RNA at 37°C for 1 h. Two microliters of cDNA from this reaction was subjected to real-time PCR, which was performed by using the iCycler iQ system (Bio-Rad). Reactions were performed in 96-well plates with a SYBR PCR kit (Qiagen), and each sample was analyzed in triplicate

and normalized to the internal control, actin mRNA.

Transfection assay in Drosophila S2 cells — Full length cDNA sequences of IMD and PRGRP-LC were amplified from immune activated mosquitoes. Primers were designed for full length transcripts including unique restriction sites and cloned into pac5.1/HisA/V5 plasmid. Coding region sequences of *Ae. aegypti* IMD, and PGRPLC were amplified by means of PCR, inserted into pAC5.1/V5/HisA vector (Invitrogen), and used to transfect *Drosophila* S2 cells. 2×10^6 cells per ml were distributed on 35-mm plates in *Drosophila* Schneider's medium (GIBCO) supplemented with 5% fetal bovine serum (GIBCO) and 1X Antibiotic-Antimycotic (GIBCO). A 1:4 DNA to Lipid ratio was used for all transfections. The cells were transfected with 1 μ g of each plasmid construct and incubated for 6h in serum-free Schneider's medium (SFM), which was then removed and replaced with complete medium. The cells were then incubated for 48 h with 20-hydroxyecdysone, followed by bacterial challenge using heat-inactivated *E. cloacae* and then incubated for an additional 5h. Total RNA was isolated from harvested cells using the Trizol method (Invitrogen), and samples were then subjected to Northern analysis. Blots were hybridized to *Drosophila* Drosomycin and Dipteracin probes. The Drosomycin blot was stripped and reprobed with Actin for loading control.

Plasmodium infection — *Plasmodium gallinaceum* cycle was maintained by transmission between mosquitoes and White Leghorn chickens. 4-7 day old transgenic or wildtype mosquitoes were fed infected blood from chickens 9 day post infection. Mosquito

midguts were dissected 7 days post infection, stained with 1% mercurochrome (Sigma) and oocysts number was counted manually. To calculate the effect of REL2 overexpression on sporozoite number, mosquitoes were infected with *P. gallinaceum* as described above. Salivary glands were dissected 14 days post infection and the sporozoite number was calculated using phase contrast optics.

4.3 Results/Discussion

4.3.1 PGRP-LC and IMD are major mediators of the Immune deficiency (IMD) pathway in the mosquito, *Aedes aegypti*.

The innate immune response is stimulated by the recognition of microbes through receptors that are heritable through the germline. These receptors (PRRs) use pattern associated molecular patterns (PAMPs) to recognize invading organisms. Recently, Choe et. al (2005) showed that the Imd adaptor protein binds to the cytoplasmic domain of PGRP-LC and this domain is required for the activation of the signal transducing events that activate Relish. This suggests that PGRP-LC acts as a signal transducing receptor (2005).

To investigate the possible role of PGRP-LC and other components of the IMD pathway in the defense against gram-negative challenge in adult *Aedes* mosquitoes, computational analysis, RNA-interference and *in vitro* transient expression analysis was employed in *Drosophila* S2 cells and adult mosquitoes. Using *Drosophila* PGRPLC as a query sequence, several sequences on assembled sequences were identified. This led to the identification of Candidate 1.1200A as the putative PGRP-LC since knockdown results of all the candidate genes chosen were similar and alignment showed considerable overlap (data not shown). Phylogenetic analysis of other mosquito PGRPs revealed that *Aedes* 1.1200 groups with three isoforms of *Anopheles* as well as *Drosophila*. Based on sequence and phylogenetic analysis (Fig. 4.1), candidate, 1.1200 will be referred to as PGRPLC henceforth.

To understand the role of *Aedes* Pattern recognition receptor, *PGRP-LC*, and IMD in the IMD pathway, we tested the expression of immune genes 5 hours post Gram-negative bacteria infection in mosquitoes that were previously depleted with *PGRP-LC*, *IMD*, and *REL2* genes. Double strand RNA against *PGRP-LC* and *IMD* were generated and introduced into mosquito thorax and after 5-7 days post injections, the output gene expression level was analyzed. The depletion of *PGRP-LC* and *IMD* by RNAi resulted in the reduction of Defensin expression which was used to measure the output of the IMD pathway (Fig. 4. 2B). This reduction mimicked that of *REL2*, previously known component of the IMD pathway. The double knockdown of *PGRP-LC* and *REL2* suggested that these two receptors might not work together in mosquitoes as apparent in *Drosophila* (data not shown).

To address the role of *Aedes* *PGRP-LC* and *IMD* in mosquito immune response, the susceptibility of *PGRP-LC* and *IMD* depleted mosquitoes was compared after bacterial challenge. After a 4-5 day recovery following dsRNA injections, the mosquitoes were challenged with Gram-negative bacteria, *Enterobacter cloacae*. Results indicate that the dsRNA mediated knockdown of *PGRP-LC* was more susceptible to Gram-negative bacteria, *E. cloacae*, challenge as compared with control luciferase mosquitoes. The susceptibility of *PGRP-LC* dsRNA mosquitoes was comparable to knockdown of other key components of the IMD pathway such as *IMD* and *REL2*, suggesting that *IMD* and *REL2* act in concert with *PGRP-LC* against Gram-negative bacteria invasion.

To investigate the *in vivo* role of PGRP-LC, mosquito REL2, PGRP-LC, and IMD (adaptor molecule of IMD pathway) was isolated and cloned into pAC5.1 expression vector. Only IMD was able to induce Diptericin expression level in *Drosophila* S2 cells. The dsRNA mediated knockdown of IMD caused mosquitoes to be more susceptible to Gram-negative bacteria, *E. cloacae*, challenge. The above data suggests that PGRP-LC, IMD and REL2 are all components in the IMD pathway. This is in agreement with recent results in which overexpression of IMD showed that IMD protein is sufficient for AMP production (Choe et al 2005).

4.3.2 Characterization of the cytoplasmic signaling in the IMD pathway.

In order to identify and investigate the cytoplasmic signaling components of the IMD pathway, we decided to start by examining the genes that have been identified as *Drosophila* homologs. The goal of these experiments was to identify the components of the IKK complex in *Aedes* and whether this complex is similar or unique to the complex identified in *Drosophila* or in mammals (Silverman et al 2000). In order to elucidate the exact components of the IKK complex, it was necessary to identify putative IKK β , IKK ϵ and or IKK δ in *Aedes* using *Drosophila* genome sequences of these genes. The mammalian signalosome consists of IKK α , IKK β and IKK γ as the structural scaffold that holds IKK α , IKK β kinases. The importance of the *Drosophila* IKK β kinase complex, which include IKK β and IKK γ have been implicated in Relish cleavage and antibacterial immunity. Similarly, we suspect that IKK β phosphorylates Rel2 and is involved in subsequent activation.

To test the involvement of putative cytoplasmic component: IKK, IKK β , FADD and TAK1, mosquitoes were infected with *E. cloacae* after treatment with *dsRNA against* IKK β , IKK, FADD and TAK1. RNA was extracted and Northern analysis was done 5 hours after infection to assess the induction of AMPs. Results showed that RNAi mediated knockdown of IKK β but not IKK γ and REL2 compromised Defensin and Cecropin expression. RNAi expression profiles also revealed that TAK1 but not FADD or Dredd also resulted in reduced expression of Defensin and Cecropin. This can be explained by the branching of the IMD pathway.

In *Drosophila*, one branch of the pathway leads to the phosphorylation of Relish via Tak1-Key-Ird5 whereas the second arm directly activates Relish through activation of Caspase Dredd and FADD. From this data, we suspect that this branch of the pathway is not homologous to *Drosophila*. Recent study showed that IKK complex specifically IKK β controls Relish by direct phosphorylation and this is not required for Relish cleavage, nuclear translocation or DNA binding (Erturk-Hasdemir et al., 2009). These finding support our results that IKK β and not IKK-gamma is involved in the *Aedes* pathway. Furthermore, the involvement and mechanism of IKK β further supports the branching of the IMD pathway where cleavage of Relish is regulated by a separate pathway and is not necessary for Relish activation. This phenomenon further supports the hypothesis that *Aedes* REL2 does not under cleavage, eliminating the need for a caspase.

4.3.3 The investigation of Caspase 8, 18, 20 and IAP2 in *Aedes* IMD pathway.

Several mutations that affect antibacterial immune response were discovered in *Drosophila* simultaneously in the 1990s. These included *imd* (immune deficiency), IKK β , *ird5*, and *Dredd*, which are structurally related to vertebrate caspase 8 (Georgel et al 2001). We decided to investigate the involvement of these genes in the *Aedes* IMD pathway.

Nonapoptotic genes such as a *Drosophila* caspase (DmDredd) and an IAP (DmIAP2) are required for Imd signaling (Kleino et al., 2005; Leulier et al., 2006; Leulier et al., 2000). We tested the involvement of caspase 8, 18, and 20 in *Aedes* in order to identify which Dredd like effector caspase was involved in the *Aedes* Imd pathway. Caspase 18 and 20 belong to a unique mosquito cluster, which also includes caspase 19; however, closer examination revealed that Caspase 19 is the result of a duplication (phylogenetic tree). Caspase 8 was chosen as a likely candidate since mosquito CASPS7 and CASPS8 form orthologous pairs, with CASPS8 being most closely related to DmIce and DmDcp1. RNAi mediated knockdown of all three caspases did not have an affect on Defensin expression (Fig. 4.3B). This indicates that perhaps these caspases are not implicated in the IMD pathway or perhaps that there is functional duplication; further supporting that perhaps a posttranslational modification event and not a cleavage event activates REL2; nonetheless future investigations should confirm experimental conditions in order to conclude if caspases play an active role in the activation of REL2.

IAPs (inhibitor of apoptosis proteins) were shown to be key components of the *Drosophila* IMD pathway (Kleino *et al.*, 2005) and several evidences suggest a crosstalk between immune and apoptotic pathways. We decided to investigate this branch of the pathway using IAP2 as the starting point.

In order to further investigate the function role of IAP2, mosquitoes were injected with dsRNA against IAP2, allowed to recover from RNAi injections for 4-7 days and were infected with *E. cloacae*. Total RNA was isolated 5h post infection and northern analysis was done. Defensin was used as a marker gene to study the role of IAP2. Results reveal that IAP2 is constitutively expressed in naïve and infected state. However, RNAi results revealed that the dsRNA mediated knockdown of IAP2 did not affect Defensin expression; however, this does not rule out the possible role of IAP2 or a similar gene in mosquito immunity (Fig. 4.3A). IAP2 maybe serving a redundant role with an unidentified gene; therefore, a single knockdown resulted in an inconclusive phenotype.

4.3.4 *REL2* gain of function exhibit an increased resistance *Plasmodium gallinacium* infection.

Because mosquitoes are obligate vectors of malaria transmission, the spread of the disease can be curtailed by rendering them incapable of transmission by gene manipulation. The ability to genetically transform pest insect species such as the human disease vector, *Aedes aegypti*, has sparked research in vector control capacity by creating genetically altered mosquitoes overexpressing genes of interest suspected to be involved in antiplasmodium mosquito immunity. Current genetic engineering of mosquitoes and other insects involves consists of three components: a gene vector commonly a

transposable element, the gene of interest, and regulatory elements which include a promoter able to control the expression of a gene in a tissue and temporal specific manner.

One proposed method to curtail the spread of malaria parasite is by rendering mosquitoes incapable of transmitting the disease through genetic transformation. Expression of transgenes in a tissue specific manner is one method. Previous reports show that the enhancement of immune response by boosting NF-kappaB-dependent basal immunity aborts the development of *Plasmodium berghei* (Frolet et al., 2006). Furthermore, the expression of a synthetic peptide, SM1, resulted in decreased number of *P. berghei* oocysts in *Anopheles* (Ito et al., 2002). Several studies have shown that the inhibition of functional activity of Rel2, and deletion of Rel1A results in the susceptibility to gram-negative bacteria and fungi, respectively (Shin et al., 2005). Additionally, silencing of the Rel2 gene nearly doubles the number of parasites in the midgut as compared to control mosquitoes (Meister et al., 2005). Little is known about the regulators of mosquito immune pathways. Previously, the gene homologous to *Drosophila* Relish was isolated and characterized from the mosquito *A. aegypti* (Shin et al., 2003). However, little else is known about the PRRs or the cytoplasmic signaling of the IMD pathway in the mosquito.

We used this technology to study the overexpression of REL2 on parasite infection. *Plasmodium gallinaceum* and *Aedes aegypti* are a natural vector and parasite experimental model system. Previous studies report that activation of basic or bacterial induced immunity in *Anopheles gambiae* and *Anopheles stephensi* leads to an increase

resistant to the rodent parasite *Plasmodium berghei* (Frolet et al., 2006). The use of transgenic approach to generate mosquitoes expressing antiplasmodium factors in the midgut has also been utilized (Ito et al., 2002).

To study the role of REL2 in mosquito immunity, we have created two transgenic mosquito strains in which the full length of the REL2-Short isoform which includes the His/Gln (Q/H)-rich and SRR putative transactivation domain(s) as well as the REL homology (RHD and IPT) domain incorporated into the mosquito genome under the control of the Vg promoter. A transformation vector pBac[3xP3-EGFP, afm] containing the Vg-REL2-Short isoform and SV40 polyadenylation region was used for mosquito germ-line transformation (see Antonova et al 2008 published data). Transformation vector was constructed by YJ Kim transgenic strain was created by V Kokoza and characterization was done by Y Antonova and KS Alvarez (Antonova et al 2009). The two transgenic mosquito strains, named REL2-A and REL2-B, were established as a result of the germ-line transformation and distinguished by means of green fluorescent glowing in the eyes at larval, pupal, and adult stages.

The incorporation of the transgene was confirmed by Southern blot and the induction of the transgene expression was confirmed by northern blot, real time pcr as well western blot analysis. Southern, Northern and Western analysis as well as all survival rate studies were conducted by Dr. Yevgeniya Antonova (Antonova et al 2008). Real time PCR analysis corresponds to the northern analysis where the expression of the REL2 transgene peaks between 12 to 24 hours after blood meal (Fig. 4.5A). REL2 transgene expression in REL2-gain of function transgenic mosquitoes peaked at 12 hours

post blood meal (hPBM) and diminished by 36 hPBM). Defensin expression followed the peak of REL2 transgene expression, maximizing 24 hPBM. Minimal expression of both REL2 transgene expression and Defensin was seen wildtype and previtolegenic mosquitoes. The survivorship of REL2 transgenics against Gram-negative and Gram-positive bacteria was tested by Yevgeniya Antonova. Results indicated that transgenic REL2-A mosquitoes were more resistant to *P. aeruginosa*, *E. cloacae* and *S. aureus* compared to wildtype mosquitoes (Antonova et al 2009).

To examine the effect of overexpression of REL2 expression on the development of the malaria parasite, *P. gallinaceum* in the mosquito *Aedes aegypti*, we assessed the number of oocysts, a stage that manifests in the midgut and the number of sporozoite present in the salivary gland. In order to assess the oocysts number, we infected REL2-A strain mosquitoes on White leghorn chickens infected with *Plasmodium*. Then, 7 days post infection, the midguts of each infected female mosquito were dissected and the number of the oocysts was counted. The results showed a significant reduction of oocyst number in REL2A mosquitoes (Fig. 4.6A). Specifically, most REL2-A mosquitoes had between 0-10 oocysts relative to the wildtype mosquitoes, which had more than 40 oocysts per midgut (Student t-test, $p < 0.05$) (Fig. 4.6B).

The results of the second strain, REL2B, were obtained and analyzed by Antonova et al (2009). Though the parasite level (oocysts and sporozoites) were similar, REL2-B was less resistant to *Plasmodium*. REL2-B had significantly fewer oocysts than wild type mosquitoes (Wilcoxon test, p values < 0.05), and exhibited a 50% reduction in

the mean oocyst number. Thus, overall data showed that overexpression of REL2 hinders development of *P. gallinaceum*.

The transgenics also exhibited significant decrease in sporozoites number in the salivary gland when the *Plasmodium* infected mosquitoes were given a second naïve blood meal at 7th days post infection to re-activate transgenic REL2 expression. The second bloodmeal mimics a mosquitoes' natural feeding pattern in the wild. REL2A transgenic strain displayed a significant decrease in sporozoite infection (Fig. 4.6C). Specifically, three independent experiments revealed a 60-67% reduction in sporozoite number (Fig. 4.6D). These data show that REL2A and REL2B transgenics exhibited increased immune-capacity.

Our experiment described above revealed that REL2A and REL2B transgenics exhibited increased immune-capacity that is likely due to presence of Defensins in the hemolymph after blood feeding as indicated by northern and western results (Fig. 4.5A and Antonova et al., 2009). Furthermore, it has been demonstrated that dragon fly's (*A. cyanea*) and flesh fly's (*P. terranova*) Defensins are toxic to *P. gallinaceum* late oocyst stages and sporozoites). Observed anti-Plasmodium effect of REL2 overexpression was probably due to the affect of Defensin on the late oocyst stages to sporozoite stage.

4.4 Concluding Remarks

In this study, we showed that PGRP-LC and IMD are imperative components the IMD pathway. RNAi and survival studies revealed that knockdown of PGRP-LC and IMD ablated Defensin expression and diminished mosquito's survival to Gram-negative

bacteria. It is not yet clear the exact mechanisms involved in PGRP-LC binding to peptidoglycan in the extracellular domain that activate intracellular signaling because its cytoplasmic domain has no known homology to characterized proteins.

In *Drosophila* as well as other insects, Imd serves as a scaffold and as a branching point in the IMD pathway. This knowledge can be used to map and characterize this pathway in mosquitoes. Additionally, the dissection of the cytoplasmic signaling exposed the involvement of IKK β . However, we could not conclude the involvement of FADD, DREDD, Caspase 8, 18, and 20 and IAP2. Currently, we do not know the function or the involvement of a caspase in the activation of REL2 in *Aedes*. Caspase has been implicated inhibiting the Imd pathway in *Drosophila* by inhibiting Dredd. A search for the Caspar target in mosquitoes could lead to the identification of a putative Caspase.

Previously, we have shown the involvement of the Relish homology, Rel2 (Shin et al., 2002; Shin et al., 2003) in the IMD pathway and in antibacterial study. In this study, we show the involvement of Rel2 in anti-*Plasmodium* response using REL2-gain of function transgenic mosquitoes (Antonova et al., 2009). Use of transgenic organisms is a powerful tool to study gene function though this can't be done until gene targets are identified though the study and characterization of signaling pathways. This study shows promising results; however, the fitness of these mosquitoes remains to be assessed. The success of the transgenic depends on population replacement which directly correlates with fitness cost. Fitness loads have attributed to insertion mutagenesis and expression of antimicrobial peptides (Marrelli et al., 2006) so therefore, the Rel2 gain of function transgenic is unlikely to exhibit fitness reduction due to gene expression. Additionally,

fitness cost occurs mostly in transgenic organism carrying ubiquitous promoters and less likely a factor when gene expression is specific in certain tissues. This is another advantage of the REL2 transgenic; however, these issues need to be addressed in further studies. Furthermore, the resistance to insecticide should be assessed for each transgenic since the environment that they will be released is often sprayed with various insecticides and pesticides. The testing of REL2 and other transgenic in laboratory is only the primary step while fitness costs and chemical resilience are also necessary.

The completion of the mosquito genome sequence along with RNAi, microarrays has allowed comparative and functional genomic approaches to study the mosquito innate immune system. Particularly, the usage of transgenic mosquitoes to study and dissect mosquito immune pathways has improved our understanding in the immune mechanisms. This study suggests that the mosquito IMD immune pathway deviates slightly from the *Drosophila* Imd pathway.

Appendix 4A: List of Primers

T7- IKKgammaF	TAATACGACTCACTATAGGGCCAGTGTCATCATGGGCGAAA
T7- IKKgammaR	TAATACGACTCACTATAGGG CTCGCAGGCAGTTCAGCTCATC
T7- IKKBeta F	TAATACGACTCACTATAGGGGAGACGTTCTGCAGGAATTG
T7-IKKBeta R	TAATACGACTCACTATAGGGCTCGCTGTTTTGGATTCCATTC
T7-TAK1 F	TAATACGACTCACTATAGGGTATGGAGCTTGCACAAGACGT
T7-TAK1 R	TAATACGACTCACTATAGGGTGGTCCACAACATCTTGCAT
T7-FADD F	TAATACGACTCACTATAGGG GAATGAAATGCATTCCGTTAGA
T7-FADD R	TAATACGACTCACTATAGGG CCCACAATAGCTTTAACGTTCTAG
PGRP-LC (1.1200) F	TAATACGACTCACTATGGGAAGATGACCACAACGACTCAAG
PGRP- LC(1.1200)R	TAATACGACTCACTATAGGGCATCTGGGAGAAAGTCTTTATC
7-Casp18 F	TAATACGACTCACTATAGGGGACCTACTTGCGATGAGTATTATG
T7-Casp18 R	TAATACGACTCACTATAGGG CTCCCACAACGAGTCCACGTAG
T7-Casp8 F	TAATACGACTCACTATAGGGGCTTACACAATTGAACGCTATTC
T7-Casp8 R	TAATACGACTCACTATAGGG GTTCGTTGCACAACCTCTCGGATG
T7 Casp 20 F	TAATACGACTCACTATAGGG GTCACCTTCGAAGATAACAAGC
T7 Casp 20 R	TAATACGACTCACTATAGGGGATGGAGGTTGTTGCCAACTC
CaspL1 F:	TAATACGACTCACTATAGG CAACCTGTGTCTCCTGAAGTG
CaspL1 Reverse:	TAATACGACTCACTATAGG CCGAGGTTGGTGAACAGATTC
FADD FL F	CAA GAA TTC GCATCGAGCTTACTGCAGAATCGAC
FADD FL R	CAA CTC GAG GTT TCA TTTCATAAGTTTTTCG
Tak1 FL F	CAA GGA TCC ACTGTGGGAAAAGGATCTTTTCGG
Tak1 FL R	CAG CTC GAG TTA CTTACATGAATTCTCCACCAG

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Fig. 4.1. Phylogenetic analysis of PGRPs in the Fly, and mosquito. Candidate 1.1200 was identified as *Aedes* PGRPLC based on its relationship with *Anopheles* and *Drosophila*. Protein sequences were taken from preassembled *Aedes* genome and BLAST sequence alignments.

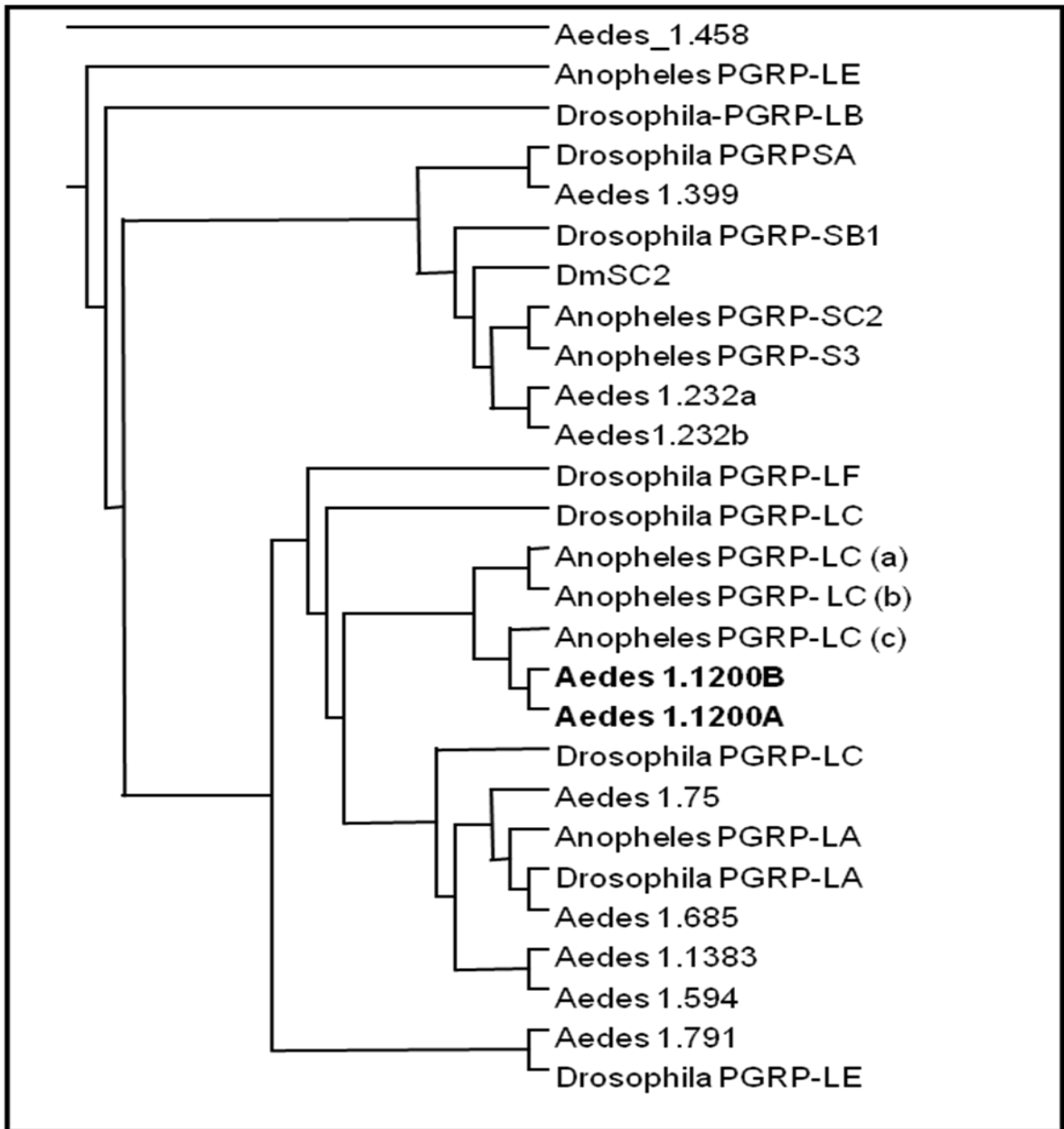


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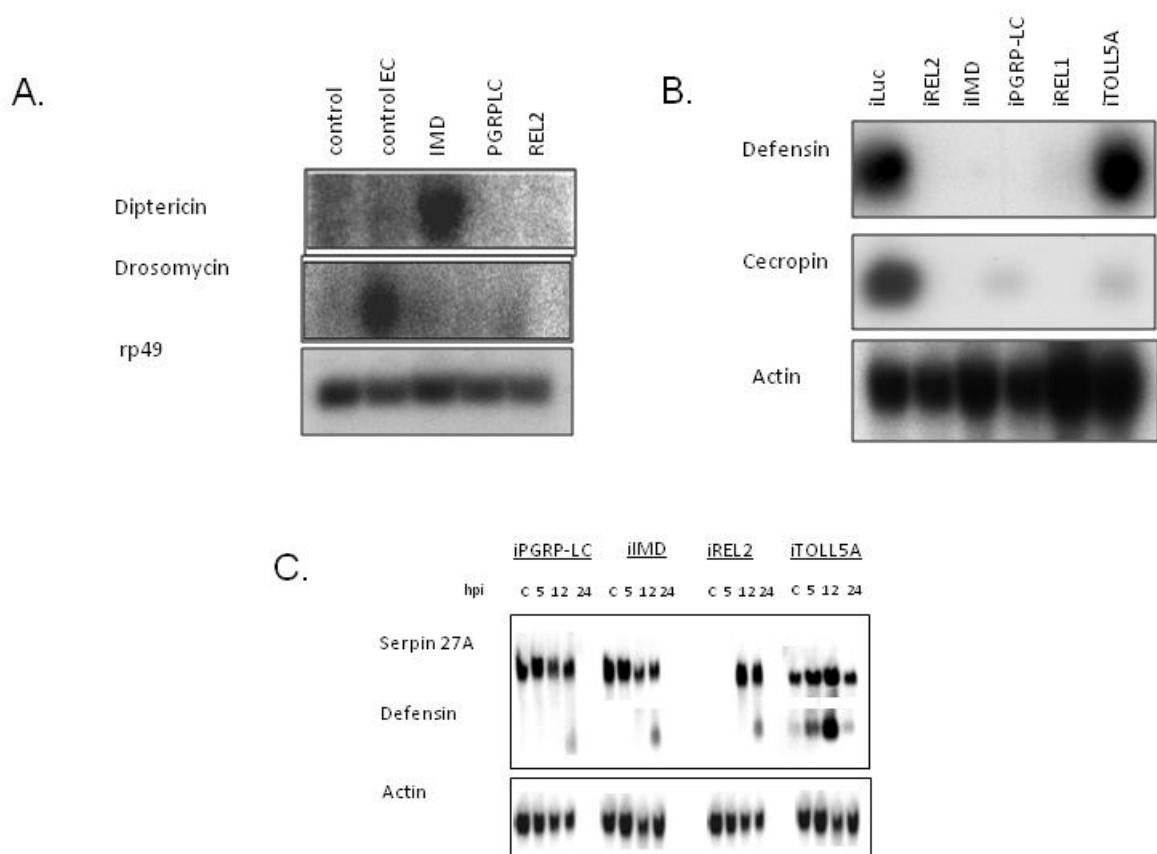


Fig. 4.2. Molecular and Functional characterization of PGRP-LC and IMD in *Aedes*. A, Ectopic expression of PGRP-LC and IMD in *Drosophila* S2 cells. IMD is able to induce Diptericin (marker of the *Drosophila* IMD pathway) expression. Ribosomal protein (rp49) was used as the RNA loading control. B and C, Role of PGRP-LC and IMD in *Aedes* IMD pathway. Northern blot analysis revealed that RNAi mediated knockdown of *Aedes* PGRP-LC and IMD compromised the immune activation of Defensin but not Serpin 27A in the mosquito, *Aedes aegypti*.

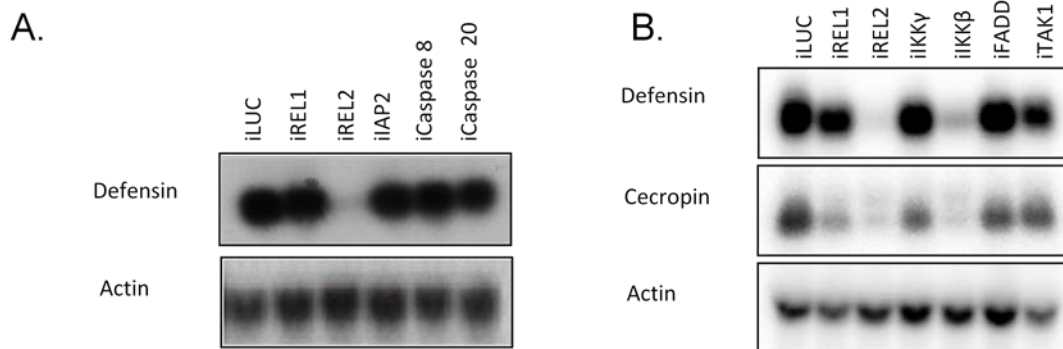


Fig. 4.3. Investigation of the cytoplasmic signaling components in the IMD pathway. A, Caspase 8, 20 and IAP2 does not affect Defensin expression. B, IKK β and TAK1 play a role in the *Aedes* IMD pathway. Mosquitoes were treated with 3-5ug of dsRNA against Caspase 8, 20 and IAP2. Northern analysis was done 4 days after treatment with dsRNA and 5 hours post *Enterobacter cloacae* infection.

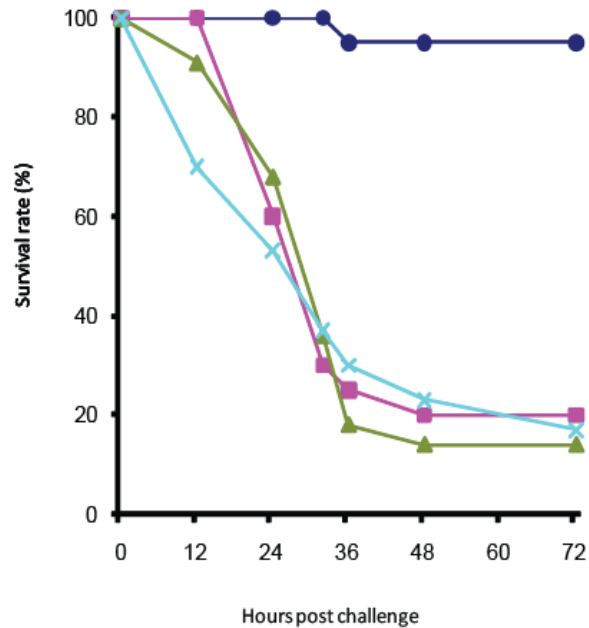


Fig. 4.4. Susceptibility of PGRP-LC and IMD dsRNA treated mosquitoes to Gram-negative bacteria infection. The iPGRP-LC and iIMD mosquitoes were significantly more sensitive to Gram-negative bacteria infection than luciferase dsRNA treated mosquitoes. Mosquitoes treated with iLUC, iPGRP-LC, iIMD and, iREL2 indicate Luciferase, PGRP-LC, IMD, and REL2 dsRNA treated mosquitoes, respectively. 3µg-5µg of dsRNA was injected in to the thorax of the adult *A. aegypti* mosquito. Septic injuries were performed by pricking mosquitoes in the rear part of the abdomen with an acupuncture needle (0.20 x 25 mm) dipped into a suspension of *E. cloacae*. Number of dead mosquitoes were calculated after 12, 24, 48 and 72 hours post infection. Results represent three independent experiments.

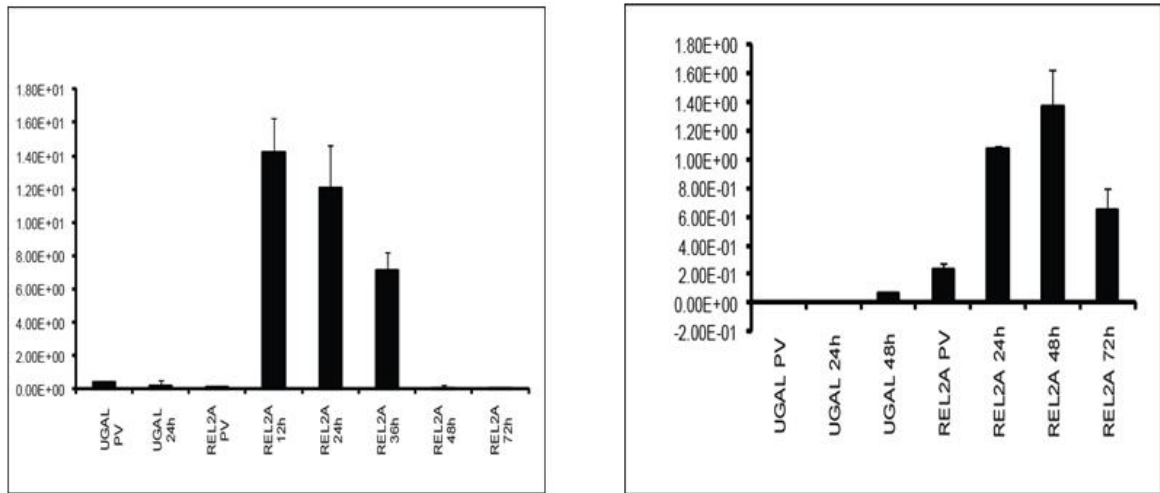


Fig. 4.5. Gene expression profile of REL2 transgene and Defensin expression in REL2 gain of function mosquitoes. *A*, Real-time PCR analysis showing the relative level of endogenous REL2 transcripts in transgenic and wildtype mosquitoes (UGAL). After blood feeding, the REL2 gain of function transgenic mosquitoes showed the overexpression of REL2 transcripts at 12 hours, whereas REL2-PV and UGAL wildtype mosquitoes did not exhibit REL2 transgene expression after a blood meal. *B*, Defensin expression follows REL2 expression, peaking at 48 hPBM. REL2A, REL2 gain of function transgenic mosquitoes, UGAL, wildtype, PV, previtellogenic.

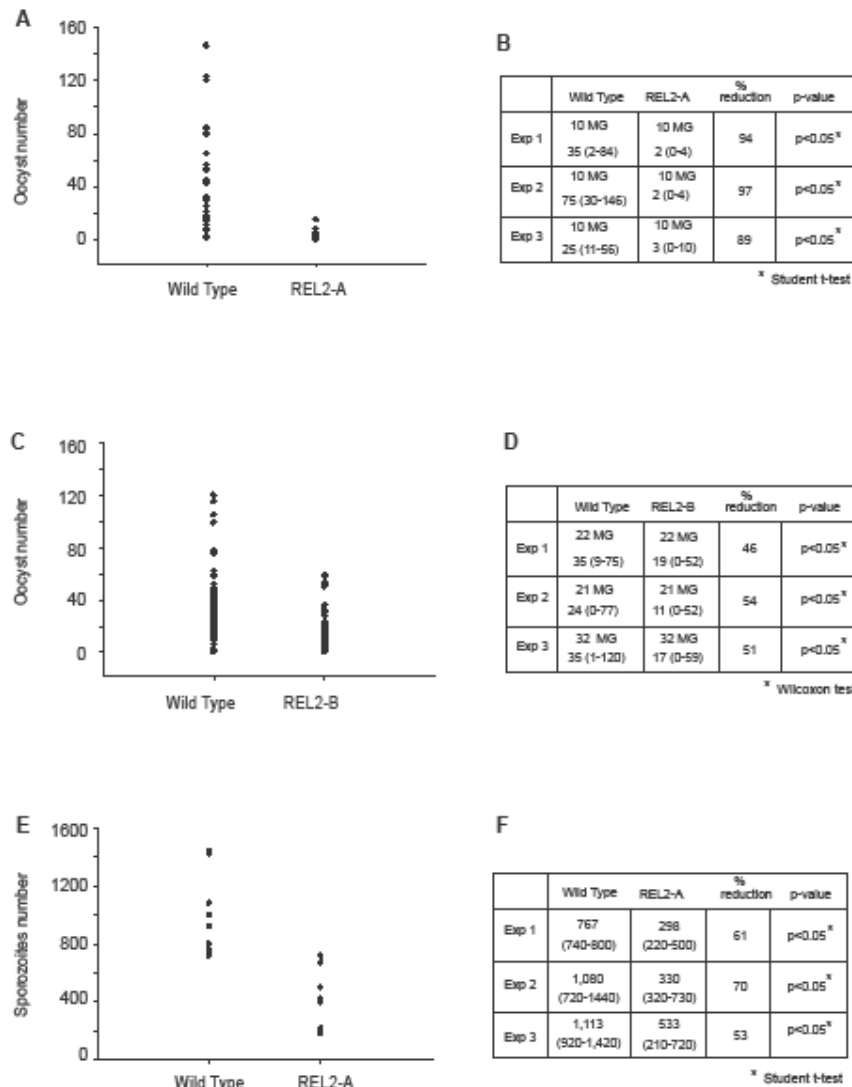


Fig. 4.6. Increased resistance of REL2 transgenic *Ae. aegypti* to *P. gallinaceum* infection. A, Decreased number of *P. gallinaceum* oocyst in the midguts of REL2-A mosquitoes. B, Numeric representation of the graph in (A) respectively; average oocyst number for REL2-A and wild type mosquitoes represents the average range of number of oocysts per experiment (shown in parentheses) C, Sporozoite number was significantly reduced in REL2-A mosquitoes. D, Numeric representation of the graph in (C); the table reflects average number of sporozoites and the range is indicated in parentheses.

**CHAPTER 5: COMPARATIVE ANALYSIS OF TOLL AND IMD PATHWAYS IN
MOSQUITOES AND THEY FLY**

ABSTRACT

Genome sequencing has facilitated a multiple species comparison which allows for the study of innate immunity of several insects and identified species-specific immune mechanisms. This comparison revealed the conservation of immune pathways; however, it also revealed species-specific gene expansion, which is probably incorporated to approach particular aspects of immune reactions. The *D. melanogaster* genome spans 120 million base pair (Mbp) encoding roughly 13600 genes. The *Aedes aegypti* genome is 1376 Mbp, while the the *Cu. quinquefasciatus* genome is 579 Mbp, which makes it approximately twice the size of *Anopheles* genome and four times larger than that of *Drosophila*. Among three sequenced mosquitoes, *Cu. quinquefasciatus* tops the gene numbers with total 18,883 genes. Conversely, the yellow fever mosquito, *Ae. aegypti*, genome encodes 15,419 genes compared to 12,457 genes in *An. gambiae*

In this genome study comparison, over 22 PGRPs have been identified in *Drosophila* compared to 11 genes in *Anopheles*, 8 in *Aedes* and 10 in *Culex*. The REL transcription factors show 1:1:1 conservation for *DmRelish/REL2*, a duplication of REL1 in *Aa*, and the loss of Dif in the mosquitoes. Additionally, an orthologous group of cysteine-rich defensins with *AeDEFD*, *DmDef*, *AgDEF1* and *CpDEFC* was also identified. The genomes of both, *An. gambiae* and *Ae. aegypti* mosquitoes contain a homologue of *Drosophila* MyD88. Five components were identified in the *Culex* Toll pathway: Cactus, MyD88, Pelle, TRAF6, and Tube (IRAK4). *Culex* Cactus, MyD88, Pelle and IRAK4 form orthologous trios with Cactus, MyD88, Pelle, and IRAK4 in

Anopheles and *Aedes*. *Culex* IRAK4 and Pelle were both identified to have a death domain as well as an IRAK domain, a serine/threonine kinase domain.

5.1 Introduction

Mosquitoes are vectors of numerous diseases, such as dengue fever, yellow fever, and malaria, which is immense important for public health. The adaptation of mosquitoes to hematophagy allows the chance for pathogen admittance. This includes a variety of bacteria (Gram-negative or -positive), fungi, parasites, and viruses. To combat infection, the mosquito relies heavily on multifaceted strategies that are conserved between species, ranging from arthropods to mammals. This chapter will enforce the phylogenomic analysis of the immune gene repertoire in three mosquito species, *Anopheles gambiae*, *Culex quinquefasciatus*, *Aedes aegypti*, as well as the fly, *Drosophila melanogaster*.

Although *Drosophila* is used to derive basic concepts of innate immunity, it is imperative to do comparative studies in order to identify and tackle the development of novel vector control methods. A multiple species comparison facilitated the study of innate immunity of several insects and identified species-specific immune mechanisms. This comparison revealed the conservation of immune pathways; however, it also revealed species-specific gene expansion, which is probably incorporated to approach particular aspects of immune reactions. This resurfacing of the immune repertoire perhaps reflects the dynamic interaction of the vector with its pathogen.

5.1.2 Overview of *Anopheles*, *Aedes*, *Culex* and *Drosophila* genome

The fly, *D. melanogaster*, serves as a model system for the study of number molecular developmental and cellular universal in higher eukaryotes including humans. The *D. melanogaster* genome spans 120 million base pair (Mbp) encoding roughly 13600 genes. *An. gambiae* is the principal vector of malaria, which causes 2.7 million deaths in sub-Saharan Africa every year. The *Aedes aegypti* genome, 1376 Mbp, is five times larger than the malaria vector, *An. gambiae* genome, which is about 278 Mbp. The *Cu. quinquefasciatus* genome is 579 Mbp, which makes it approximately twice the size of *Anopheles* genome and four times larger than that of *Drosophila*. Among three sequenced mosquitoes, *Cu. quinquefasciatus* has ultimate the gene numbers with total 18,883 genes (Arnesburger et al. unpublished data). Conversely, the yellow fever mosquito, *Ae. aegypti*, genome encodes 15,419 genes compared to 12,457 genes in *An. gambiae* (See Table 1) (Holt et al., 2002; Nene et al., 2007).

Based on phylogenetic analysis, it revealed the genes implicated in the innate immunity, with 285 in *Drosophila*, 338 in *Anopheles*, 353 in *Aedes*, and 251 in *Culex*, which are involved in the reactions such as melanization, phagocytosis, and anti-*Plasmodium*. The orthologous genes was identified throughout the immune gene repertoires with 4951 orthologous trios (1:1:1) and 886 mosquito specific orthologous pairs (absent from *Drosophila*). The orthologous pairs likely serve corresponding functions in the each organism. However, the mosquito specific orthologous genes direct

to the difference in pathogen encounter and subsequent development of immunity in response to these organisms.

5.2 Methods

Automated gene annotation for *Culex* – Three automated gene prediction pipelines was run independently by BROAD, JCVI, and Vectorbase. These were later merged by BROAD into a single initial consensus gene set CpipJ1.1, later updated to gene set CpipJ1.2. The three centers used different parameters to create each gene set, thus maximizing the probability of discovered genes. The methodologies used by each center are described below. Updates to the gene set are curated by VectorBase and can be accessed at www.vectorbase.org. *Culex* gene, intron, and exon statistics of the most recent gene set (CpipJ1.2) are shown in Table 2, along with similar statistics for the *An. gambiae*, *Ae. aegypti* and *D. melanogaster* genomes.

Automated gene annotation for *Aedes* – Two independent automated pipelines for structural annotation resulted in the prediction of 17,776 and 27,284 predicted gene models. A single high confidence gene set consisting of 15,419 gene models (AaegL1.1) was generated by comparing the 265,000 expressed sequence tags and dipteran protein and cDNA sequences. This approach involved identifying similarities in the protein coding region of Human and *Drosophila melanogaster* genes using tBLASTN, followed by the homology assisted gene prediction using Fgenesh+. Gene predictions were then further annotated manually as described below. The assembly was lead by Nene et al (2007). The annotation of the *Aedes* genes were done as a collaborative process where the Alex Raikhel lab (Sang Woon Shin, Guowu Bian, Kanwal Alvarez, and Alexander Raikhel) annotated the IMD and Toll pathway members, inhibitors of apoptosis, and

caspsases. The predicted sequences of the IMD pathway members were sent to SW Shin and each gene was confirmed by KSA and SWS using the methodologies discussed below.

Database Search – Predicted gene sequences were used to as query sequences to perform BLASTP, and tBLASTN searches. Results were organized by ascending E-values. Results with the lowest E-values were used to identity each putative gene.

Protein domain determination – Predicted gene sequences were also analyzed using PROSITE, SMART, and Conserved domain database at NCBI (CDD) for conserved protein domains. For some genes, manual analysis of proteins was done to identify the gene. Information for manual analysis was based on information from literature.

5.3 Results and Discussion

5.3.1. Recognition: PGRPs, GNBPs, TEPs, and LRR

The surfaces of microorganisms bear repeating patterns of molecules such as peptidoglycan, β -1,3-glucans, and lipopolysaccharides (LPS) collectively referred to as pathogen associated molecular patterns (PAMPs). Molecular interaction between PAMPs and PRR (pathogen recognition receptor) induce systemic immune response such as phagocytosis, encapsulation, and synthesis of antimicrobial peptides (Davidson et al.). These receptors include hemolymph circulating receptors, as well as membrane bound receptors of host cells. The mosquito PRRs are classified as: peptidoglycan recognition proteins (PGRPs), gram-negative binding proteins (GNBPs), thioester containing proteins (TEPs), scavenger receptors (SCRs), C-type lectins (CTLs), galactosidase binding lectins (GALE), and fibrinogen like domain immunoproteins (FBNs).

Sequence homology analysis suggests that PGRPs are likely involved in innate immunity and are conserved from insects to humans. *Drosophila* studies show that the immune system of the fly is able to distinguish between classes of microorganism, which leads to a pathogen specific immune response. The Imd pathway is induced by Gram-negative bacteria and some Gram-positive bacteria. It is now recognized that the peptidoglycan (PGN), which comprising of the inner layer of the bacteria, triggers the induction of the IMD pathway. The PGRP family is characterized by a common PGRP domain which is evolutionarily related to bacteriophage type II amidases. Some PGRPs have retained this domain and are known as catalytic PGRPs whereas others PGRPs

have lost this function and serve as microbial sensors. Over 22 PGRPs have been identified in *Drosophila* compared to 11 genes in *Anopheles*, 8 in *Aedes* and 10 in *Culex*. Orthologous trios could be formulated with mosquito genes, PGPPS1, PGRPLB, PGRPLC, PGRPLA, and PGRPLD. The PGRP-LE orthologue was identified in *Aedes* and *Culex* but not in *Anopheles*.

Aedes PGRP-LC was cloned and the annotation was later confirmed. The *Aedes* preassembled genome was used to identify PGRP-LC. One fragment of sequence, 1.1200, was isolated based on homology searches by the template of *Drosophila* PGRP-LC. The PGRP domain as well as a transmembrane domain was identified in fragment 1.1200 using PROSITE and CDD domain search. Lastly, a ClustalX alignment along with a phylogenetic tree (chapter 4, Figs) confirmed sequence 1.1200 as *Aedes* PGRPLC.

There are minor discrepancies in number of PGRP domains across different mosquito species. For example, *AaPGRPLA* appears to be a single domain PGRP domain gene like PGRP-LA in *Dm* and unlike PGRPLA in *Ag* that possesses two PGRP domains. The exact gene architecture of *AaPGRPLC* remains elusive since there are several gaps of unsequenced regions of the gene. As a result, *AaPGRPLC* was initially only predicted to have one instead of three PGRP domains; however, complete sequencing and analysis of this gene is likely to reveal multiple domains. Additionally, several what looks like splice variants of PGRPLC exist in *Drosophila*, and *Anopheles* but not in *Aedes* and *Culex*.

5.3.2. Signal culmination and induction of antimicrobial peptides.

Immune signaling cascades culminate in the translocation of the nuclear transcription (NFκB) factors. The REL transcription factors show 1:1:1 conservation for *DmRelish/REL2*, a duplication of REL1 in *Aa*, and the loss of Dif in the mosquitoes.

AMPs fall into three major classes, which can be described as peptides containing cysteine disulfide bonds, linear peptides which form α -helices, or proline- and/or glycine-rich peptides (Bulet and Stocklin, 2005). *D. melanogaster* possesses a wide range of AMPs including metchnikowin, drosocin, defensin, dipterocins, attacins, cecropins, and drosomycins. Four attacins have been identified in *Dm*; however, only one of these glycine-rich AMPs has been found in each of the mosquitoes. An orthologous group of cysteine-rich defensins with *AeDEFD*, *DmDef*, *AgDEF1* and *CpDEF1*. Gambicins, which have so far only been identified in the two mosquitoes, are cysteine-rich peptides that form four disulfide bridges. Cecropins are much more widespread among insects; however, these α -helical peptides are relatively divergent between the mosquitoes and *Dm*. Aggambicin has been shown to be induced in the mosquito after challenge with *E. coli* but not *Plasmodium*, contrasting *AgCEC1* which shows the opposite effect (Christophides et al., 2002). A putative dipterocin and a putative holotricin (Glycine-Rich Repeat Protein: GRRP) were identified in the *Aa* genome.

Culex AMPs were identified using the above mentioned characteristics. We identified a Defensin C (CPIJ001276) in the *Culex* genome. This mRNA sequence was approximately 240 amino acids long and it matched with *Aedes* Defensin D with an E-value of $3e^{-36}$ using tBLASTN. HMM search revealed Defensin domain in the sequence CPIJ001276. We later identified this domain using the characteristic CVC motif which is typical of Defensins (Bulet and Stocklin, 2005). Multiple Cecropins were also identified in *Culex* including Cecropin A, Cecropin N, Cecropin E, and Cecropin B1. These were all approximately 140 amino acids long and have the typical cysteine and glycine residues. These all had a significant tBLASTN E-values. The HMM search confirmed the identification of Cecropins. Each putative cecropin protein was classified as being in the Cecropin family. Additionally, one mRNA sequence was identified as Diptericin (CPJ007542). For a complete list of the AMPs annotated in the *Culex* genome, see Table 2.

5.3.3 Annotation of the IMD pathway in *Aedes* and *Culex*

The IMD pathway culminates in the activation of another NF- κ B transcription factor, Relish, which in *Drosophila* activates AMPs such as Diptericin. Signaling by this pathway mimics the mammalian TNFR1 (tumor necrosis factor receptor) cascade. IMD, a death domain protein that is most similar to the mammalian receptor interacting protein 1 (RIP1), interacts with the cytoplasmic domain of the receptor PGRP-LC (Choe et al., 2005). The central step resulting in the activation of TAK1, a MAP3K, is its ubiquitination by *Drosophila* Ubc13/Uev1A ubiquitin-conjugating complex (Zhou et al.,

2005). TAB2 has been suggested to function in a step between IMD and TAK1 (Zhuang et al., 2006). However, the requirement of TAK1 in Relish activation has been questioned (Delaney and Mlodzik, 2006). *Drosophila* FADD acts downstream of IMD linking it with DREDD (Naitza et al., 2002). *Drosophila* IKK complex (IKKb/IKKg) and DREDD, a *Drosophila* homologue of mammalian Caspase 8, are required for the signal-dependent phosphorylation and endoproteolytic cleavage of Relish (Stoven et al., 2003). After endoproteolysis of Relish, the N-terminal NF-kappaB module translocates to the nucleus where it drives the expression of immune genes while the C-terminal Ikb domain remains in the cytoplasm (Stoven et al., 2003).

Rather than creating novel cascades, mosquito signal transduction components show robust conservation by maintaining sequence identity and function despite evolutionary challenges. Using predicted genes IDs from an HMM, the IMD pathway was annotated in both *Aedes* and *Culex*. *Aedes* IMD pathway was confirmed to have 6 members: Caspar1/2, FADD, IKK1A/B (IKK β), and IKK2 (IKK γ), IMD, TAB2, and TAK1. Predicted genes sequences were used as initial sequences to confirm the identity of each gene. CLUSTAL analyses as well as BLAST searches were used as methodologies to confirm these genes. Interestingly, analysis revealed that two *Aedes* Caspar genes; however, sequence is incomplete since the first methionine, the initiation amino acid of a peptide, was not found. It is unclear whether it is caused by a sequence error or the result of a misassembled contig. The genome sequences of *Ae. Aegypti*, and *An. Gambiae*, contain 1:1 orthologs of *Drosophila* IMD pathway components: Imd,

FADD, TAK1, DREDD (CaspaseL1) and IKKg (IKK2). Two genomic loci of *Ae. aegypti* encode almost identical proteins, which are orthologs of *Drosophila* IKKb (IKK1). The IMD gene was also found in the *Aedes* genome. This sequence harbors the characteristic death domain (residues: 178-244) that is identical to *Anopheles* IMD in amino acid content and length, each being 282 amino acids long. We also identified a *Culex* IMD protein approximately 259 amino acids long harboring a death domain that is 71% identical to *Anopheles* and *Aedes* IMD. Furthermore, we also found TAK1, a MAP3K in both *Aedes* and *Culex* genome. Both *Aedes* and *Culex* TAK1 was confirmed to have an N-terminus protein kinase domain approximately 200 amino acids long. *Aedes* TAK1 forms an orthologous pair with *Anopheles* TAK1. Based on sequence similarity, protein domain structure, as well as similarity to *Anopheles* and *Aedes* based on BLAST analysis, we can infer that *Culex* TAK1 and IMD will mostly complete orthologous trios.

We also identified FADD in both the *Aedes* and *Culex* genomes. FADD is a death domain protein involved in the IMD pathway. Its immunity role has been shown extensively in *Drosophila* and recently, its importance was recognized in the *Aedes* (Cooper et al 2009). *Aedes* FADD is approximately 260 amino acids long. A death domain, which is approximately 80 amino acids long, was identified in the *Ae*FADD.

5.3.4 Toll pathway annotation in *Cu. quinquefasciatus*

Upon activation of the *Drosophila* Toll pathway, the TIR (Toll-interleukin 1-Resistance) domain of the Toll receptor recruits a cascade of signaling adaptors and kinases leading to the phosphorylation of Cactus, which causes the release of the REL transcription factors, Dorsal and DIF that results in their nuclear translocation (Hoffmann and Reichhart, 2002). In mosquitoes, REL1, a homologue of *Drosophila* Dorsal, has been reported for *Anopheles gambiae* and *Aedes aegypti* (Barillas-Mury et al., 1996; Shin et al., 2005). Transgenic analysis has shown that the REL1/Cactus cassette mediates the anti-fungal immune response in *Ae. aegypti*, indicating an evolutionary conservation of REL1/Dorsal in mosquito immunity (Bian et al., 2005). The genomes of both, *An. gambiae* and *Ae. aegypti* mosquitoes contain a homologue of *Drosophila* MyD88 that interacts with Toll through its TIR domain and required to activate expression of Drosomycin (Tauszig-Delamasure et al., 2002). Two proteins, Tube and Pelle are required to transmit the signal from Toll to the Dorsal/Cactus complex in *Drosophila* (Hecht and Anderson, 1993). Tube possesses an interaction motif belonging to the death domain (DD) super-family, whereas Pelle has a kinase domain as well as an N-terminal DD. Homologues of Pelle have been found in mammals in the form of the interleukin-1 receptor associated kinases (IRAKs) (Janssens and Beyaert, 2003). IRAKs are multidomain proteins, consisting of an N-terminal DD and a kinase domain (Kollewe et al., 2004). Four different IRAKs, IRAK-1, IRAK-2, IRAK-M, and IRAK-4, have been identified in the human and murine genome (Janssens and Beyaert, 2003). Experiments

with knock-out mice revealed an vital role of IRAK-4 in the TLR/IR-1R signaling (Suzuki et al., 2003). Inactivation of kinase function of IRAK-4 strongly diminishes IL-1-induced NF- κ B activation, revealing an essential function of its kinase domain (Li et al., 2002). A single gene orthologue of *Drosophila* Pelle exists in genomes of *An. gambiae* and *Ae. aegypti*. Though a clear homologue of *Drosophila* Tube was not found, a predicted protein from the genomes of both mosquitoes containing the N-terminal DD and an additional kinase domain phylogenically grouped with mammalian IRAK4. The presence of Tube (DD)-IRAK4 (kinase domain) in mosquito genomes suggests that IRAK4 was inherited during the evolution of the Toll/TLR immune response, while kinase domain of *Drosophila* Tube may be degenerated during evolution.

Five components were identified in the *Culex* Toll pathway: Cactus, MyD88, Pelle, TRAF6, and Tube (IRAK4). *Culex* Cactus, MyD88, Pelle and IRAK4 form orthologous trios with Cactus, MyD88, Pelle, and IRAK4 in *Anopheles* and *Aedes*. MyD88 was identified by known Death and TIR domain, which are precisely located from residues 52-119 and 167-296. *Culex* IRAK4 and Pelle were both identified to have a death domain as well as an IRAK domain, a serine/threonine kinase domain. The death domain spans from residues 14-105 and IRAK domain consists of residues 202-457. Both of *Cq*Pelle and *Cq*IRAK4 form their own subcluster in a phylogenetic tree with *Aedes* and *Anopheles* (see Chapter 3, Fig. 2). Multiple sequence alignment and domain structure analysis revealed that *Culex* IRAK4 death and IRAK domain are significantly identical to other insects; specifically, the IRAK domain is 56% identical to *Aedes* and 58% to *Culex*.

It is predicted that *Cq*IRAK4 which harbors the IRAK kinase domains functions similarly to *Ae*IRAK4 in that the later is a prominent factor in *Aedes* Toll pathway.

5.3.5 Concluding Remarks

With this comparative analysis, we have provided evidence of functional conservation of intracellular immune signaling components (Toll and IMD pathway) and evolutionary diversification of proteins that compose the insect innate defense repertoire in two species of the mosquito, *Ae. aegypti* and *Cu. quinquefasciatus*. The differences in conservation are likely due to a distinct need for specialized molecular interactions and interactions with diverse microorganisms encountered by the host insect. For example, *Drosophila* Myd88, Pelle, and TRAF which form a macromolecular complex with Toll/interleukin 1 receptor form an orthologous pairs in *Anopheles*, *Aedes*, and *Culex*. However, an overall expansion is observed in GNBPs, CLIPs, NFκBs, PPO, and CTLs; the latter of which are involved in anti-*Plasmodium* defense. Additionally, the summary of putative immune gene count (Table 5.2) suggests that mosquitoes (*Aedes*, *Anopheles*, and *Culex*) have a larger arsenal in their innate immune defense than *Drosophila*. This also supports the mosquitoes encounter a larger and more diverse repertoire of microorganisms and therefore, require genes specific for each type of defense.

This comparative analysis has also uncovered interesting genes and gene families for future research. For example, the existence of 1:1 ortholog of *Drosophila* PGRP-LE in *Aedes* and *Culex* but not in *Anopheles* is interesting and may allow us to study whether or not PGRP-LE has similar functions in *Drosophila* as in these mosquitoes. Additionally,

Anopheles CASPL1 in *Aedes* (not in *Culex* or *Drosophila*) may allow us to test the distinct function of CASPL1 in *Anopheles* and *Aedes* but not in *Culex*. We also identified a unique Interleukin receptor associated kinase (IRAK) family member in the three mosquito species separate from *Drosophila*. The death domain of IRAK4 is ortholog to *Drosophila* Tube death domain; however, the mosquito IRAK4s contains an IRAK domain that forms a distinct cluster in the IRAK4 tree (see Chapter 3, Fig. 3.2). The proposed existence of IRAK4 as well as its function needs to be examined in *Anopheles* and *Culex*.

It is noteworthy that the functions of *Aedes*, and *Culex* immunity related genes are based mostly on sequence similarity to *Drosophila*, *Anopheles*, and other species. Functional analyses using reverse genetic techniques are necessary to confirm the presence and function of these genes. Nevertheless, the framework of information established from this work as well as the large scale annotation projects should act as a foundation for future studies of these disease vectors.

5.4 References

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Species	Genome size	Number of genes	Average gene size (bps)	Number of exons	Number of introns
<i>C. quinquefasciatus</i>	579 Mb	18,883	5,673	71,094 bps	52,211 bps
<i>Ae. aegypti</i>	1.38 Gb	15,419	15,488	63,650 bps	51,076 bps
<i>An. gambiae</i>	278 Mb	12,457	5,145	52,595 bps	38,051 bps
<i>D. melanogaster</i>	168 Mb	14,039	5,253	65,706 bps	43,588 bps

Table 5-1. Comparison of the mosquito and fly genome. Genome information compiled from Arensburger et al. 2010 (manuscript submitted). Abbreviation: megabases (Mb), gigabases (Gb), base pairs (bps).

Table 5-2. Summary of potential immune components in mosquito and the fly. Genes are divided by overall gene function and number of genes per category are listed. Total number of genes are indicated in parentheses. Last column indicates number of orthologs trios among mosquitoes in that category. Information gathered from <http://cegg.unige.ch/Insecta/immunodb>.

Immune involvement	Gene families	<i>D. melanogaster</i>	<i>A. gambiae</i>	<i>A. aegypti</i>	<i>C. quinquefasciatus</i>	<i>Ag:Ae:Cp</i>
<u>Recognition</u>	PGRPs	(22) : -LC (a,x,y), -LE, -LA, LD, LB, -SA*, -SB(1,2), -SD*, -SC2, -SC1 (a,b)	(11): -LC1-3, LA1,2, S1-3, LB 1,2,LD	(8):-LA, LB, LC, -LE, -S1, S4, S5	(10): -LA, LB, LC, LD, LE, S1, S4, S5	1
	GNBPs	(3): 1,2*,3*	(7): 1-5, GNBPA1,2	(7): 1,3,4,5 6 GNBPA1, 2	(10): PA1, PA2, B1, B3, B4A-D, B5, 6A/B	3
<u>Signal Transduction</u>	CLIPs	easter, snake, Psh	(63): A,B,C,D,E	(65): CLIPB29	(77): CLIPA, B, C, D, E	0
	IMD pathway	(5) IMD, FADD, Dredd, TAK1, IKKb, IAP2	(5): IMD, FADD, IKK1,2, TAK1, CASPL1	(8):IMD, FADD, IKK, TAK1, CASPL1, Caspar1/2	(6): FADD, IKK1,2, IMD, TAK1,	0
	Toll pathway	(4): MyD88, Tube, Traf6, Pelle,	(5): Cactus, Myd88, Traf6, IRAK4, Pelle	(5): Cactus, Myd88, IRAK4, Pelle, Traf6,	(5): Cactus, Myd88, Traf6, Pelle and IRAK4	0
	NFkBs	(3): Dif*, Dorsal, Relish	(2): Rel1, Rel2	(3): Rel1a, Rel1b, Rel2	(4): Rel1A, RelB, RelC, Rel2	0
	Serpins	30	17	23	52	12
	Toll	(9): Toll 1-9	(10): Toll1A, B, 5A, B, 6-11	(12): Toll1A, B, 4, 5A, B, 6-8 9A, 9B, 10, 11	(11): Toll1A/B, 6-8, 9A/B, 10, 11	0
<u>Effector molecules</u>	AMPs	(21): Attacin(4), Cecropin(5), Drosomycin, Diptericin, Defensin (1), Metchnikowin, Drosocin,	(10): Gambicin, Cecropin (1-4), Defensin (1-4), Gambicin	(18): Attacin, Defensin, Diptericin, Cecropin, Gambicin, GRRP	(7) Attacin, Gambicin, cecropin (A1,A2,B1, B2), Defensin	1
	<u>Antibacterial</u>	PPOs	(3): 1,2,3	(9): PPO 1-9	(10): PP0 1-10	(9): PP0 1-7, 9, 10
<u>Antiplasmodium</u>	Lysozymes (LYS)	(13): S,X,C,P,D,B,E	(8): LYSC 1-8	(7): LYSC 4, 6, 7A, B, 9-11	5	1
	TEPs	(6): 1,2,3,4, 5	(15): 1-4, 9-15	(8): 13, 15, 20-25	(10): 15, 23(A,B), 24 (A,B), 26-30	0
	CTL	34	26	39	54	4
<u>TOTAL</u>		156	188	218	250	

Table 5-2. Summary of potential immune components in mosquito and the fly.

Appendix B: List of Publications

Zhen Zou, Sang Woon Shin, **Kanwal S. Alvarez**, Vladimir Kokoza and Alexander S. Raikhel. (2010). Distinct melanization pathways in the mosquito *Aedes aegypti*. **Immunity**: 32: 41-53.

Yevgeniya Antonova, **Kanwal S. Alvarez**, Yu Jung Kim, Vladimir Kokoza and Alexander S. Raikhel. (2009). The role of NF- κ B factor REL2 in the *Aedes aegypti* immune response. **Insect Biochemistry and Molecular Biology**: 39(4): 303-314.

Zhen Zou, Sang Woon Shin, **Kanwal S. Alvarez**, Guowu Bian, Vladimir Kokoza, and Alexander S. Raikhel. (2008). Mosquito RUNX4 in the immune regulation of PPO gene expression and its effect on avian malaria parasite infection. **Proc Natl Acad Sci U S A**: 105(47): 18454–18459

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