AMERICAN UNIVERSITY OF BEIRUT

FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF CLIPA7 IN *ANOPHELES GAMBIAE* IMMUNITY

by

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AN ABSTRACT OF THE THESIS OF

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Malaria is one of the most devastating vector-borne diseases in the world. It is caused by parasites of the genus *Plasmodium* that are transmitted to humans by the bite of female *Anopheles* mosquitoes. Studies in the major African vector *A. gambiae* revealed that mosquitoes are not passive to *Plasmodium* infection, rather they mount potent immune responses which are particularly efficient against ookinete stages of the parasite. Clip domain serine proteases and their homologs are important components of mosquito immune responses and have been particularly studied in the context of melanization and complement regulation. In this project, we characterized the role of the clip domain serine protease homolog CLIPA7 which was previously identified as a negative regulator of the melanization response to *P. berghei* ookinetes. CLIPA7 has an unusual protein structure that contains, in addition to the clip domain, several other protein-protein interaction domains including a proline rich domain, a tropoelastin and prion-like domains which indicate that it might play a broader role in mosquito immunity compared to other homologs. We revisited the role of CLIPA7 in defense against *P. berghei* infections and showed that its knockdown result in a more pronounced melanization response against ookinetes than previously reported. This response is dependent on the complement-like protein TEP1 protein which is a key player in mosquito immunity. Our data also revealed that CLIPA7 is not involved in resistance to *S. aureus* and *E. coli* infections, however, *CLIPA7* Kd mosquitoes exhibited drastically enhanced tolerance to *S.* aureus but not to *E. coli* infections. Biochemical analysis revealed that CLIPA7 regulates the activation cleavage of both CLIPA8 and CLIPA14, which are key positive and negative regulators of the melanization response, respectively. This suggests that CLIPA7 plays a complex role in the regulation of the clip cascades. Genetic and biochemical analysis of clip domain serine proteases and their homologs is needed to build the functional clip domain protease network and understand how and at what level it integrates with the melanization and complement responses.

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ABBREVIATIONS

A. gambiae	*Anopheles gambiae*
AMP	Anti-microbial peptide
APL1C	*Anopheles Plamodium*-responsive leucine-rich repeat protein 1C
B. bassiana	*Beauveria bassiana*
cDNA	Complementary DNA
clip-SPHs	Clip-domain serine protease homologs
CO_2	Carbon dioxide
CTL	C-type lectin
ddH_2O	Double distilled water
DIF	Dorsal-related immunity factor
dkd	Double knock down
Dscam	Down syndrome cell adhesion molecule
dsRNA	Double-stranded RNA
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FREP	Fibrinogen-related protein
GAM	Gambicin

GNBP	Gram-negative binding protein
GFP	Green fluorescent protein
IACUC	Institute Animal Care and Use committee
Ig	Immunoglobulin
IKK	IκB kinase
Imd	Immune deficiency
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Kd	knockdown
LB	Luria Broth
LRIM1	Leucine-rich repeat immune protein 1
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
M. sexta	*Manduca sexta*
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
O.D.	Optical density
PAMP	Pathogen-associated molecular pattern
PAP	Prophenoloxidase-activating proteinase

P. falciparum	*Plasmodium falciparum*
P. berghei	*Plasmodium berghei*
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGRP	Peptidoglycan recognition protein
PPO	Prophenoloxidase
PO	Phenoloxidase
PRR	Pattern recognition receptor
Psh	Persephone
RNAi	RNA interference
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
S. aureus	*Staphylococcus aureus*
Sf9	*Spodoptera frugiperda* 9
SP	Serine protease
SPE	Spz processing enzyme
SPH	Serine protease homolog
Spz	Spaetzle
SRPN or spn	Serine protease inhibitor or serpin
STAT	Signal transducers and activators of transcription

TEP	Thioester-containing protein
WHO	World Health Organization

CHAPTER I

INTRODUCTION

A. Introduction

Malaria is still one of the most devastating diseases in the world causing high mortality each year; around 438,000 deaths in 2015 alone (WHO,2015). It is caused by flagellates of the genus *Plasmodium* that are transmitted by the bite of few species of *Anopheline* vector mosquitoes.

Plasmodium parasites undergo multiple cycles of development inside the mosquito vector before being transmitted to another host. The developmental cycle takes three weeks. Briefly, in the first 24 hours following a bloodmeal, the gametocytes differentiate into male and female gametes soon after blood ingestion and undergo fertilization to produce a diploid zygote that later become a motile ookinete (Billker et al., 1997; Billker et al., 1998). The latter then invades the epithelial cells of the midgut and resides at the basal lamina where it comes in contact with the mosquito hemolymph. Ookinetes encounter a dramatic loss in their numbers due to several immune effector responses elicited in the hemolymph and targeting them for killing mainly by lysis (Figure 1 b). The surviving ookinetes differentiate then into resistant oocysts that undergo meiosis followed by multiple rounds of mitosis within 10 days post infection. Within the next 4 days, matured oocysts rupture to produce thousands of haploid sporozoites that start to migrate in the hemolymph towards salivary glands (Figure 1 a). Only sporozoites that invade the salivary glands

are capable to be transmitted by the mosquito bite to another host, thus propagating the infection.

Mosquitoes are not passive to *Plasmodium* infection, and mount potent immune response especially against ookinete stages (Blandin and Levashina 2004). Even though *Plasmodium* parasites dramatically suffer at the transition stage of ookinete to oocyst bottleneck, few numbers of escaped oocysts are capable of reestablishing the infection again (Figure 1 b). Transgenic and para-transgenic approaches that would enhance ookinete killing are likely to render mosquitoes refractory to infection. So, understanding the immune response involved at this stage is crucial and may lead to novel transmission blocking interventions in the future.

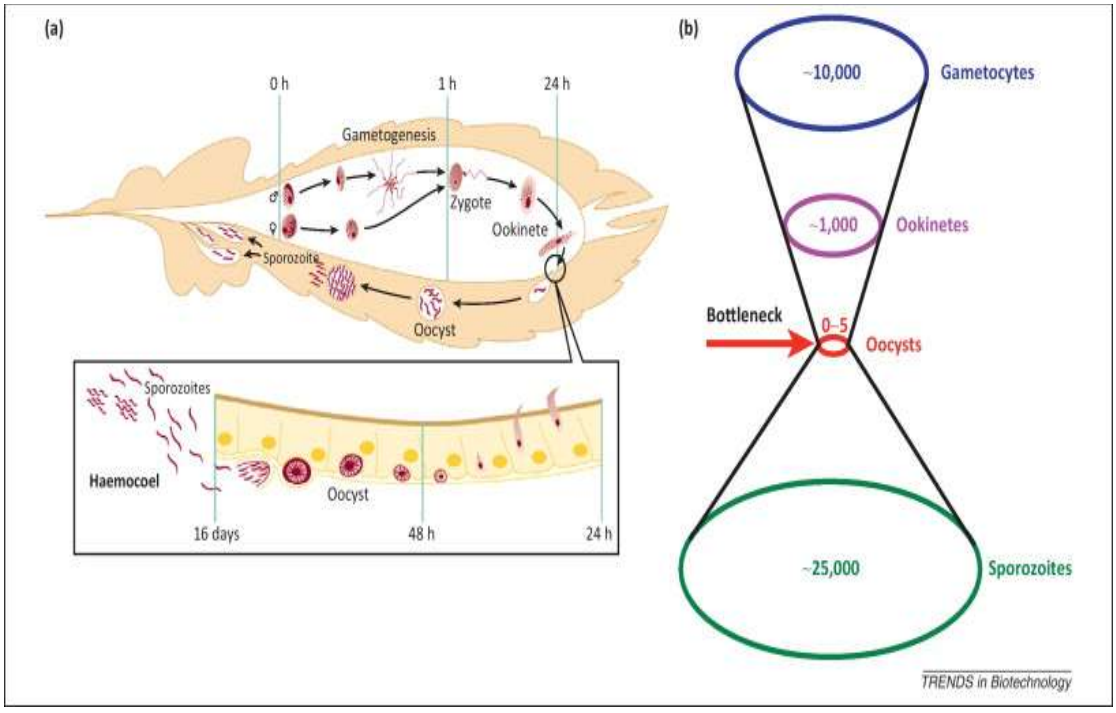


Figure 1 . Life cycle of *Plasmodium* parasites in the *Anopheles* mosquito vector. (a) malaria parasite life cycle in the mosquito starts after a blood meal from an infected host. Rightly after infection gametocytes differentiate by gametogenesis into male and female gametocytes (star-shaped) and soon they undergo fertilization to produce diploid zygotes. The latter then differentiate into motile ookinetes that traverse the midgut epithelia 24hr post infection and reside in the basal lamina where they become in contact with the mosquito hemolymph. Then they differentiate into resistant oocysts that undergo several rounds of meiosis and mitosis to produce

thousands of sporozoites 2 weeks post infection. Sporozoites are released after the rupture of oocysts into the hemolymph and they invade the salivary gland, then the mosquito is ready for transmitting the infection into new hosts. (b) *Plasmodium* parasites in the *Anopheles* mosquitoes undergo several bottlenecks during their complex development in the mosquito midgut. They encounter dramatic losses in their numbers and they become vulnerable mainly at the ookinetes to oocysts transition stage [Adapted from(Wang and Jacobs-Lorena 2013)]

Innate immunity in insects serves as the first and only line of defense against pathogen infection. Unlike mammals, insects do not have adaptive immunity, but still, they can differentiate between different classes of intruders, and elicit an immune response that is specific to control particular pathogenic infections (Ferrandon et al., 2007). In general, the immune responses are initiated upon infection when the pattern recognition receptors (PRRs) recognize pathogens specific components known as pathogen associated molecular patterns (PAMPs). Afterwards, the signal is modulated and amplified by a cascade of several clip-serine proteases interacting with each other, and eventually leading to activation of multiple effector immune responses like melanization, phagocytosis, AMP production and coagulation (Yassine and Osta 2010).

Several tools have been used to study the function of immunity genes and their involvement in defense against infection. Invitro genome wide expression profiling was used to identify the genes that are being upregulated or suppressed following various types of infection such as bacterial and malaria challenges, as well as in response to stress, such as injury and oxidative stresses (Dimopoulos et al., 2002). Gene silencing by RNAi proved to be a powerful technique used for identifying gene function in mosquitoes where classical genetics is not as straight forward as in drosophila (Blandin et al., 2002; Holt et al., 2002).

A pilot study using cDNA microarray based on 4000 EST chips identified several immunity genes that are upregulated upon bacterial challenges and which in turn induced cross protection against *Plasmodium* infections (Dimopoulos et al., 2002). Later, RNAi pioneering studies in *A. gambiae* mosquitoes identified several proteins with opposing effects implicated upon Plasmodium infection; TEP1, LRIM1 and APLC1 were identified as potent anti-parasitic factors (Blandin et al., 2004; Osta et al., 2004; Riehle et al., 2006). While CTL4 and CLTMA2 were identified as potent parasitic protective factors (Osta et al., 2004).

B. Innate immunity pathways in insects

Three major pathways; Toll, Imd and JAK/STAT are identified in insect immunity. Each pathway responds to particular classes of pathogens or stressor, and then activates a specific immune response. They mainly activate the production of antimicrobial peptides in the fat body as well as other immunity genes and genes involved in wound healing (Lemaitre and Hoffmann 2007). In *Drosophila* the Toll pathway is activated mainly upon infection with Gram-positive bacteria and fungus, while, Imd is activated in response to Gram- negative bacteria (Lemaitre et al., 1995). Both toll and Imd induce a humoral immune response and trigger the release of drosomycin and cercopins antimicrobial peptides respectively in the hemolymph (Bulet and Stocklin 2005). On the other hand, JAK/SAT pathway is involved in cellular immune defense. it is activated upon infection or septic injury (Agaisse and Perrimon 2004). Also, it is involved in inducing inflammation and in activation of phagocytosis and melanization (Sorrentino et al., 2004; Wertheim et al., 2005).

The role of Toll was first identified in dorsoventral patterning in *Drosophila*, then it was subsequently identified as a key player in drosophila immunity (Hashimoto et al., 1988; Lemaitre et al., 1996). Toll is a transmembrane domain protein with a leucine rich repeat. It does not bind directly to PAMPs, rather it is activated by specific PRRs that relay the signal to a cascade of clip serine proteases (CLIPs). CLIPs amplify the signal and trigger the cleavage of spätzle (SPZ) by Spätzle Processing Enzyme (SPE), triggering SPZ binding to toll. Toll triggers the activation of downstream signaling cascades that activate the transcription factor DIF (NF-κB like transcription factor) by degrading its inhibitor cactus. DIF translocates to the nucleus and induces the expression of Drosomycin AMP and other immunity related genes [reviewed in (Lemaitre and Hoffmann 2007)]. Unlike *Drosophila*, *Anopheles gambiae* does not have a homologue for DIF. However, Toll in mosquitoes activates the transcription factor Rel1, orthologous to *Drosophila* Dorsal, that is also sequestered by cactus in the cytoplasm and degraded upon phosphorylation (Figure *2*). Upon Cactus silencing, mosquitoes show complete refractoriness to *Plasmodium berghei* and substantial loss in *Plasmodium falciparum* parasite (Garver et al., 2009). Cactus Kd triggers the upregulation of key immunity genes including Tep1, LRIM1 and APL1C; they constitute the components of the mosquitoes complement system which play an important role in triggering lysis and melanization of *Plasmodium* parasite (Frolet et al., 2006; Riehle et al., 2008).

Imd has been discovered by a mutation in flies rendering them highly susceptible to infections, mainly by Gram-negative bacteria. PGRP-LE and PGRP-LC are the PRRs of the Imd that directly bind to DAP-type PGN of Gram-negative bacteria cell wall. It was shown recently that another PGRP called PGRP-SD acts on PGRP-LC to enhance the localization of PGN to bacterial surface (Iatsenko et al.,

2016).After bacterial recognition, Imd receives the signal from its receptor and activates a signal transduction cascade that leads to the activation of the transcription factor Relish after the degradation of its c-terminal ankyrin inhibitory domain (Stoven et al., 2003). Furthermore, Relish is phosphorylated by IkB kinase (IKK) which is needed for the efficient recruitment of RNA polymerase II (Erturk-Hasdemir et al., 2009). Relish translocates to the nucleus and activates the production of AMPs like diptericin and cecropins. In *A. gambiae,* Imd pathway activates the transcription factor REL2 (Figure *2* **)**. Unlike Relish, Rel2 undergo alternative splicing and give rise to two isoforms REL2-F and REL2-S (Meister et al., 2005). In addition, REL2 has broader defense activity compared to Relish which solely triggered in response to Gram-negative bacteria; REL2-F is involved in response to Gram-positive bacteria and fungi, while REL2-S is involved in defense against Gram-negative bacteria (Meister et al., 2005). Importantly, the IMD/REL2-F plays an anti-parasitic role and its activation reduces the number of *P. berghei* oocyst in mosquito midgut. In addition, REl2-F activates the expression of TEP1 and LRIM proteins which might explain the role of REL2 in anti-parasitic defense (Garver et al., 2009).

The JAK/STAT pathway plays a major role in anti-parasitic defense in *A. gambiae*. There are two STAT genes in *A. gambiae* mosquitoes (AgSTAT-A and AgSTAT-B) that are expressed after bacterial or *Plasmodium* infection. AgSTAT-A is involved in the activation of Nitric oxide synthase (NOS) while AgSTAT-B was shown to regulate the expression of AgSTAT-A(Figure *2*). Silencing the STAT suppressor SOCS increases NOS expression and reduces *Plasmodium* infection in the midgut (Gupta et al., 2009). It was suggested that nitric oxide production after *Plasmodium* infection is needed for Tep1-mediated parasite attack. It seems that NO-labelled parasites guide TEP1 to recognize the parasites and kill them by lysis and

melanization (Garver et al., 2013). Some strains of *P. falciparum* that express Pfs47 surface molecule disrupt JNK signaling pathway which is required to trigger midgut epithelia nitration. As a result, these parasites escape complement attack. (Molina-Cruz et al., 2013; Ramphul et al., 2015).

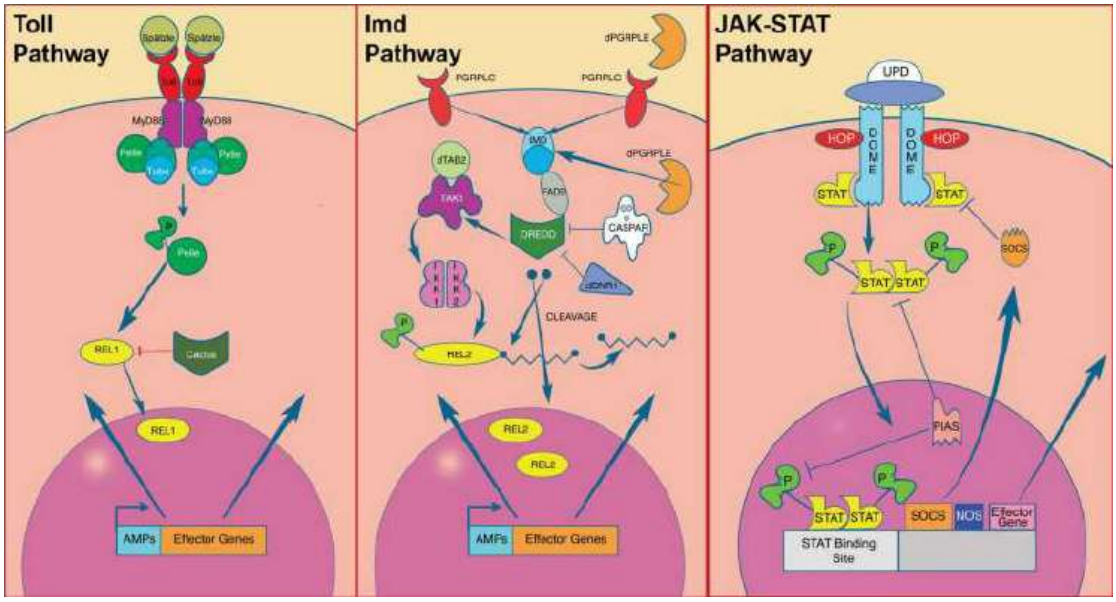


Figure 2. Toll, Imd, and JAK-STAT pathways activation in *A. gambiae* mosquitoes upon pathogen infection. Toll is activated upon bacterial or *Plasmodium* infections resulting in the activation of intracellular cascade leading to the activation of Rel1 transcription factor. Then Rel1 translocates to the nucleus and activates the expression of immunity genes and the production of AMP. (first lane). PGRPLC receptor of the Imd pathway in *A. gambiae* is activated by the recognition of bacterial and parasitic associated molecular components leading to the activation of Rel2 transcription factor that is present in two isoforms. REL2-F is implicated in defense against *Plasmodium* parasites and Gram-positive bacteria, while REL2-S is implicated in defense against Gram-negative bacteria. It is suggested that Imd pathway is the most efficient compared to other pathways in defense against the human malaria parasite *P. falciparum* (second lane). JAK -STAT pathway seems to play an important role in defense against *Plasmodium* parasites and viral infections. Upon activation, two Stat transcription factor translocate to the nucleus and induce the expression NOS and other immunity-related genes (third lane) (Adapted from Clayton, et al., 2014).

C. Pattern Recognition Receptors (PRRs)

In mosquitoes, as in other insects, immune responses are initiated when pattern recognition receptors bind to PAMPs present on microbial surfaces. Several PRRs have been identified in the genome of insects and these include PGRPs, GNBPs, CTLs, FREPs and Ig-domain proteins.

1. Peptidoglycan Recognition Receptors (PGRPs)

In *Drosophila*, PGRP-SA was the first PGRP to be discovered upstream of Toll receptor (Gobert et al., 2003). By genetic screening of different *Drosophila* mutants, *semm* mutant flies (gene encoding for PGRP-SA) showed high susceptibility to Gram-positive bacterial infections and loss of drosomycin AMP production (Michel et al., 2001). Moreover, GNBP1, a protein that specifically bind to LPS and β-(1,3) glucan, was identified to cooperate with PGRP-SA upon Gram-positive bacterial infections. It was suggested that GNBP1 is needed for breaking down Lys-type PGN for efficient detection by PGRP-SA (Kim et al., 2000; Wang et al., 2006). Hence, PGRPSA/GNBP1 complex is critical for detection of Gram-positive bacteria (Gobert et al., 2003). However certain Gram-positive bacteria were shown to activate Toll pathway through PGRP-SD; PGRP-SD mutants showed extreme susceptibility to Gram- positive bacteria (Bischoff et al., 2004). In addition, PGRP-SD was shown to cooperate with PGRPSA/GNBP1, for efficient detection of Gram-positive bacteria upstream of Toll receptor (Wang et al., 2008). However, a more recent study revealed that PGRP-SD is an important DAP-Type PRR that act upstream of the Imd pathway and is not required for Toll activation (Iatsenko et al., 2016).

Other genetic screens identified that PGRP-LC mutants are susceptible to Gram- negative bacterial infection and have similar phenotype to Imd mutants. (Gottar et al., 2002) PGRP-LC is a transmembrane receptor that binds exclusively to DAP- Type PGN. PGRP-LC mutants did not severely inhibit diptericin production as Imd pathway mutants, and lead to the discovery of another PGRP, PGRP-LE that cooperates with PGRP-LC for activating Imd pathway (Takehana et al., 2004). Mutation of both receptors showed a severe loss in diptericin production suggesting that PGRPLE and PGRPLC act in synergy for activating Imd pathway upon Gram-negative infection (Kaneko et al., 2006). More recent studies revealed that Imd pathway activation in Fat body relies mostly on PGRP-LC (Neyen et al., 2012). PGRP-LE plays a major role in the midgut in defense against Gram-negative bacteria (Bosco-Drayon et al., 2012; Neyen et al., 2012). In *A. gambiae* seven PGRPs have been identified (Waterhouse et al., 2007). PGRP-LC was shown to be involved in defense against both Gram-type bacteria. PGRP-LC also involved in controlling bacterial proliferation in the midgut of *A. gambiae* following a blood meal, and hence indirectly protect against parasitic infections (Meister et al., 2009).

2. Gram negative binding proteins (GNBPs)

Gram-negative binding proteins are a class of PRRs that mainly detect fungal components and activate Toll pathway leading to the production of drosomycin. In addition to the previously mentioned GNBP1, GNBP3 is involved in recognition of β-(1,3) glucan, and was shown to activate Toll pathway upon fungal infection (Gottar et al., 2006). GNBP3 mutant flies showed similar susceptibility to bacterial infection as the controls indicating that GNBP3 is critical for fungal detection, and is not required

for anti-bacterial defense. GNBP3 mutant flies abort drosomycin production upon infection with dead spores, however the latter is expressed upon infection with live spores (Gottar et al., 2006). This lead to the discovery of a clip-domain serine protease called Persephone (Psh) that acts as an enzymatic sensor and that is cleaved and activated directly by the fungal PR1 virulence factor. Active Psh cleaved SPE which in turn cleaved SPZ and leading to Toll pathway activation (Gottar et al., 2006). This indicates that two parallel pathways are involved in Toll pathway activation upon fungal infection. In *A. gambiae* mosquitoes, an anti-parasitic role has been suggested for GNBP3 and GNBP4. GNBP4 is involved in defense against *P. berghei* and *P. falciparum* infection, as well as Gram-positive and Gram-negative bacteria (Warr et al., 2008).

3. C Type lectins (CTLs)

There are 23 members of C-type lectins superfamily in *Anopheles gambiae* which are classified according to their carbohydrate binding specificity (Waterhouse et al., 2007). In *A. gambiae* two CTLs, CTL4 and CTLMA2 have been identified as potent agonists for malaria development. Silencing of either protein triggered a strong melanization response against ookinetes of *P. berghei* (Osta et al., 2004). In addition, the knock down of either protein showed strong susceptibility to Gram-negative bacterial infections (Schnitger et al., 2009). Biochemical analysis revealed that CTLs formed an obligate disulfide heterodimer complex needed for defense against Gram-negative bacteria but not Gram-positive bacterial infection (Schnitger et al., 2009). In a subsequent study, it was shown that the melanization response triggered by CTL4 Kd is dependent on Tep1 (Povelones et al., 2011). A recent study revealed that CTL4

and CTLMA2 Kd mosquitoes trigger also the melanization of *P. falciparum* but in an infection-intensity dependent manner. Also the function of these genes has diverged in *A. albimanus* where they play a *Plasmodium* antagonistic role (Simoes et al., 2017).

4. Fibrinogen-related proteins (FREPs)

Fibrinogen related protein family is the largest PRR family in *Anopheles gambiae*, including 59 members as compared to 14 in *Drosophila* (Waterhouse et al., 2007). Structurally FREPs are characterized by a fibrinogen-like domain located at the c- terminal region. it is highly involved in pathogen recognition in invertebrates while it plays an essential role in coagulation activation in vertebrates. One of these FREPs, FBN9 shows the most potent antimicrobial activity against diverse array of pathogens. It is implicated in defense against *P. berghei* as well as *P. falciparum*, in addition to bacterial and fungal infection (Dong and Dimopoulos 2009). Similar to FBN9, FBN8 is an another FREP that is involved in defense against *P. falciparum* and *P. berghei* (Dong et al., 2006aa). On the other hand, FREP1 was found to be secreted by midgut epithelia cells and facilitates the anchoring of plasmodium ookinetes and sexual stages to the peritrophic matrix (Zhang et al., 2015). FREP1 knock out mosquitoes by CRISPR/Cas9 showed reduced permissiveness to *P. berghei* and *P. falciparum* but associated with fitness cost (Dong et al., 2018).

5. Immunoglobulin (Ig) domain proteins

The Ig domain containing receptors in *Anopheles gambiae* provided an expanded recognition capacity with around 140-150 genes identified in *Drosophila* and *A. gambiae* (Dong et al., 2006b). Of these, The AgDSCAM (Down Syndrome Cell Adhesion Molecule) undergoes around 31000 alternative splice variants, and is capable of recognizing multiple classes of pathogens which add more complexity to mosquito immune responses (Dong et al., 2006b). Moreover, AgDSCAM undergoes pathogen-specific alternative splicing which is regulated by Toll and Imd pathways (Dong et al., 2012).

D. Signal modulation by Clip Serine proteases (CLIPs)

Clip-domain serine proteases are important components of insect immunity. They are involved in multiple physiological processes in which they play a role in signal modulation and amplification (Figure 4). They are involved in activation of Toll, melanization, coagulation and Tep1 complement pathways (Jiang and Kanost 2000; Kanost et al., 2001). There are 42 CLIPSs in *Drosophila*, 125 in *M. sexta* and 54 CLIPs in *A. gambiae* (Waterhouse et al., 2007; Cao et al., 2015). CLIPs are made up of one or more amino-terminal clip domains that are proposed to be involved in protein-protein interactions, and a catalytic protease domain that is connected to the clip domain by a linker motif. CLIPs are present as zymogens in the hemolymph of insects and they are activated by cleavage upon infection or injury with the clip and catalytic domains remaining attached to each other by a disulphide bond (Figure 3).

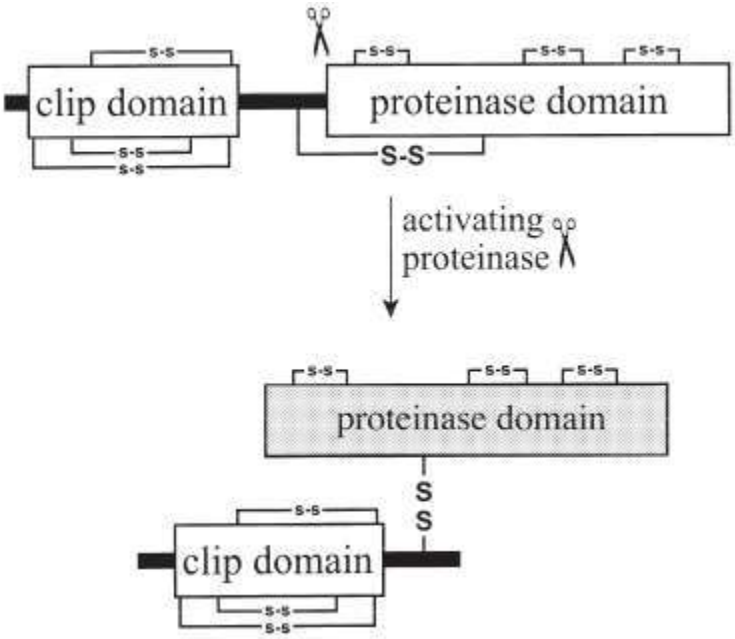


Figure 3. Mechanism of Clip serine proteases activation. CLIPs are present as zymogens in the hemolymph of insects. They are sequentially activated by cleavage. Proteolytic cleavage occurs at the site between the clip domain and the proteinase domain in the linker region. both domains remain attached to each other by a disulfide bond [Adapted from (Jiang and Kanost 2000)]

CLIPs function in cascades and they sequentially cleave each other in order to amplify the immune response. CLIPs are negatively regulated by serpins (serine protease inhibitors) that bind covalently and irreversibly to the active site of CLIPs and abort their activities (Figure 4). In *A. gambiae* CLIPs are divided into 5 subfamilies (CLIPA, B, C, D and E). CLIP B, CLIPC and CLIPD have catalytic protease activity and they are called catalytic Serine Proteases (cSPs), while CLIPA and CLIPE have one or more mutation in the catalytic triad of the protease domain which renders them inactive, and hence are called SPHs (Serine Protease Homologues) (Waterhouse et al., 2007). SPHs play an essential role in insect immunity, some SPHs act as cofactors for catalytic CLIPs that involve in the activation of Prophenoloxidase (PPO) needed for melanization response activation

(Yu et al., 2003). While other SPHs act as negative regulators of the melanization pathway to prevent exaggerated melanotic immune response (Volz et al., 2006).

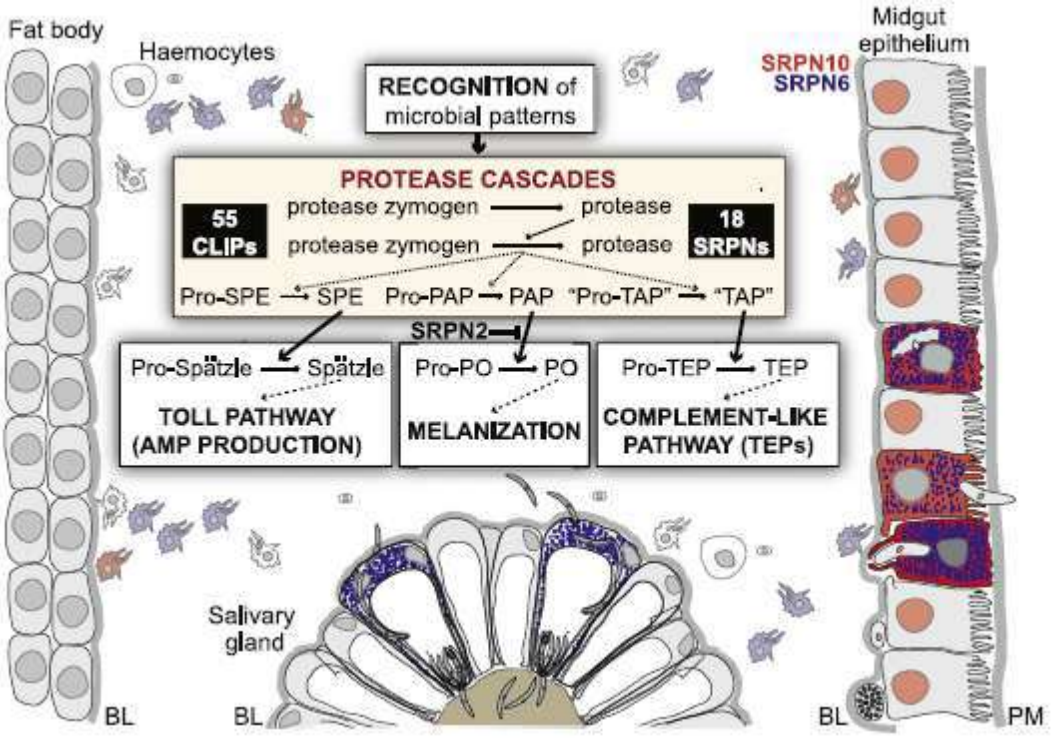


Figure 4. CLIPs regulation of multiple immune effector responses in *Anopheles* mosquito immunity. Upon pathogen recognition by PRRs, a cascade of clip serine proteases is activated leading to modulation and amplification of the initial signal. CLIPs then mediate the activation of several immune effector responses; 1- they trigger the production of Anti-microbial peptides (AMP) which is mediated by Toll activation. 2- they regulate the activation of melanization by triggering the cleavage of Prophenoloxidase. 3- they also regulate TEP1 activation, thus triggering lysis and phagocytosis immune response. Due to the multiple roles associated with CLIPs, it is tightly regulated by serpins to ensure proper regulation and activation of the elicited immune effector response (Adapted from Gulley et al., 2013).

1. Activation of Drosophila Toll by CLIPs

Toll pathway in *Drosophila* have been regulated by various CLIPs. Some CLIPs are shown to be activated in response to developmental cues while others are

shown to be linked to immunity pathways (Figure 4). Snake and Easter are activated in the early stages of *Drosophila* development and involved in dorso-ventral patterning. Snake was shown to sequentially activate Easter which in turn triggers the proteolytic cleavage of spätzle that bind to and activate Toll receptor (Stein and Nusslein-Volhard 1992; DeLotto and DeLotto 1998). On the other hand, Grass and Spirit were shown to play an essential role in *Drosophila* immunity. Both CLIPs are activated in response to Gram-positive bacterial infection by the PGRP-SA/GNBP1 complex. Grass was shown to sequentially activate Spirit which in turn lead to the cleavage of spätzle processing enzyme (SPE). SPE triggers the proteolytic cleavage of spätzle and hence activates Toll receptor leading to drosomycin production (Michel et al., 2001; Kambris et al., 2006). Similar to Grass/Spirit, Persephone (Psh) was shown to induce the activation of toll receptor by triggering the cleavage of SPE (Jang et al., 2006). However, Psh is activated in response to fungal infection, independent of PRRs, by directly recognizing fungal PR1 virulence factor (Ligoxygakis et al., 2002).

2. CLIPs in the melanization response pathway

Melanization is one of the potent immune responses in insects, it is directly activated upon microbial, fungal and parasitic infections, and is involved in encapsulating and killing of invading pathogens (Fuchs et al., 2014). In addition, it has an important role in wound healing and tissue sclerotization (Andersen 2010). The melanization reaction is initiated by the Prophenoloxidase (PPO) enzyme which is cleaved and activated soon after infection by a catalytic CLIP (Figure 4***)***. In fact a network of CLIPs (cSP and cSPH) controls PPO activation(Jang et al., 2008). Because melanin is highly toxic and hyper reactive compound, CLIPs are tightly

regulated by serpins leading to limited proteolytic activation of PPO, hence preventing melanization overactivation which may lead to fitness cost.

Manduca sexta has been the best model used for studying the PPO activating cascade at the biochemical level because of the large volume of hemolymph that can be extracted from larvae in contrast to that in flies and mosquitoes. In *M. sexta*, three key CLIPs called PPO activating enzymes (PAP-1, PAP2 and PAP3) are activated upon bacterial and fungal infections (Jiang et al., 1998; Jiang et al., 2003aa; Jiang et al., 2003bb). The response is initiated by bacterial recognition receptors βGRP1 and βGRP2 that transmit the signal to a non-clip modular serine protease HP14 (Ma and Kanost 2000; Jiang et al., 2004). HP14 activates the cSP HP21 which in turn cleaves PAP-2 and PAP-3 that are needed for PPO cleavage and melanization activation (Wang and Jiang 2007). Moreover, two SPHs; SPH1 and SPH2 are needed as cofactors for PAP-2 and PAP-3 for the efficient cleavage and activation of PPO, and it is suggested that SPHs facilitate the interaction between PAP and PPO because PAP alone is not efficient in activating PPO (Yu et al., 2003; Gupta et al., 2005). PAP-1 is another cSP that is activated by a separate pathway independent of Hp14-Hp21 which is not fully elucidated yet. The cSP Hp6 was shown to cleave PAP-1 which in turn triggers the cleavage of PPO activating the melanization response. Interestingly, Hp6 cleave HP8, an SPE homolog, which then catalyzes the cleavage of spätzle and activates toll pathway (An et al., 2009). This suggest the presence of a cross talk between the two pathways, toll and melanization pathways in *M. sexta* (Figure 5). Furthermore, biochemical analysis showed that active PAP-1 can in turn cleave and activate HP6 leading to a positive feedback loop (Wang and Jiang 2008). In addition, recombinant PAP-3 injected into *M. sexta* was shown to cleave SPH1 and SPH2 enhancing its own cleavage in a positive feedback loop (Wang et al., 2014). Several

serpins act at different layers of the CLIP cascade to regulate this pathway, and some exhibit redundant roles in inhibiting CLIPS. (Ligoxygakis et al., 2002; Michel et al., 2005; Scherfer et al., 2008). This tight regulation by serpins ensures that the pathway is not overactivated.

In *Drosophila*, MP1 and MP2 are two cSP that were shown to be involved in PPO activation in spn27a mutant flies that showed spontaneous melanization response (De Gregorio et al., 2002; Ligoxygakis et al., 2002). MP1 and MP2 are the targets of spn27 which act as a negative regulator of the melanization response (Tang et al., 2006). However, the PRRs and modular serine proteases acting upstream of MP1-MP2 cascade are still unknown (Figure 5). Biochemical analysis revealed that MP1 and MP2 active forms are not capable of cleaving PPO directly, which suggests the presence of a putative PPAE downstream of MP1 and MP2 that is required for PPO cleavage (Jang et al., 2008). Another target of spn27a is the cSP Hayan. Hayan mediates the melanization response during wound healing and its silencing showed high susceptibility to sterile injury (Nam et al., 2012). Moreover, Hayan is involved in the melanization response upon infection by acting as a PPO activating enzyme. Its silencing abolishes the melanization response independent of MP1 and MP2. In addition to spn27a, two other SRPNs (spn28 and spn77) were shown to regulate the melanization response in *Drosophila* at different levels. Interestingly, silencing of Hayan reverses the phenotype of any serpin Kd, suggesting that Hayan is an essential key in the melanization response, but there is no evidence of its interaction with MP1-MP2 (De Gregorio et al., 2002; Ligoxygakis et al., 2002; Scherfer et al., 2008). In addition, spn27 is shown to be regulated by an acute immune factor expressed upon toll activation (Ligoxygakis et al., 2002) This suggests the presence of a crosstalk between both melanization and toll activation pathways.

In *Anopheles gambiae,* malaria vector mosquitoes, several CLIPs were shown to have a role in the regulation of the melanization response (Figure 5). CLIPA8 is an essential and potent positive regulator of melanization (Volz et al., 2006). it is activated soon after bacterial infection and enhances PPO activity in a TEP1 dependent manner (Schnitger et al., 2007; Povelones et al., 2013). TEP1 seems to act as an upstream positive regulator of melanization. It binds to the surface of *Plasmodium* and induces their melanization or lysis depending on the mosquitoes genotype. A network of cSPs and SPHs function downstream of TEP1 however, the full panel of CLIPs involved in it and their interactions are still under investigation. The SPH CLIPA2 acts as a negative regulator of melanization response (Volz et al., 2006). Its silencing exhibits an increased resistance to *Plasmodium* parasite manifested by increased ookinete melanization. In a subsequent study it was shown that CLIPA2 seems to regulate the activity of TEP1 and thus its knockdown enhances TEP1 activity leading to PPO activation upon systemic *E. coli* infections (Yassine et al., 2014; Kamareddine et al., 2016). In contrast to CLIPA2, SPCLIP1 is an another SPH belong to the CLIPE subfamily that seems to be needed for the conversion of the inactive full form Tep1(Tep1-F) to the active processed Tep1(TEP1-C) that binds to the surfaces of *Plasmodium* ookinetes. Its silencing abolished CLIPA8 cleavage and reduced Tep1 mediated PPO activation upon bacterial systemic infections (Povelones et al., 2013). Similar to CLIPA2, SPCLIP1 seems to belong to the TEP1 complex needed to initiate lysis and melanization. Another SPH, CLIPA14, acts like CLIPA2 as a negative regulator of the melanization response. Interestingly CLIPA2/CLIPA14 dKd shows a more dramatic increase in the melanization response, manifested by increased resistance to plasmodium parasites, than silencing each gene alone. This suggested that both CLIPs are acting in synergy to control the melanization response

(Nakhleh et al., 2017c). Regarding the role of cSP in melanization response, several catalytic SPs were identified to play a key role in activating the response. Of these, CLIPB17, CLIPB8, CLIPB3 and CLIPB4 were identified as positive regulators of melanization response against *Plasmodium* parasites (Volz et al., 2005; Volz et al., 2006). Mosquito CLIPs are tightly regulated by serpins to prevent exaggerated melanotic response. For instance, SRPN2 is an inhibitor of CLIPB9 and silencing SRPN2 triggers the spontaneous activation of PPO leading to significant melanization of *Plasmodium* ookinetes (Michel et al., 2005).

On the other hand, two CLIPBs were shown to be involved in the lytic pathway of *Plasmodium* parasites in Tep1 dependent manner; CLIPB14 and CLIPB15 Kd increased the number of live oocysts in susceptible mosquitoes. However, the mechanism of action of these CLIPs remain to be addressed.

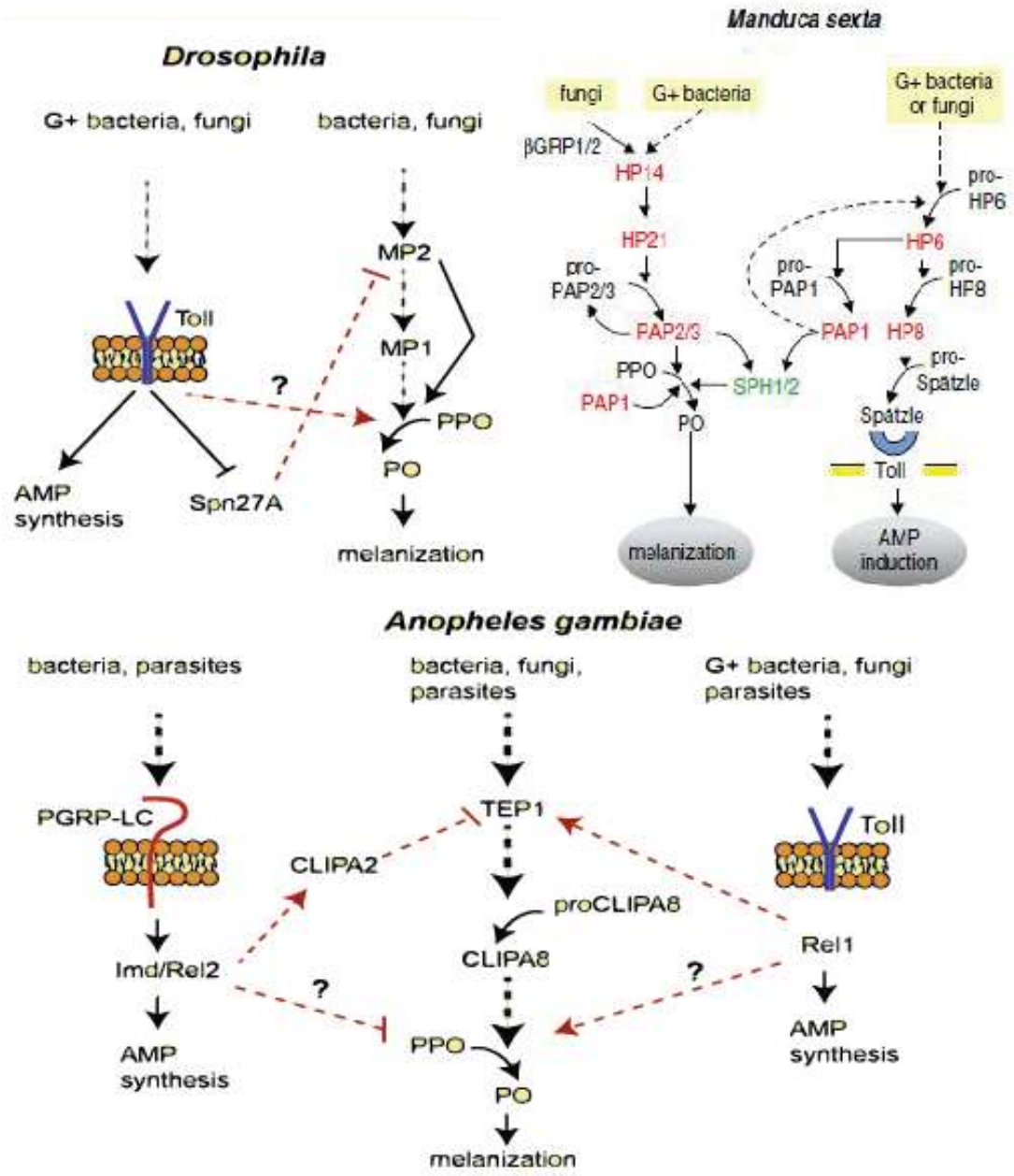


Figure 5. Clip serine proteases cascade in insect immune responses. Microbial recognition by the yet unidentified PRRs lead to the activation CLIPs cascades and resulting in PPO or pro-spätzle activation. The diagrams summarize data from the following: *Drosophila, Manduca sexta and Anopheles gambiae* [reviewed in (Nakhleh et al., 2017d)]

E. Mosquito immune effector mechanisms

1. Antimicrobial peptides (AMPs)

In *Anopheles gambiae,* there are 11 identified AMPs. *Anopheles* shares only cecropins and defensin families of their AMPs with *Drosophila* (Waterhouse et al., 2007). Although defensin in drosophila acts against Gram-positive bacteria only, in *Anopheles* it has broad activity against *Plasmodium* and bacteria (Dimopoulos et al., 1997; Richman et al., 1997); it acts mainly on Gram-positive bacteria and against some classes of filamentous fungi with no effect on Gram-negative bacterial infection and yeast except for some strains of *E. coli* bacteria (Vizioli et al., 2001bb). Furthermore, cecropin in *Anopheles* showed a broader activity against multiple classes of pathogens; it acts in defense against Gram-positive and Gram-negative bacteria, in addition to fungus and yeast (Vizioli et al., 2000). A novel antimicrobial peptide Gambicin, unique for *Anopheles*, is highly induced upon *Plasmodium* infection. It is active against ookinetes stages, Gram- positive, Gram-negative bacteria and filamentous fungus infections (Vizioli et al., 2001aa). Defensin, Cecropin and Gambicin are predominantly expressed in the thorax, abdomen and the anterior compartment of the midgut.

2. TEP1- mediated killing

TEP1 is a key protein involved in multiple immune effector mechanisms. It is involved in triggering phagocytosis of bacteria and lysis of parasites (Blandin et al., 2004; Moita et al., 2005). It is also involved in triggering melanization of *Plasmodium* ookinetes mainly in refractory strains of mosquitoes (Blandin et al.,

2004). TEP1 belong to the thioester- containing protein family. It is structurally similar to C3 complement protein in mammals. TEP1-F is activated by cleavage, hence forming TEP1-C that attack nucleophilic components on bacterial surfaces through its thioester bond. TEP1 is secreted by hemocytes to the hemolymph of mosquitoes and it seems to be spontaneously activated upon its release (Levashina et al., 2001). TEP1-C is stabilized by the LRIM1 and APL1C proteins. Structurally, LRIM and APL1C proteins are made up of LRR domains and they interact together forming an obligate disulfide bond and a protein complex (Fraiture et al., 2009; Povelones et al., 2009). It was suggested that LRIM1/APL1C complex guides TEP1-C to the surface of pathogens and prevents its auto immune attack. The knock down of either protein showed similar phenotype to TEP1 Kd that is manifested by an increased number of oocysts in the mosquito midgut (Osta et al., 2004; Riehle et al., 2006). In addition, it was shown that TEP1 plays a major role in defense against *Beauveria bassiana* in which it interferes with its invasion and retards it growth (Yassine et al., 2012). The consumption of TEP1 is also regulated by CLIPs including SPCLIP1 and CLIPA2 SPHs. it was shown that both CLIPs colocalize with TEP1 on the surface of bacteria and ookinetes. SPCLIP1 is involved in triggering the conversion of circulating TEP1-F in the hemolymph to TEP-1C on the surface of pathogens (Povelones et al., 2013). On the other hand, CLIPA2 is involved in regulating the activity of TEP1 and it controls its accumulation on bacterial surfaces. It was shown that CLIPA2 Kd enhances the formation of TEP1-C that largely accumulate on the surface of *Plasmodium,* bacteria and fungi (Yassine et al., 2014). This indicates that both SPHs, LRIM1 and APL1C are tightly regulating TEP1 protein which highlight a key role of TEP1 in mosquito immunity. Some strains of *P. falciparum* that are expressing Pfs47 surface protein are capable of evading TEP1

complement attack complex, thus escaping lysis and melanization immune response (Molina-Cruz et al., 2013).

3. Melanization

Melanization is a potent effector mechanism in arthropods. It is involved in wound healing, tissue sclerotization and melanization of invading pathogens. Melanization is a humoral immune response that function independent of hemocytes in small insects including adult mosquitoes (Christensen et al., 2005). It is highly implicated as a humoral defense against malaria parasites in *A. gambiae* refractory strains, in contrast to susceptible strains that depend mainly on lysis pathway to abort *Plasmodium* development (Volz et al., 2006). Upon microbial infection, PRRs recognize bacterial components and activate a cascade of serine proteases that in turn triggers the proteolytic cleavage of PPO zymogen and its conversion to active PO. PPO is the key enzyme in this response (Nappi and Christensen 2005); it catalyzes the oxidation of phenolic products in the hemolymph resulting in melanin formation. Melanin polymerizes on the surface of pathogens and forms a black coat surrounding the infecting agent. Thus it results in killing of pathogens by aborting gas and nutrients exchange with the surrounding environment (Soderhall and Cerenius 1998). In addition, melanization is involved in encapsulating and isolating dead pathogens to prevent excessive and constitutive activation of immune effector responses. Melanization reaction is tightly regulated by CLIPs to prevent exaggerated activation of melanotic immune response which might lead to tissue damage or result in fitness cost (Volz et al., 2006).

F. Contribution of Melanization to insect immunity

Several studies have provided considerable evidences supporting the role of melanization in defense against pathogen infections. Studies on *M. sexta,* showed that some pathogens evolved strategies to escape and counteract the host melanization response. For example, some pathogenic bacteria like *P. luminescens* which belong to *Photorhabdus* species encode the expression of antibiotic molecules which is secreted to the hemolymph and inhibits PO activity and melanization activation. (Eleftherianos et al., 2009).

In *Drosophila*, genetic studies combined with enzymatic assays of PO activity suggested that PPO is been activated upon bacterial infection. However, knocking down of MP1 and MP2 exhibited similar tolerance to Gram-positive, Gram- negative and fungal infections as the control groups. Also, it showed no effect on bacterial resistance upon *E. coli* infection as compared to controls. This indicated that melanization activation is not required for *Drosophila* defense against bacteria and fungi even though PPO is activated upon such infections (Leclerc et al., 2006). It was suggested that using a larger panel of bacterial infection might explain this discrepancy because some bacteria might be efficiently neutralized by other predominant effector mechanisms such as AMP and phagocytosis. In a subsequent study, flies harboring mutants of both PPO1 and PPO2 genes showed decreased tolerance and resistance to *S. aureus* infection. In addition, infection with other bacteria like *Listeria monocytogenes*, *Enterococcus faecalis* and *Candida albicans* showed only reduced tolerance with no effect on the resistance of flies compared to the controls (Binggeli et al., 2014). However, these phenotypes were absent from flies

carrying single mutants of either PPO genes. This suggested that melanization response seems to play a significant role against particular infections in *Drosophila*.

In *Anopheles gambiae*, abolishing PPO activity upon CLIPA8 Kd showed similar susceptibility to *E. coli* and *S. aureus* as compared to controls. This indicates that melanization activation in *A. gambiae* is not needed in defense against bacterial infections (Schnitger et al., 2007). However, using a broad panel of bacteria is needed for assessing the role of melanization in anti-bacterial defense, especially that *E. coli* and *S. aureus* are naturally occurring in *A. gambiae* microbiota. On the other hand, melanization plays a potent role in *A. gambiae* in defense against *Plasmodium* infections. It is highly induced in mosquito refractory strains upon *P. berghei* infection (Volz et al., 2006). It was also triggered in CTL4 Kd and CLIPA2/CLIP14 dKD susceptible mosquitoes, and resulted in extensive loss in the number of oocysts (Osta et al., 2004; Nakhleh et al., 2017c). Nevertheless, wild type fields caught mosquitoes rarely melanized *Plasmodium* ookinetes. This indicates that melanization response plays a significant role in defense against *Plasmodium* infection and its induction in susceptible strain seems to retard *Plasmodium* transmission and infection. In addition, abolishing PO activation by silencing CLIPA8 renders the mosquitoes highly susceptible to *B. bassiana* infection which suggests a potent role of melanization in defense against fungal infections(Yassine et al., 2012).

CHAPTER II

AIMS OF THE PROJECT

Several immunity genes in *Anopheles gambiae* mosquitoes were shown to play important roles in defense against malaria parasites. Of special interest among these are cSPs and SPHs that regulate several immune responses including melanization, coagulation, Toll pathway activation and complement regulation. The main complexity in these immune responses resides in understanding the fine regulation of the CLIP cascades that control effector molecule activation, be it PPO or the protein TEP1. This complexity is indirectly reflected in the large number of genes encoding cSPs and SPHs in several insects, especially in mosquitoes. Hence, a thorough functional and molecular characterization of both cSP and SPHs is required in order to build a comprehensive protease network with clear connectivities. CLIPA7 has been previously identified in the context of a functional genetic screen as an SPH that acts as negative regulator of *Plasmodium* melanization (Volz et al., 2006). In **specific aim1** of my project, I studied the functional contribution of CLIPA7 to immune defense against infection by: First re-investigating the impact of CLIPA7 on *Plasmodium* melanization to test whether there has been any changes in the phenotype as a result of bottlenecks occurring in the colony after several years of inbreeding. Second, I investigated the role of CLIPA7 in modulating mosquito tolerance and resistance to bacterial infections by monitoring survivals to infection and bacterial proliferation in *CLIPA7* kd mosquitoes. In **specific aim2**, I mainly focused on characterizing the role of CLIPA7 at the molecular level by combining RNAi with western blot analysis to test whether silencing CLIPA7 impacts the level of cleavage of other key SPHs, such as CLIPA8, CLIPA28 and CLIPA14, in the mosquito

hemolymph after bacterial infections. The ultimate goal from this aim is provide some insight into the hierarchical position of CLIPA7 in the SPH network.

CHAPTER III

RESULTS

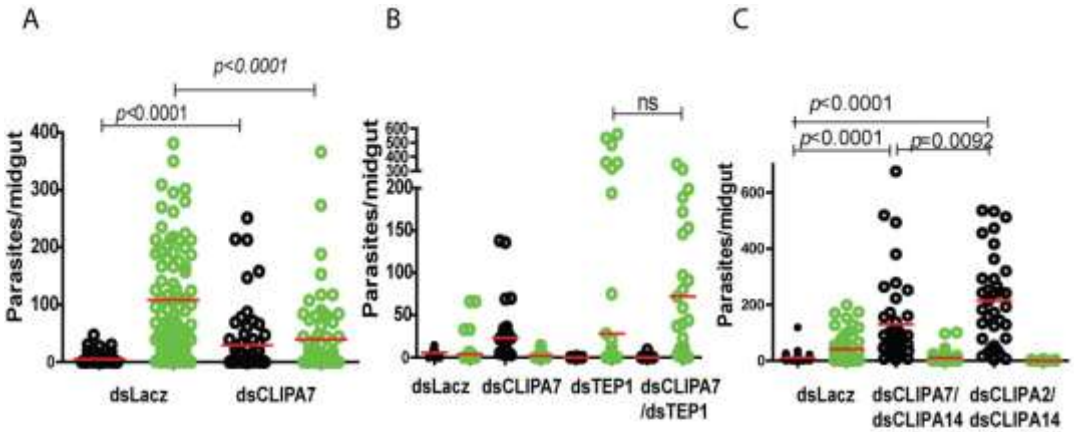
A. CLIPA7 Kd triggers *Plasmodium* ookinete melanization in susceptible mosquitoes.

A previous systematic functional genetic screening of a panel of *A. gambiae* CLIPs by RNA interference (RNAi) identified several CLIPs, both cSPs and SPHs, involved in the melanization of *P. berghei* ookinetes residing in the basal labyrinth of the mosquito midgut epithelium (Volz et al., 2006). Based on the RNAi phenotypes two functions have been ascribed to mosquito SPHs in the context of melanization. The positive regulators of the response which include CLIPA8, whose knockdown abolished parasite melanization and the negative regulators of the response which include CLIPA2, CLIPA14 and CLIPA7, whose knockdown triggered parasite melanization to different degrees. While CLIPA2 (Yassine et al., 2014) and CLIPA14 (Nakhleh et al., 2017b) have been recently subjected to in-depth characterization, the role of CLIPA7 in mosquito immune defense was not rigorously addressed. CLIPA7 is an unusual SPH owing to its structure that contains, in addition to the clip and protease-like domains, several other protein-protein interaction domains including a proline rich domain, a tropoelastin and prion-like domains (Volz et al., 2006), which suggest that CLIPA7 is involved in interactions with multiple proteins.

Although the CLIPA7 Kd was previously shown to trigger the melanization of a small percentage of parasites (Volz et al., 2006), we repeated these assays to determine whether the strength of the response has changed since the mosquito colony is likely to go through several bottlenecks after years of in-breeding. Our data revealed that CLIPA7 Kd in susceptible G3 strain mosquitoes triggered the

melanization of 43 % of *P. berghei* ookinetes. Further, 31% of the analyzed midguts contained not a single live oocyst (Figure 6 A). Ookinete melanization observed in the two other negative regulators CLIPA2 (Yassine et al., 2014) and CLIPA14 (Nakhleh et al., 2017b) was shown to be TEP1-dependent. Due to the key role of TEP1 in the melanization response we addressed whether TEP1 is also required for ookinete melanization in *CLIPA7* Kd mosquitoes using genetic epistasis analysis. The results revealed that TEP1 is indeed required for ookinete melanization since *CLIPA7*/*TEP1* dKd mosquitoes showed only basal level melanization similar to *LacZ* kd controls (Figure 6 B**)**

CLIPA2 and CLIPA14 were shown to act synergistically to negatively regulate *P. berghei* melanization since their simultaneous knockdown triggered a more robust melanotic response than the individually silenced genes (Nakhleh et al., 2017b). Since the RNAi phenotype of CLIPA14 is stronger than that of CLIPA2, we wanted to address whether *CLIPA7*/*CLIPA14* dKd exhibits a more stronger melanization phenotype than that observed for *CLIPA2*/*CLIPA14* dkd mosquitoes. The results revealed that ookinete melanization in *CLIPA2*/*CLIPA14* dkd mosquitoes was more pronounced in inducing ookinete melanization, suggesting that CLIPA2 and CLIPA14 play a more important regulatory role in the melanization response than CLIPA7 (Figure 6 C).



Gene Kd	n	% melanization	prevalence (%)
Lacz	96	4.4	93.75
CLIPA7	64	43	68

Gene Kd	n	% melanization	prevalence (%)
Lacz	45	22.9	77.3
CLIPA7/CLIPA14	43	92.28	54.8
CLIPA2/CLIPA14	46	99.5	42.9

Figure 6. *CLIPA7* Kd mosquitoes trigger ookinete melanization in a TEP1-dependent manner. Scatter plots representing the distribution of *P. berghei* parasites scored in the midguts of the indicated mosquito genotypes seven days post-infection. Green circles represent live GFP-expressing *P. berghei* oocysts and black circles represents dead melanized ookinetes. (A) CLIPA7 kd in wildtype mosquitoes, (B) genetic epistasis analysis with TEP1, (C) genetic epistasis analysis with CLIPA14. Data were pooled from five independent biological experiments for A and from two biological experiments for B and C with the Red lines indicating the median number of parasite. Statistical analysis was performed using the Mann-Whitney test and p-values less than 0.05 were considered significant.

B. Measuring the effect of *CLIPA7* kd on lifespan of naïve mosquitoes

Melanization is known to trigger a fitness cost in insects. For instance, *SRPN2* kd in *A. gambiae* triggered the appearance of spontaneous pseudotumors in tissues, associated with a reduction in the mosquito lifespan (Michel et al., 2005). To determine whether *CLIPA7* Kd in naïve mosquitoes exhibits any fitness cost, we measured the survival of these mosquitoes over a period of 3 weeks as compared to *LacZ* Kd controls. The results showed that *CLIPA7* Kd mosquitoes had similar survival pattern as controls which suggested that CLIPA7 have no fitness cost at least

on mosquito lifespan (Figure 7 A). To ensure that CLIPA7 is efficiently silenced we measured the relative abundance of its mRNA transcripts by qRT-PCR. The data revealed that the RNAi silencing efficiency was 96% (Figure 7 B).

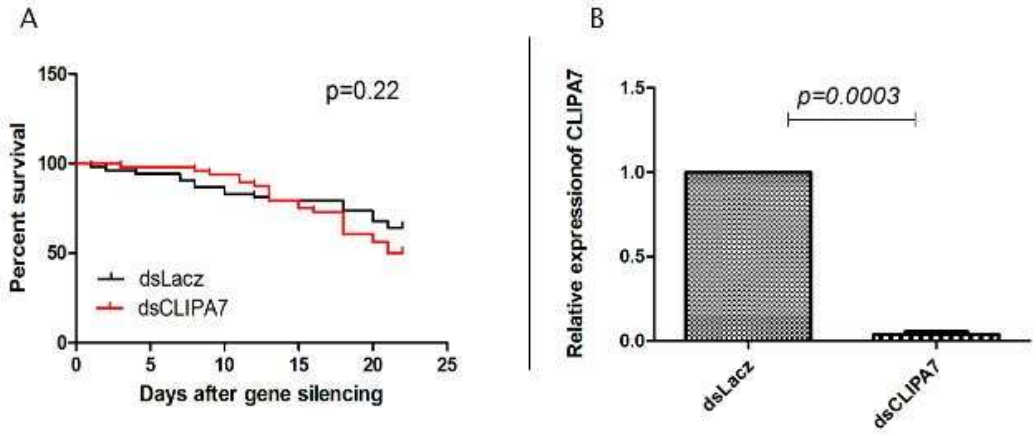


Figure 7. *CLIPA7* Kd have no effect on the fitness of naive mosquitoes. (A) Survival assay of the indicated mosquito genotypes. One representative experiment is shown from two independent biological experiments. Statistical analysis was performed using The Kaplan-Meier survival test and the statistical significance was calculated using the Log-rank test. (B) Efficiency of CLIPA7 silencing by RNAi. Shown are mean expression levels relative to the *LacZ* kd control mosquitoes for which the mean value was adjusted to a value of "1". Data is from two independent biological experiments. Statistical analysis was performed using unpaired t-test.

C. Effect of *CLIPA7* kd on mosquito tolerance and resistance to bacterial infections

The RNAi phenotypes of several mosquito negative-regulatory SPHs with respect to tolerance and resistance to bacterial infections were highly variable. For instance, while *CLIPA2* kd mosquitoes exhibited significantly enhanced tolerance to *E. coli* infections (Yassine et al., 2014), *CLIPA14* kd mosquitoes showed basal level tolerance to *E. coli* but reduced tolerance to *S. aureus* infections, despite the fact that both kd exhibited a dramatic reduction in *E. coli* CFUs. To determine whether CLIPA7 plays a role in anti-bacterial defense, we monitored the survivals of *CLIPA7*

kd mosquitoes after infection with either *E. coli* or *S. aureus* bacteria. Our data revealed that *CLIPA7* kd mosquitoes had similar survival patterns as *LacZ* kd controls with respect to *E. coli* infections (Figure 8 A). However, *CLIPA7* Kd mosquitoes showed consistently enhanced survival compared to controls upon *S. aureus* infection (Figure 8 B**)**. Interestingly, *CLIPA7* kd did not influence the CFU levels of either bacterial species relative to controls (Figure 8 C, D), indicating that it does not alter mosquito resistance to infection.

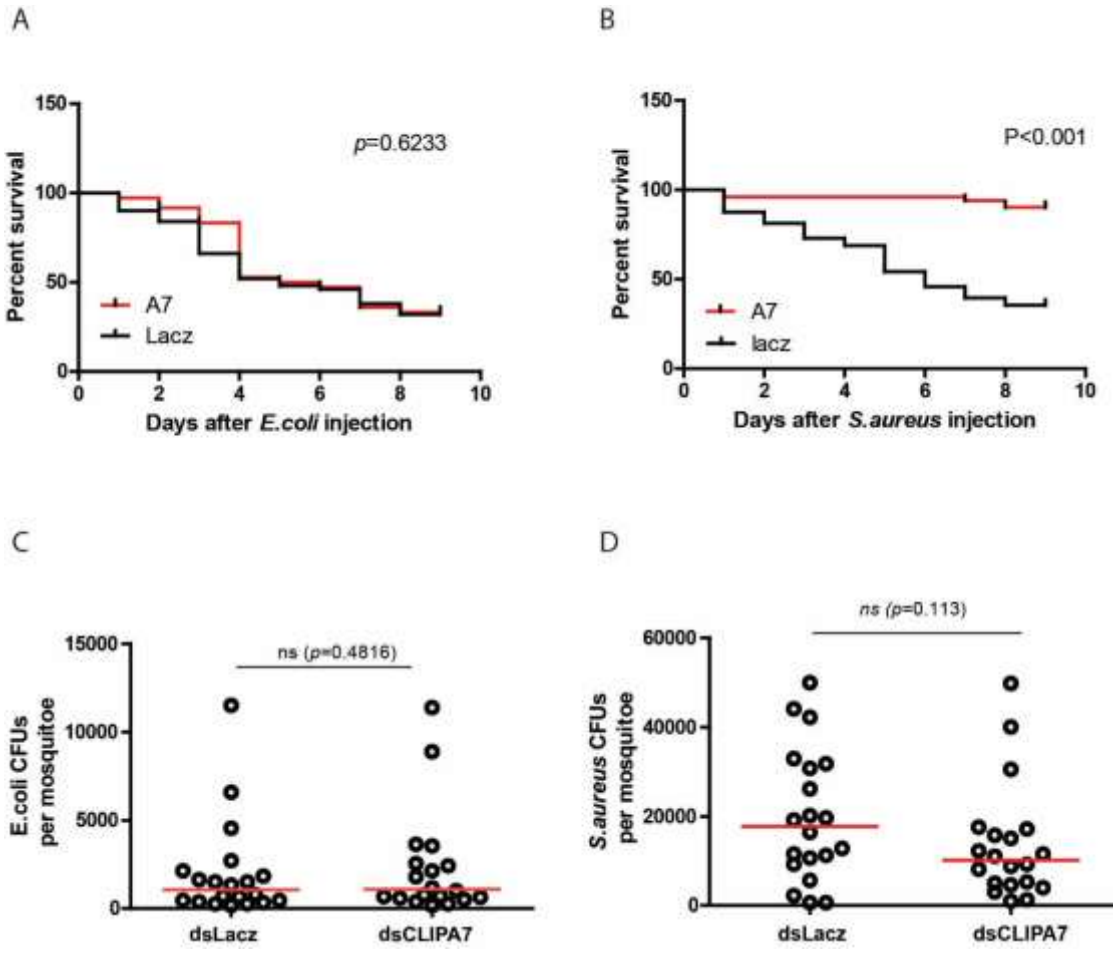


Figure 8. *CLIPA7* Kd enhances mosquito tolerance to *S. aureus* but not *E. coli* infections. Mosquito survival assays following infection with (A) *E. coli* (OD_{600} = 0.4) and (B) S. aureus (OD_{600} = 0.4). One representative experiment is shown from four independent biological experiments. Statistical analysis was performed using the Kaplan-Meier survival test and the statistical significance was calculated using the Log-rank test. Bacterial proliferation assays conducted on whole mosquitoes following infection with (C) *E. coli* (OD_{600} = 0.8) and (D) *S. aureus* (OD_{600}= 0.4).

Batches of 8 mosquitoes were grinded in 1x PBS medium at 24 and 48 hrs post- *E. coli* and *S. aureus* infections, respectively. Colony forming units (CFU) were scored on antibiotic treated LB plates. Each point on the scatter plot represents the mean CFU per mosquito per batch. Data were collected from four independent biological experiments. Red lines represent the median of CFUs distribution. Statistical analysis was performed using the Mann-Whitney test and p-values less than 0.05 were considered significant.

D. CLIPA7 is not induced by bacterial infections

In order to test the effect of septic infection on the expression of *CLIPA7*, mosquitoes were infected with *E. coli* or *S. aureus* and CLIPA7 mRNA expression was monitored in cDNA prepared from whole mosquitoes at the indicated time points by qRT-PCR. The results showed that neither *E. coli* nor *S. aureus* infection enhanced the expression of CLIPA7 (Figure 9 A, B). In addition, the expression of CLIPA7 is not significantly altered after *B. bassiana* infection (Figure 9 C). However, whether CLIPA7 is subject to post-transcriptional control awaits the production of specific antibodies to measure the temporal variations in CLIPA7 protein levels in the hemolymph of infected mosquitoes.

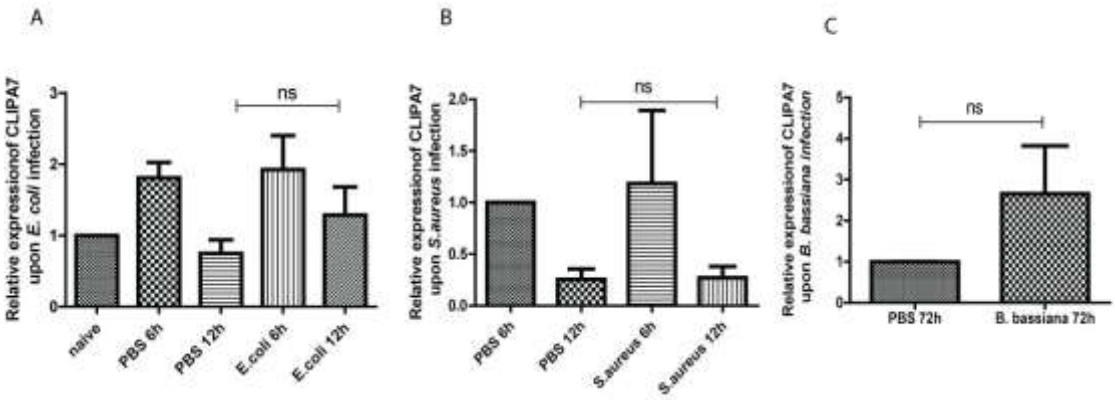


Figure 9. CLIPA7 is not induced by systemic bacterial infections. Expression levels of *CLIPA7* in mosquitoes infected with (A) *E. coli*, (B) *S. aureus* and (C) *B. bassiana* at the indicated time points after infection. Shown are mean expression levels relative to the naive control mosquitoes in (A) or relative to mosquitoes injected with PBS in (B,C) for which the mean value was adjusted to a value of "1".

Data shown is from 3 independent biological experiments. Error bars indicate SEM of three independent experiments. Statistical analysis was performed using unpaired t-test and means were considered significantly for $p < 0.05$

E. Hierarchical position of CLIPA7 in the SPH network

Although non-catalytic, mosquito SPHs seem to be activated in a hierarchical manner. We have previously shown that SPCLIP1 controls the cleavage of CLIPA8 (Povelones et al., 2013) and more recently CLIPA8 was shown to control the cleavage of CLIPA28, a novel positive regulator of the melanization response (Osta MA, unpublished data). Also SPCLIP1, CLIPA8 and CLIPA28 are all required for the activation cleavage of CLIPA14 [(Povelones et al., 2013) and (Osta MA, unpublished)]. This reveals that SPHs are arranged in a hierarchical module where some members like CLIPA14 may also act in a negative feedback loop to control the cleavage of upstream components (Figure 12). To determine the putative position of CLIPA7 in this module we scored the effect of its RNAi knockdown on the cleavage patterns of CLIPA8, CLIPA28 and CLIPA14 by western blot analysis. To that purpose, CLIPA7 kd mosquitoes were infected with *S. aureus* and hemolymph was extracted 1 hour post-infection and protein quantified by Bradford analysis. Western blot analysis revealed that CLIPA7 kd significantly enhanced CLIPA8 cleavage in the hemolymph which is consistent with its role as a negative regulator of melanization (Figure 10 A); band quantification revealed more than two fold increase in CLIPA8 cleaved form (CLIPA8-C) compared to LacZ (Figure 10 A`) . This suggests that CLIPA7 negatively regulates the activation cleavage of CLIPA8 in the hemolymph following systemic infections.

As mentioned above, CLIPA28 was recently shown to act downstream of CLIPA8. However, *CLIPA7* kd did not affect CLIPA28 cleavage (Figure 10 B), indicating that it specifically act at the level of CLIPA8. Although there seems to be a trend toward slightly enhanced cleavage of CLIPA28 in *CLIPA7* kd mosquitoes, band quantification revealed that the difference with control was not significant (Figure 10 B`).

CLIPA14 is the most potent negative regulator of the melanization response identified so far. Biochemical studies also show that CLIPA14 is the most downstream in the SPH module [(Nakhleh et al., 2017b) and (Osta MA, unpublished)]. Interestingly, western blot analysis revealed that CLIPA7 kd almost completely abolished the cleavage of CLIPA14 (Figure 10 C); band quantification revealed that the cleaved form of CLIPA14 (CLIPA14-C) is reduced by approximately seven folds with respect to *LacZ* kd control (Figure 10 C`). This suggests that CLIPA7 is strongly required for the activation of CLIPA14 following systemic infections.

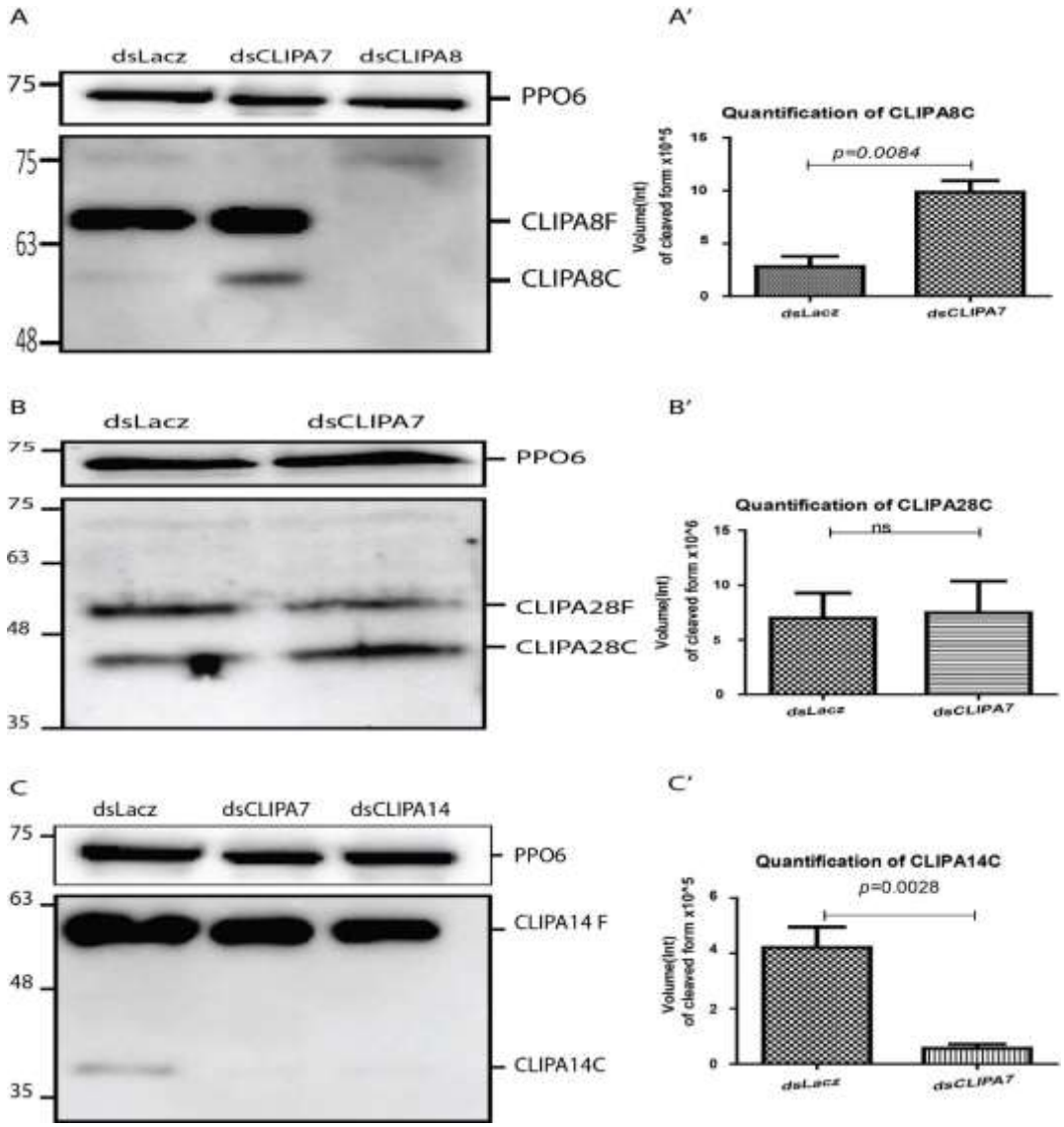


Figure 10. **CLIPA7 negatively regulates the cleavage of CLIPA8 but is required for CLIPA14 cleavage**. Western blot analysis of hemolymph extracts collected 1 hour after *S. aureus* infection of the indicated mosquito genotypes. The membranes were probed with antibodies against (A) CLIPA8, (B) CLIPA28 and (C) CLIPA14. Protein quantification was performed using the Bradford protein assay and 2.5, 2.3 and 1.95 µg of hemolymph proteins were loaded per lane in figures A, B and C, respectively. PPO6 was used as further evidence of equal loading. Each image is representative of three independent experiments. Protein quantification of the cleaved forms of the respective proteins in A`, B` and C` was calculated using image lab. Error bars indicate SEM of three independent experiments. Statistical analysis was performed using unpaired t-test and means were considered significantly different for *p*-value < 0.05.

Since CLIPA7 and CLIPA14 are both negative regulators of the melanization response we hypothesized that co-silencing both genes might trigger a more potent

cleavage of the positive regulators CLIPA8 and CLIPA28. However, western analyses revealed that the cleavage patterns of the latter proteins were similar in *CLIPA7* kd and *CLIPA7*/*CLIPA14* dkd mosquitoes (Figure 11 A&B), indicating that there is no synergy between these proteins.

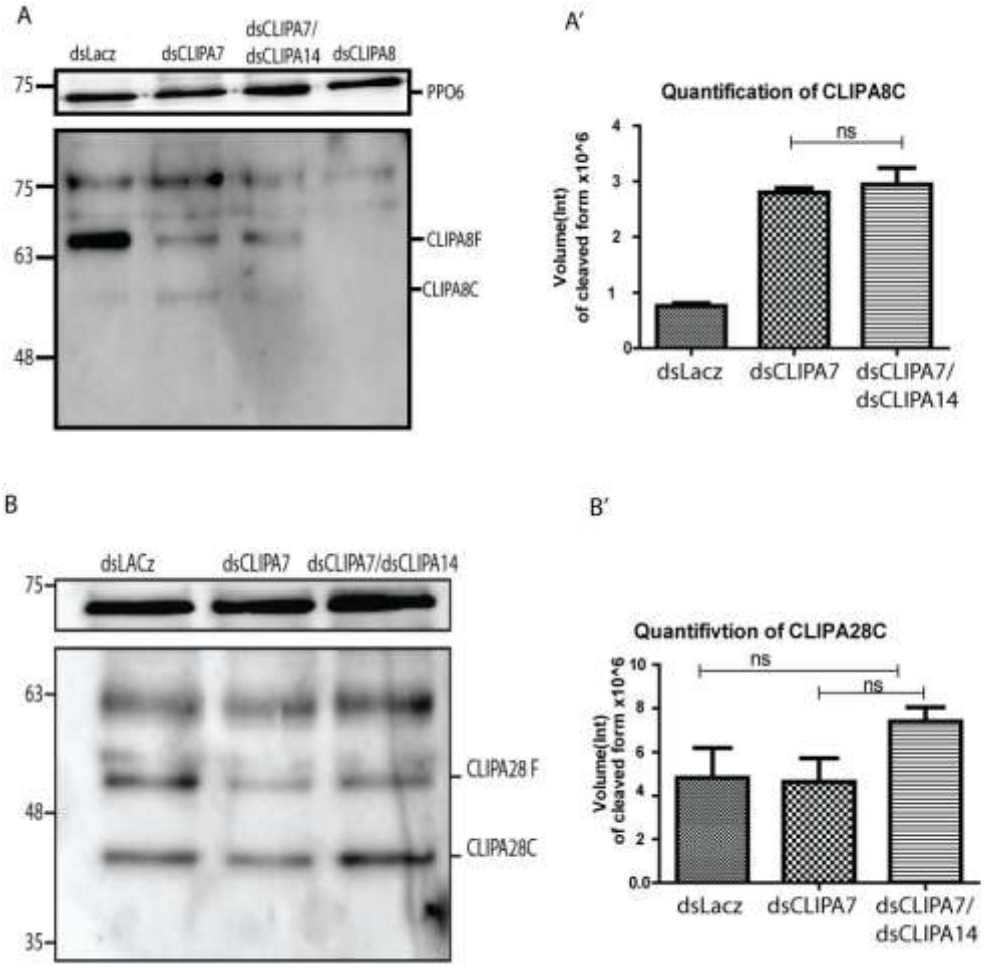


Figure 11. CLIPA7 negatively regulates the cleavage of CLIPA8 and it acts upstream of CLIPA14. Western blot analysis of hemolymph extracts collected 1 hour after *S. aureus* infection of the indicated dsRNA treatments. The membranes were probed with antibodies against CLIPA8 (A) and CLIPA28 (B) and PPO6 was used as loading control. Protein quantification was performed using the Bradford protein assay and 2.59 and 2.9µg, of hemolymph proteins were loaded per lane in figures A and B respectively. The image is representative of two independent experiments. Protein quantification of the cleaved forms of the respective proteins in A` and B` was calculated using image lab. Error bars indicate SEM of two independent experiments. Statistical analysis was performed using unpaired t-test and means were considered significantly for *p*-value < 0.05

CHAPTER IV
DISCUSSION

SPHs play important roles in mosquito immunity despite being non-catalytic. Several SPHs have been identified so far which exhibit positive or negative regulatory roles in the melanization response. Some SPHs like CLIPA2 and SPCLIP1 may also constitute nodes at which cross-talk occurs between the complement (Povelones et al., 2013) and melanization (Yassine et al., 2014; Kamareddine et al., 2016) responses. Biochemical data from our lab also points to a hierarchical mode of activation of SPHs [(Povelones et al., 2013; Nakhleh et al., 2017a); Osta MA, unpublished] indicating that these molecules may impose a multilayered control on the melanization cascade that is not limited to the final step of PPO activation as suggested from studies in other insects (Lee et al., 2002; Yu et al., 2003; Lu and Jiang 2008). The full spectrum of SPH involved in mosquito immunity is still unknown and the activation cleavage patterns of even several functionally characterized candidates like CLIPA2, SPCLIP1 and CLIPA7 remain to be determined. These cleavage profiles are important read outs which when combined with gene silencing experiments permit to define hierarchy among SPHs. In this thesis project we aimed to further characterize CLIPA7 at the functional and molecular levels to highlight specifically two major points: First, its contribution to immune responses and second, its relative position in the SPH module. While the first has been achieved, the second has been partly addressed because we were not able to produce the antibody against CLIPA7, to characterize its cleavage pattern, before the termination of this thesis project due to time limitations.

CLIPA7 has been previously identified in the context of a functional genetic screen of CLIPs as an SPH that negatively regulates the melanization of *Plasmodium* ookinetes (Volz et al., 2006); At that time, it was reported that *CLIPA7* kd triggered the melanization of 16% of invading *P. berghei* ookinetes. First, we repeated this assay to determine whether there are changes in the RNAi phenotype of CLIPA7 since mosquito colonies are expected to go through several bottlenecks while inbred which would influence allelic distributions and hence phenotypes of certain genes. Indeed, here we found that *CLIPA7* kd triggered a more pronounced melanization reaching to 43% of ookinetes as compared to our previous study. These changes in RNAi phenotypes that occur between two distant periods of time have been previously observed for CLIPA2 (Yassine et al., 2014) and CLIPA14 (Nakhleh et al., 2017a), indicating that inbred colonies undergo genetic variations with time that may influence immune phenotypes. Ookinete melanization in *CLIPA7* kd mosquitoes requires TEP1 function. The fact that TEP1 was also shown to be required for parasite melanization in CLIPA2 and *CLIPA14* kd mosquitoes further confirms its role as a key player in mosquito melanization. TEP1 seems to be acting upstream of the SPH module since the cleavage patterns of several SPHs including CLIPA8, CLIPA28 and CLIPA14 are controlled by TEP1. Unfortunately antibodies are lacking to examine effect of TEP1 on the remaining SPH involved in melanization such as CLIPA2, SPCLIP1 and CLIPA7. Co-silencing CLIPA2 and CLIPA14 gave an additive effect with respect to the intensity of ookinete melanization as previously described (Nakhleh et al., 2017a), however such an effect was not observed in *CLIPA7*/*CLIPA14* dkd mosquitoes, which indicates that CLIPA14 role dominates over that of CLIPA7.

The examination of CLIPA7 role in antibacterial immunity revealed that CLIPA7 is not involved in resistance to infection as the CFU counts of *E. coli* and *S. aureus* in infected *CLIPA7* kd mosquitoes were similar to those in *LacZ* kd controls. However, CLIPA7 kd mosquitoes exhibited drastically high tolerance to *S. aureus* but not *E. coli* infections. These results suggest that *CLIPA7* kd mosquitoes can cope better with the consequences of *S. aureus* infections compared to control. A plausible explanation to that phenomenon is the possible involvement of CLIPA7 in other physiological responses such as tissues repair or coagulation. If these responses are enhanced they are likely to provide the host with increased fitness. Since CLIPs are involved in the coagulation response (Kurata et al., 2006), one would posit that if CLIPA7 exhibits a negative regulatory role over the CLIP cascade involved in coagulation then increased coagulation in CLIPA7 kd may enhance bacterial trapping, preventing their spread to tissues, hence reducing tissue damage. In fact there is evidence that certain CLIPs constitute nodes through which melanization cross-talks with other immune responses [reviewed in (Nakhleh et al., 2017d)]. This might not be surprising in the case of CLIPA7 since it is an unusual SPH which, in addition to the clip-domain, has several other protein-protein interaction domains that may permit interactions with more than one protein.

The cleavage pattern of SPHs helps defining their hierarchy in the SPH module that controls melanization. We have previously shown that CLIPA8 cleavage requires SPLCIP1 and more recent data indicate that CLIPA8 in its turn is required for the cleavage of CLIPA28 (Osta MA, unpublished); hence, in this functional module SPCLIP1 is most upstream followed by CLIPA8 and then CLIPA28. Since CLIPA7 kd enhances the melanization response we hypothesized that its knockdown might enhance the cleavage of CLIPA8 and CLIPA28 which are both positive

regulators of the response. Interestingly, while *CLIPA7* kd enhanced CLIPA8 cleavage no effect was observed on CLIPA28. These data suggest that CLIPA7 might be negatively regulating a catalytic CLIP (cSP) that specifically cleaves CLIPA8 but not the one that cleaves CLIPA28. This could explain why enhanced CLIPA8 cleavage did not result in increased CLIPA28 cleavage despite being upstream of it. Despite the evidence for a hierarchy in the SPH module, the cSPs that cleave these SPHs and the levels at which SPHs and their catalytic counterparts interact within the broader clip network are interesting questions that remain to be addressed. We have recently shown that both CLIPA8 and CLIPA28 control the cleavage of CLIPA14 which seems to be most downstream in the module (Osta MA, unpublished). Interestingly, *CLIPA7* kd abolished CLIPA14 cleavage after bacterial infection which was rather unexpected since CLIPA7 is a negative regulator as inferred from its RNAi phenotype and from its effect on CLIPA8 cleavage. It is also difficult to interpret this result because it infers that CLIPA7 should have similar RNAi phenotype as CLIPA14 with respect to ookinete melanization yet this is clearly not the case as the latter has a significantly stronger phenotype (Nakhleh et al., 2017a). It is possible that the full form of CLIPA14 is also functional in melanization, however only *in vitro* reconstitution assays can test this hypothesis. It is tempting to speculate that CLIPA7 might positively or negatively regulate the cleavage patterns of SPHs based on the nature of the cSP it interacts with. This also need to be addressed using rigorous *in vitro* reconstitution assays with recombinant SPH and cSP. These assays will also require that the cSPs that control the cleavage of these SPHs in the hemolymph be identified first. The fact that co-silencing *CLIPA14* and *CLIPA7* did not enhance CLIPA8 cleavage suggests that CLIPA14 has a more dominant role in this regard.

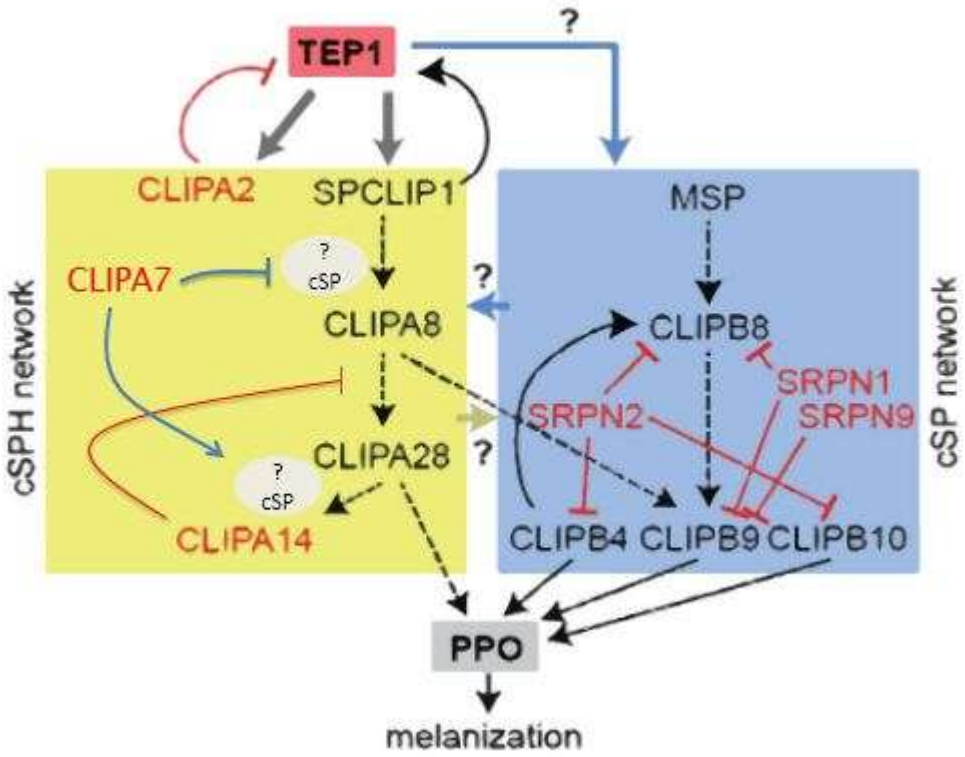


Figure 12. schematic representation of CLIPs cascade involved in the regulation of melanotic immune response

In conclusion, despite their non-catalytic nature a large number of mosquito SPHs have been shown to exhibit strong RNAi phenotypes in the context of immunity in particular melanization. Interestingly, many of these phenotypes are even stronger than those observed for catalytic cSPs, which might indicate the more specific and targeted function of SPHs as compared to the redundancy in function of cSPs. Serpins are historically known to be the key regulators of serine protease cascades. Adding an additional layer of control through SPHs will probably allow to better fine tune these cascades and add more stringency on the substrate specificity of cSPs. Understanding these interactions within protease networks is a crucial aim to draw a comprehensive picture of insect immunity owing to their importance in driving several key immune responses.

CHAPTER V

MATERIALS AND METHODS

A. Ethics statement

All the studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (U.S.A). the Institute of Animal Care and Use committee at the American University of Beirut (IACUC) approved the Animal protocol (permit number 11-09-199), which agrees with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (U.S.A)

B. Materials

1. Anopheles gambiae mosquito strain

Anopheles gambiae mosquitoes of the G3 susceptible strain were used in all experiments. For maintenance and mosquito production, adults were fed on a Balb/c mouse anaesthetized with 100 µl mixture of 0.3 mg xylazine and 4.2mg ketamine. Mosquitoes were maintained at 27°C, 80% humidity and 12 day-night cycle. Larvae were fed on tropical fish food and adults were maintained on 10 % sucrose.

2. Bacterial, fungal, and P. berghei parasite strains

GFP-expressing and ampicillin resistant *E. coli* strain OP-50 and tetracycline-resistant *S. aureus* strain were grown at 37°C on Luria-Bertani (LB) medium

supplemented with 100 μg/ml ampicillin and 50 μg/ml tetracycline, respectively. Bacteria were pelleted by centrifugation at 4000 rpm and washed 3 times with (1x) PBS. The pellet was then re-suspended in (1x) PBS and adjusted to the appropriate OD_{600} that is needed for mosquito challenges.

Wildtype *B. bassiana* were grown on potato dextrose agar plates at 25°C in a 75-80% humid environment. The spores were extracted from the agar plates by scrapping in ddH2O. Fungal structures were collected and passed through a funnel containing sterile glass wool in order to separate the spores from other mycelial structures. The spores were collected and washed two times with ddH2O and centrifuged at 4000 rpm. The spores were then counted and diluted to the desired concentration needed for infecting the mosquitoes that is done through spraying.

Recombinant *P. berghei* parasite expressing GFP was passaged in Balb/c mice. The level of parasitaemia in the infected mouse was determined by a blood smear prepared from a drop of blood extracted from the mouse tail. The smear was fixed with methanol and then stained with 10% giemsa for 45 minutes. Mice showing 4-5 % parasitemia were used to infect the mosquitoes. Feeding the mosquitoes on infected mice was carried in darkness at 20°C.

C. Mosquitoes infection

1. Bacterial infections

Individual female mosquitoes that were injected with dsLacz or dsCLIPA7 were then infected with freshly prepared *E. coli* or *S. aureus* bacteria at OD_{600}= 0.4. Then, mosquitoes survivals were scored over a period of 9 days. This experiment was

repeated four times and the Kaplan-Meier test was performed on each experiment. Statistical significance of the observed differences was calculated using the Log-rank test. Difference were considered significant for *p*-value< 0.05. Concerning the bacterial proliferation assay, mosquitoes were infected with *S. aureus* at OD_{600}= 0.4. Then 48 hours post infection, five batches of 8 mosquitoes each were grinded in 400 µl (1x) PBS on ice. The homogenates were then plated on tetracycline treated agar plates at the serial dilutions (1:100,1:1000 and 1:10000) and incubated at 37°C for 12 hours. The Data was reported as the number of CFUs developed per mosquito per batch. The experiment was repeated three times. Statistical analysis was performed using Mann-Whitney test on the pooled data and the differences between tested groups was considered significant for *p*-value< 0.05. The same was performed on *E. coli* infection except that OD_{600}=0.8 was used to challenge mosquitoes and homogenates were plated on ampicillin treated plates at serial dilutions (1:50,1:100 and 1:1000).

2. P. berghei infection

Three to four days post silencing, mosquitoes were fed on a mouse infected with *P. berghei* with around 4-5% parasitaemia. Feeding was carried in a humid container at 20°C. One or two days post infection mosquitoes were anesthetized on CO2 and non-fed mosquitoes were removed. Guts of infected mosquitoes were dissected 7 to 9 days post-infection, then fixed in 4% paraformaldehyde for 50 minutes, and then washed three times with (1x) PBS. The guts were then mounted on glass slides using the ProLong® Gold antifade reagent. Live oocysts (GFP fluorescent) and melanized black ookinetes were counted under a fluorescent

microscope. Statistical analysis was performed using Mann-Whitney test and differences between tested groups were considered significant if p-value<0.05.

D. Molecular biology

1. Gene silencing by RNA interference

RNAi Gene silencing was carried by injecting specific dsRNA into the thorax of female mosquitoes as described in (Blandin et al., 2002). Before dsRNA production, the amplicon of interested (prepared from cDNA and inserted into a plasmid) should be amplified by PCR using T7 primers (shown below) and then purified and extracted with phenol:chloroform. For dsRNA production the Promega transcript aid kit was used and dsRNA was produced according to the manufacturer instructions. Then the produced dsRNA was purified and extracted with phenol:chloroform:isoamylalcohol, and then precipitated with isopropanol. The pellet was then resuspended in nuclease free water and the concentration of dsRNA was measured using nanodrop. The final concentration was adjusted to 3.5 µg/µl, and 69nl of corresponding dsRNA was injected to each female mosquitoes.

List of primer used for dsRNA production (sequence 5'-3'): The T7 promoter sequence is underlined**.**

LacZ-F TAATACGACTCACTATAGGGAGAATCCGACGGGTTGTTACT

LacZ-R TAATACGACTCACTATAGGGCACCACGCTCATCGATAATTT

TEP1-F TAATACGACTCACTATAGGGTTTGTGGGCCTTAAAGCGCTG

TEP1-R TAATACGACTCACTATAGGGACCACGTAACCGCTCGGTAAG

CLIPA8-F

TAATACGACTCACTATAGGGAACAACGAACCCGTAGAATATG

CLIPA8-R

TAATACGACTCACTATAGGGGGTTAGCGCCTCGATACC2.

CLIPA7-F

TAATACGACTCACTATAGGGGCCGATCTGATGGAGACGGTCAAC

CLIPA7-R

TAATACGACTCACTATAGGGGAACATTGGCACGTTCACGTACAC

CLIPA14-F

TAATACGACTCACTATAGGGCGGCATCATCGACATCCGTGTC

CLIPA14-R

TAATACGACTCACTATAGGGGTTGCTGTCGGCGACACGCTCCT

2. RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted from 15 mosquitoes infected with either *E. coli* or *S. aureus* according to the supplier's instructions, and contaminant genomic DNA was removed by DNase I treatment. The pellet was resuspended with nuclease free water and the concentration was measured using Nanodrop. First strand cDNA was produced from 1 µg total RNA using the iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad). QRT-PCR was performed in a CFX96 Real-Time Detection System (Bio-Rad) using the SYBR Green JumpStart^{TM} Taq ReadyMix (Sigma-Aldrich) according to the manufacturer's instructions. Relative gene expression values were calculated using the Comparative C_T Method. The S7 ribosomal gene was used to normalize the relative expression of CLIPA7 between tested groups. The experiment was repeated three times and statistical analysis was

performed using unpaired t-test and differences were considered significant if *p-value*< 0.05. Primers used for Real-time PCR are the following:

CLIPA7-F 5`GTACGFTGAGTTCCCGTGGAT 3`

CLIPA7-R 5` ACGTTGATCACTTGGTCCAGC 3`

E. Protein biochemistry

1. Hemolymph extraction and western blotting

Mosquito hemolymph was extracted after 1 hour of *S. aureus* infection by the classical proboscis clipping technique in (1x) PBS containing protease inhibitor cocktail. Protein quantification was performed using Bradford reagent and the concentration of proteins was adjusted to be the same between different samples. The quantified samples were loaded into reducing SDS-PAGE gel (10%) and were then transferred to Immuno-Blot PVDF membrane. After protein transfer, the membrane was blocked in (1x) PBS containing 5% skimmed milk and 0.05% tween 20 at room temperature for 1h. Then the membrane was probed overnight with specific primary antibody at 4°C. Three primary antibodies were used at the respective dilutions α-CLIPA8 (1:100), α - CLIPA28 (1:2000) and α - CLIPA14 (1:3000). α -PPO6 antibody was used as loading controls. After incubation with primary antibodies, the membrane was washed with (1x) PBS (3 times, 10min each), followed by incubation with peroxidase conjugated α - mouse (1:6000) or α -rabbit (1:12000) secondary antibody for 1 hour at RT. Membranes were then washed with (1x) PBS and incubated with Western ECL Substrate for 2 min and images were taken in BioRad ChemiDoc^{TM} MP Imaging System machine. Each experiment was repeated three times. Quantification of bands was done using Image Lab and statistical analysis was

performed using unpaired t-test. The difference between the tested groups and the controls was considered significant if p-value<0.05.

F. CLIPA7 protein expression and purification for antibody production

1. CLIPA7 transfection in SF9 cells

Cells were already transfected with pIEx-10 plasmid containing the CLIPA7 full-length ORF using established protocols in our lab (Kamareddine et al., 2016; Nakhleh et al., 2017b). To test for successful transfection, PCR reaction was performed on genomic DNA extracted from the cells. First, cells were detached from the flask and incubated with CTAB 2% at 65 °C. The supernatant was then collected and the DNA was precipitated with 70% ethanol. A PCR reaction was performed using CLIPA7 specific primers to check for the presence of CLIPA7-pIEx-10 plasmid.

(CLIPA7-F
TAATACGACTCACTATAGGGGCCGATCTGATGGAGACGGTCAAC
; CLIPA7-R
TAATACGACTCACTATAGGGGAACATTGGCACGTTCACGTACAC)

2. CLIPA7 protein purification.

Cells that are stably secreting CLIPA7 protein were cultured in 20 T75 tissue culture flasks. For each flask, 10ml of conditioned medium (CM) (containing 8.85ml serum-free Sf-900 II SFM Medium, 1ml FBS, 100µl PenStrep and 50µl G-418 selective agent) was added. CM was changed every 5 days until the cells became

confluent. Then CM was collected every 5 days for two rounds for protein purification. CM were centrifuged to remove dead cells and then incubated overnight with 100ml bed volume (for each 50ml media) of biotin beads on a shaker at 4°C. Next day the beads were washed 4 times in the chromatography column with wash buffer (1xPBS+0.05 TritonX-100). The last wash was done with (1x) PBS only. Bound proteins were then eluted twice, each time with 1ml elution buffer (95% formamide +10mM EDTA in water) heated to 65°C. The eluents were then concentrated down to 50 µl using centrifugal filter units with 10 KDa cutoff and exchanged with PBS on desalting columns followed by Bradford protein quantification. Then a volume corresponding to 1 µg was loaded on SDS-PAGE gel and stained with coomassie blue to check for the purity of recombinant CLIPA7 protein. Also, western blot analysis was performed to detect the amount of CLIPA7 purified from the CM using anti-Strep tag II primary antibody (1:2000), and peroxidase conjugated anti rabbit secondary antibody (1:12000) (Figure 13)

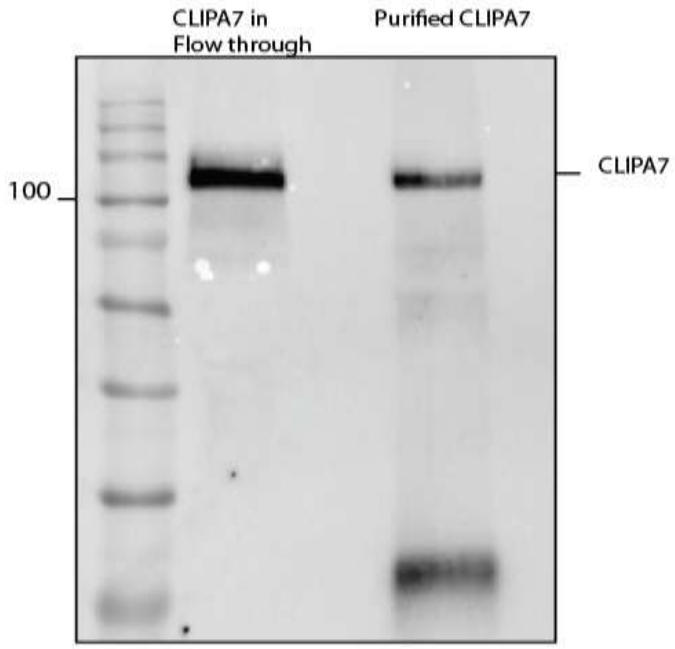


Figure 13. CLIPA7 protein is not efficiently purified from the collected CM. Western blot analysis of CLIPA7 purified from the CM and CLIPA7 remaining in the flow through is shown. The membrane was probed with α–strep tag primary antibody (1:2000) and re-probed with α-rabbit secondary antibody (1:12000).

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