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THE ROLE OF CLIP-DOMAIN SERINE PROTEASE HOMOLOGUE CLIPA14 AND TWO CARBOXYPEPTIDASES CP1 AND CP2 IN *A. GAMBIAE* IMMUNITY

by
JOHNNY NAJA NAKHLEH

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Approved by:

Dr. Mike Osta, Associate Professor Department of Biology, American University of Beirut	Advisor
Dr. Dani Osman Faculty of Sciences III Lebanese University	Member of Committee
Dr. Khouzama Knio, Professor Department of Biology American University of Beirut	Member of Committee
Dr. Laure El Chamy, Assistant Professor Department of Life and Earth Sciences Saint Joseph University	Member of Committee
Dr. Zakaria Kambris, Associate Professor Department of Biology American University of Beirut	Member of Committee

Date of dissertation defense: September 4th 2018

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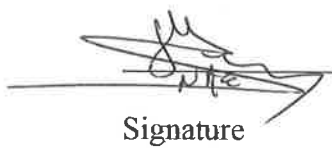

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Date

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AN ABSTRACT OF THE DISSERTATION OF

Johnny Naja Nakhleh for Doctor of Philosophy
Major: Cell and Molecular Biology

Title: The role of clip-domain serine protease homologue CLIPA14 and two carboxypeptidases, CP1 and CP2 in *A. gambiae* immunity

Functional genetic and molecular studies in the malaria vector *Anopheles gambiae* showed that mosquitoes are not passive to *Plasmodium* infections, rather *A. gambiae* mounts potent immune responses against malaria parasites as well as bacterial and fungal infections. Gene discovery tools and systematic functional genetic screens by RNAi helped identify key immunity genes, among which are clip domain serine proteases (CLIPs) that play key roles in diverse aspects of insect immunity.

CLIPs are unique to arthropods. They are composed of one or more clip domains at the N-terminus, and a serine protease domain at the C-terminus containing the catalytic His-Asp-Ser residues. A group of CLIPs is non-catalytic [also known as serine protease homologs *(*SPHs)], whereby the serine and sometimes the aspartic acid residues are substituted by another amino acid. CLIP cascades trigger several downstream effector responses including, antimicrobial peptide synthesis, coagulation and melanization. Melanization is a potent insect immune response characterized by the deposition of melanin on microbial surfaces leading to their elimination. Genetic screens by RNAi identified several catalytic CLIPs and SPHs with strong RNAi phenotypes. Among SPHs, CLIPA8 was initially shown to be essential for the melanization of ookinetes as well as bacteria and fungi. Later, SPCLIP1 and CLIPA2 were found to act as positive and negative regulators of the mosquito melanization response indirectly by amplification of complement. TEP1 is a hallmark effector molecule in mosquito immunity required for defense against *Plasmodium,* bacteria and fungi. These results altogether reveal that cSPHs play intricate roles in the regulation of the melanization and complement responses, and point to a cross talk between complement and melanization.

To identify novel SPHs with immune functions, a proteomic approach was adopted whereby key proteins involved in the melanization response including CLIPA2 and CTL4-

CTLMA2 complex were immunoprecipitated and interacting proteins identified by mass spectrometry. This led to the identification of CLIPA14 as a key gene involved in the regulation of the melanization response to *P. berghei* ookinetes and whose characterization constitutes a major part of this thesis.

We first studied the contribution of CLIPA14 in anti-*Plasmodium* immunity in *Anopheles gambiae*. Our data revealed that *CLIPA14* kd triggers a potent melanotic response against *P. berghei* ookinete, in a TEP1-dependent manner. Additionally, *CLIPA14* kd mosquitoes elicited significantly increased resistance to systemic and oral infections. Phenoloxidase enzyme also exhibited a dramatic increase in activity in the hemolymph of *CLIPA14* kd mosquitoes in response to systemic bacterial infections. Co-silencing *CLIPA14* and *CLIPA2*, a negative regulator of melanization, further increased ookinete melanization and hemolymph phenoloxidase activity, suggesting that these two SPHs act in concert to control the melanization response.

TEP1 is required so far for the cleavage of all SPHs that show clear cleavage patterns. Based on that observation, we adopted a high throughput proteomic approach to identify novel CLIPs whose cleavage is TEP1-dependent. This approach is based on degradomics which aims to identify differentially cleaved substrates in hemolymph of *E. coli*-infected *TEP1* kd compared to *LacZ* kd controls. Interestingly, among the top 10 TEP1-dependent cleaved substrates are two carboxypeptidases (termed CP1 and CP2). To this date, there is no evidence that carboxypeptidases are involved in insect immune responses, and since CP1 and CP2 proteolytic cleavage is regulated by the mosquito complement, this prompted us to characterize the contribution of CP1 and CP2 to mosquito immunity. We showed that CP1 and CP2 are required for anti-*Plasmodium* defense, whereby its RNAi phenotype is similar to that of *TEP1*, and that *CP1* and *CP2* kd affect TEP1 recruitment to ookinete surfaces. However, the exact mechanism by which these carboxypeptidases affect mosquito immunity needs to be further addressed.

This work is expected to shed additional knowledge on cSPH functions as well as on carboxypeptidases as immune proteins involved in the regulation of complement activity and melanization response.

CONTENTS

FIGURES

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ABBREVIATIONS

Ae. aegypti	*Aedes aegypti*
A. albimanus	*Anopheles albimanus*
A. gambiae	*Anopheles gambiae*
A.stephensi	*Anopheles stephensi*
AMP	Anti-microbial peptides
APL1C	*Anopheles Plamodium*-responsive leucine-rich repeat protein 1C
Asp	Aspartic acid
BSA	Bovine serum albumin
CBM 39	Carbohydrate binding module 39
cDNA	Complementary DNA
CDS	Coding sequence
CEC	Cecropin
CFU	Colony forming unit
CHT1	Chitinase 1

CP1	Carboxypeptidase 1
CP2	Carboxypeptidase 2
CRD	Carbohydrate recognition domain
CRISPR	Clustered regularly interspaced short palindromic repeat
cSPH	Clip serine protease homologue
cSP	Clip serine protease
CTL	C-type lectin
CTLD	C-type lectin-like domain
DAP	Diaminopimelic acid
DCE	Dopachrome conversion enzyme
DDC	Dopa decarboxylase
DEF	Defensin
DHI	Dihydroxyindole
DIF	Dorsal related immunity factor
Dkd	Double knockdown
D. melanogastser	*Drosophila melanogaster*

DOME	Domeless receptor
DOPA	Dihydroxyphenylalanine
Dscam	Down syndrome cell adhesion molecule
dsRNA	Double stranded RNA
DUOX	Dual oxidase
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FBG	Fibrinogen like domain
FBN	Fibrinogen domain immunolectin
FREP	Fibrinogen related protein
GAM	Gambicin
GFP	Green fluorescent protein
GNBP	Gram-negative binding protein
GPI	Glycosylphosphatidylinositol
HDF	Hemocyte differentiation factor
HdMv	Hemocyte-derived microvesicles

His	Histidine
HPX2	Heme peroxidase 2
Imd	Immune deficiency
Ig	Immunoglobulin
IRID	Infection-responsive with immunoglobulin domain
IRSF	Immune-responsive splicing factor
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Kd	Knockdown
KO	Knockout
LB	Luria-Bertani broth
LIC	Ligation-independent cloning
LL3	LITAF-like 3 transcription factor
LRIM	Leucine-rich repeat immune protein
LRRD	Leucine-rich repeat domain
MCP	Metallocarboxypeptidase

ModSP	Modular serine protease
NF-κb	Nuclear factor- κb
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX5	NADPH oxidase 5
OD	Optical density
PAH	Phenylalanine hydroxylase
PAMP	Pathogen associated molecular patterns
PAP	Prophenoloxidase activating proteinase
P. berghei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
PBS	Phosphate buffer saline
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PM	Peritrophic matric
P. mondon	*Penaeus mondon*

PPAE	Prophenoloxidase activating enzyme
PPAF	Prophenoloxidase activating factor
PPO	pro-Phenoloxidase
PRR	Pattern recognition receptors
Psh	Persephone
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
RCL	Reactive center loop
RNAi	RNA interference
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SAE	Spätzle activating enzyme
Ser	Serine
SFV	Semliki Forest Virus

SP	Serine protease
SPE	Spätzle processing enzyme
SPH	Serine protease homologue
Spz	Spätzle
SRPN	Serine protease inhibitor
STAT	Signal transducers and activators of transcription
TAFI	Thrombin-activatable fibrinolysis inhibitor
TBV	Transmission-blocking vaccines
UPD	Unpaired
WASP	Wiskott-Aldrich syndrome protein

CHAPTER I
INTRODUCTION

A. The life cycle of malaria parasite in its mosquito vector

The *Plasmodium* parasite undergoes a complex life cycle within its mosquito vector before being transmitted to the vertebrate host. After a female *Anopheles gambiae* ingests an infected blood meal, *Plasmodium* life cycle is initiated. Blood ingestion triggers gametogenesis of female (macrogamete) and male (microgamete) gametocytes [1] [2]. Fertilization leads to zygote formation which further differentiates into a motile ookinete that invades the midgut epithelium 12 hours after blood ingestion. Afterwards, ookinetes reside at the basal side of the midgut epithelium beneath the basal lamina and develop into oocysts. Oocysts further mature and develop within the next 10 days, whereby meiosis followed by numerous rounds of mitotic divisions produce thousands of haploid sporozoites. Approximately two weeks post infection, oocysts rupture releasing sporozoites into the hemocoel of the mosquito, from which they migrate to and invade the salivary glands. In the lumen, sporozoites mature for a few days, after which the mosquito becomes infectious to a new host [3] (figure 1).

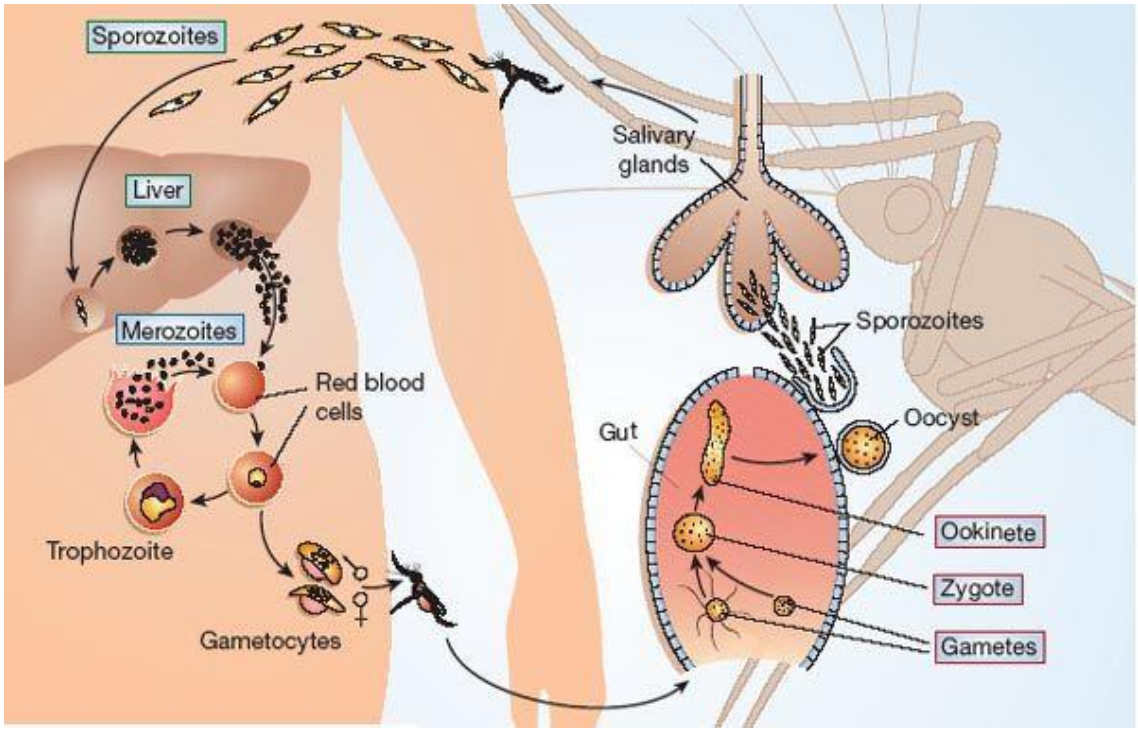


Figure 1. The life cycle of *Plasmodium* parasite.
The *Anopheles gambiae* mosquito releases the sporozoites from its salivary glands into the blood stream of the host following an infectious bite causing an infection. The sporozoites migrate to the liver where they develop into merozoites that are released into the host's bloodstream where they invade the red blood cells. In RBCs, merozoites mature into rings, then trophozoites and finally schizonts, which rupture releasing more merozoites to infect new red blood cells. Some merozoites differentiate into female and male gametocytes that further develop into gametes in the mosquito gut undergoing fertilization to produce a zygote that turns into a motile ookinete. Ookinetes traverse the midgut epithelium and develop into oocysts. Oocysts then undergo meiosis followed by numerous rounds of mitotic division to produce thousands of sporozoites that are released into the hemolymph and invade the salivary glands (Adapted from [3]).

B. Mosquito innate immunity

Invertebrates lack an adaptive immune system. Their physical barriers such as tracheal wall, cuticle of the exoskeleton and the midgut epithelium, constitute the first line of defense against pathogens [4]. Inevitably, when these physical barriers are breached, invading microorganisms encounter both local defenses mounted by epithelia, and systemic defenses mounted by hemocytes and fat body cells. Mosquito immune effector mechanisms in the hemolymph include synthesis of antimicrobial peptides, complement activation, phagocytosis, coagulation and melanization (figure 2) (reviewed in [4, 5] [6] [7]). These mechanisms are initiated upon binding of pathogen associated molecular patterns (PAMPs) to pattern recognition receptors. Hemocytes contribute to phagocytosis and melanization whereas the fat body cells are mostly involved in antimicrobial peptide synthesis and melanization.

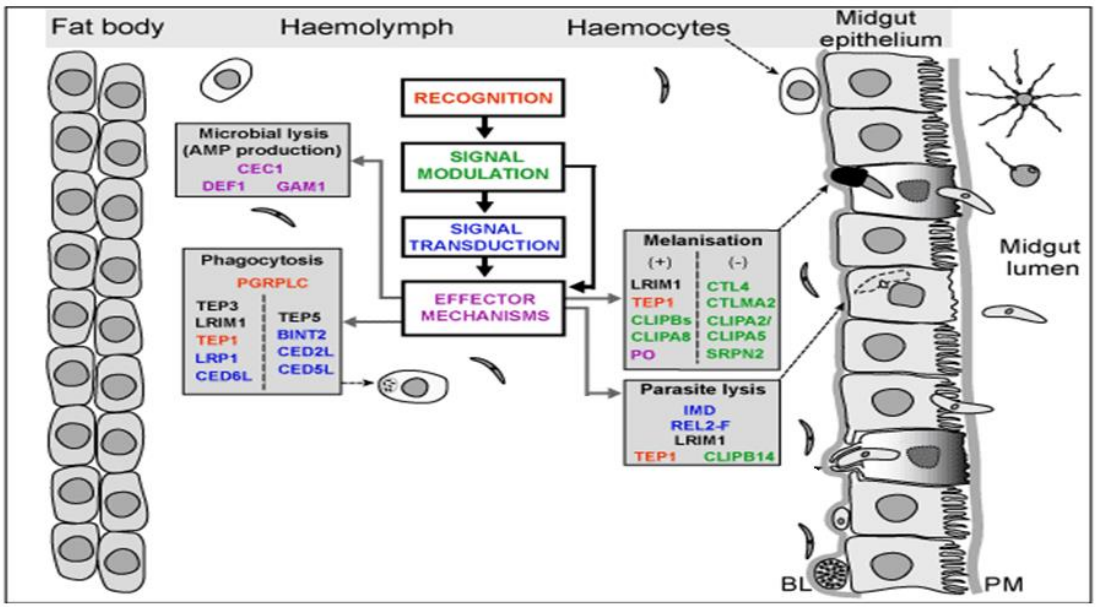


Figure 2. Schematic representation of the immune effector mechanisms mounted by the mosquitoes against pathogens.
Recognition of the invading parasite through PRRs promotes signal modulation that either directly activates various immune effector mechanisms, or triggers signal transduction that results in the activation of the immune responses such as AMP production, lysis, phagocytosis and melanization. (Adapted from Kafatos.openwetware.org).

1. Non-self recognition through pattern recognition receptors

Immune responses are initiated when soluble or membrane bound pattern recognition receptors (PRRs) recognize pathogen associated molecular patterns (PAMPs) [8]. Bioinformatic analysis of the *Anopheles gambiae* genome revealed several putative PRRs that include peptidoglycan recognition proteins (PGRPs), Gram-negative binding proteins (GNBPs), fibrinogen-related proteins (FREPs) and immunoglobulin superfamily proteins and C-type lectins [8] [9].

a. Peptidoglycan recognition proteins (PGRPs)

PGRPs are specific for bacterial recognition [10] [11] [12]. Based on size, PGRPs can be divided into two subclasses, soluble short PGRPs (PGRPS) and transmembrane or intracellular long PGRPs (PGRPL) [8, 13]. PGRPs are characterized by having at least one C-terminal domain similar to bacterial type 2 amidase sequence [14]. PGRPs retaining the amidase activity scavenge peptidoglycans and result in dampening of the immune responses, [15].

In *Drosophila* PGRPs are implicated in activation of both Toll and Imd pathways [16-23]. PGRP-SA, a short PGRP that circulates in the hemolymph, senses gram negative bacteria resulting in Toll pathway activation [24]. Initially, it was proposed that PGRP-SA functions with PGRP-SD and gram-negative binding protein 1 (GNBP1) to bind gram-positive bacteria peptidoglycan (PGN) thus activating the Toll pathway[19] [25]. However, a recent study provides a firm evidence that PGRP-SD functions upstream of Imd pathway, whereby PGRP-SD mutant flies exhibited impaired Imd activation and increased susceptibility to DAP-type bacteria [26]. PGRP-LC is expressed in the gut, hemocytes and fat body, it functions mainly in the hemolymph. PGRP-LC is in fact, the receptor of the Imd pathway that binds directly to the PGN muropeptide of Gram-negative bacteria [27, 28]. PGRP-LE also acts in concert with PGRP-LC in activating the Imd pathway in the midgut [29]. PGRP-LE was also shown to be implicated in the melanization response through activation of the proPO cascade [12] [30].

In *A. gambiae*, PGRP-LC recognizes Gram-negative and Gram-positive bacteria resulting in AMP production. Additionally, PGRP-LC was shown to be overexpressed post blood feeding due to the increase in midgut bacterial communities, which in turn indirectly interferes with malaria parasite development invading the midgut [31]. PGRL-LD was shown to contribute to mosquito immunity against parasite infections through preserving the peritrophic matrix structural integrity whereby the knock down of *PGRP-LD* in *A. stephensi* resulted in compromised peritrophic matrix (PM) integrity concomitant with increased parasite susceptibility [32].

b. β glucan recognition proteins (βGRPs)

Gram negative binding proteins (GNBPs) are highly homologous to β-1,3-glucan recognition proteins (βGRPs), hence, they are grouped in one family, the GNBP/βGRP family [33]. GNBPs are known to bind to fungal and bacterial cell wall components [34]. All members of this family have a conserved N-terminal carbohydrate recognition domain corresponding to a carbohydrate binding module 39 (CBM39) [35], and a C-terminal β-1,3-glucanase like domain which lacks the gulcanase activity since the two Glutamic acid residues were replaced with non-charged residues [36].

GNBPs/βGRPs were studied in several insect species. Two βGRPs, βGRP1 and βGRP2 for instance were identified in *Manduca sexta* [37] [38]. Both βGRP1 and βGRP2 bind curdlan, whereas only βGRP2 can interact with both gram-positive and gram-negative bacteria in addition to fungi. Another βGRP, found in *Armigeres subalbatus* was shown to bind

different species of bacteria regardless of its gram type [39]. βGRPs that have no CBM39 have been identified in *Helicoverpa armigera* [40], *Sodoptera frugiperda* [41] and termites [42], but not in *D. melanogaster*. *Drosophila* has three GNBPs/βGRPs members, GNBP1, GNBP2 and GNBP3. Studies showed that GNBP1 forms a complex with PGRP-SA culminating in the recruitment and activation of a modular serine protease (ModSP). ModSP initiates a Clip-domain serine proteases cascade resulting in the activation of Spätzle (Spz) leading to the activation of the toll signaling pathway [43] [44]. Additionally, there is evidence that GNBP1 is involved in defense against Gram-positive bacteria. GNBP1 is proposed to present a processed form of Gram-positive peptidoglycan (PG) to PGRP-SA, thus initiating downstream signaling [45]. GNBP3 recognizes β-1,3-glucans from the fungal cell wall, and eventually activates the toll pathways similarly to GNBP1 [46] [47]. GNBP3 was also shown to trigger melanization in Toll-independent manner [48]. The *Anopheles gambiae* genome contains 7 members of the GNBP gene family, *GNBPA1, GNBPA2, GNBPB1, GNBPB2, GNBPB3, GNBPB4* and *GNBPB5*. The GNBP B subfamily is mosquito specific [49]. The only mosquito GNBP to contain a putative trans-membrane domain at the N-terminal end is GNBPA1 suggesting that it may be a cell surface molecule. Additionally, GNBPB1, B2 and B4 contain putative GPI-anchor sequences suggesting a potential mechanism for rapid protein release or secretion. Several studies showed that *GNBPs* are regulated after *Plasmodium* and bacterial infections [9]. A study in 2008 by Warr *et al.* characterized the role of six GNBPs against bacterial and *Plasmodium*

infections. Interestingly, *GNBPB4* was shown to be upregulated when the mosquitoes were challenged with *E. coli, S. typhimurium* and *S. aureus*, suggesting that this gene might play an important role in innate immunity. Additionally, mosquitoes silenced for *GNBP4* showed compromised survival compared to control groups when infected with the aforementioned bacterial strains. Mosquitoes silenced for *GNBP4* and infected with *P. berghei* showed increased numbers of live *P. berghei* oocysts compared to control groups, a phenotype observed in *TEP1* kd mosquitoes. Furthermore, GNBPB4 protein was found to co-localize to ookinetes in mosquito midguts which indicates either direct interaction with the ookinetes or an indirect interaction as a component of a protein complex emphasizing a possible main role of GNBPB4 in the defense against *Plasmodium*. The role of other GNBPs is still not very well characterized in the literature; more studies are needed to further characterize these roles.

c. Fibrinogen-related proteins (FREPs)

Fibrinogen related proteins are expressed in both vertebrates and invertebrates. FREPs are characterized by the presence of the fibrinogen like domain (FBG) in their C-terminal region. In vertebrates, FREPs are essential for the coagulation process while they are linked to immunity and pathogen recognition in invertebrates. Among mammalian FREPs, ficolins are the most important class mediating immune functions such as phagocytosis and complement activation [50] [51]. In *Anopheles gambiae,* FREPs are the largest group of PRRs including 59 putative family members [8]. A study in 2006 showed

that three FBNs, FBN8, FBN39 and FBN9 are involved in anti-*Plasmodium* defense. Silencing *FBN8, FBN39* and *FBN9* resulted in increased *P. falciparum* and *P. berghei* numbers compared to control groups [52]. FBN9 was shown to interact with the surfaces of Gram-positive and Gram-negative bacteria in addition to those of *P. berghei* and *P. falciparum* ookinetes [53]. Simoes *et al.* exploited a transgenic approach to further study the defense specificity of FBN9, whereby they overexpressed FBN9 in fat body tissue after a blood meal through a vitellogenin promoter. *Vg-FBN9* transgenic mosquitoes showed increased resistance to *P. berghei* only and not to *P. falciparum*. Additionally, *Vg-FBN9* mosquitoes were more resistant to infections with both Gram-positive and Gram-negative bacteria [54].

FREP1 is a protein secreted by the midgut epithelium and integrated as tetramers within the peritrophic matrix. It was shown to directly bind *Plasmodium falciparum* parasites facilitating parasite invasion through the midgut epithelium [55]. A recent study showed that FREP1 knockout using CRISPR/Cas9 system resulted in a profound suppression of *Plasmodium berghei* and *Plasmodium falciparum* infections [56].

d. Immunoglobulin (Ig) domain proteins

Immunoglobulin superfamily proteins bind to non-self or self molecules mediating pathogen recognition and cell surface adhesion respectively. Ig superfamily proteins are thoroughly characterized in mammals and their functions encompass cell-cell recognition,

structural organization and regulation of muscle in addition to their contribution to immunity [57] [58] [59], however, they have not been adequately addressed in invertebrate systems. All Ig superfamily proteins contain at least one Ig domain which is responsible for recognizing non-self molecules and triggering immune responses. Transcriptome analysis in *Anopheles gambiae* identified 138 proteins having at least one Ig domain among of which are 6 infection-responsive with immunoglobulin domain (IRIDs) proteins. Silencing *IRID3* and *IRID4* in *A. gambiae* increased the hemolymph bacterial load by 6.3 and 5.6 times respectively compared to control groups, implying that these IRIDs contribute to mosquito immune homeostasis. Additionally, silencing *IRID6* resulted in a two-fold increase in *P. falciparum* and *P. berghei* infection intensity compared to control mosquitoes, a phenotype similar to that of *TEP1* kd mosquitoes [60].

Another Ig domain containing PRR is Down syndrome cell adhesion molecule Dscam. Dscam acts as a hypervariable receptor that mediates bacterial phagocytosis and exhibits anti-*Plasmodium* effects [52]. Studies done in Sua5B cells (*A. gambiae* cell line) showed that splicing factors Caper and immune responsive splicing factor 1 (IRSF1) seem to control the repertoire of the pathogen-specific *Dscam* splice variants through NF-κB mediated transcription [61].

e. C-type lectins

C-type lectin-like domain superfamily (CTLD) includes both C-type lectins with and without Ca^{2+}-dependent carbohydrate recognition domains (CRDs) [62]. In vertebrates, proteins with CTLDs are classified in 17 different groups, based on differences in their domain architecture. Most invertebrate CTLDs belong to group VII, the only common group between vertebrates and invertebrates [62, 63]. In invertebrates, CTLs are involved in complement activation [50], cellular encapsulation [64], opsonization [65, 66], nodule formation [67], and melanization [64, 68, 69].

Bioinformatic analysis in *Anopheles gambiae* identified 23 different CTLD members based on their primary sequence signatures that conferred different carbohydrate specificity. These members were further classified into mannose binding CTLs (CTLMAs), galactose binding CTLs (CTLGAs) and CTLs lacking a sugar binding domain [8, 9, 70].

Functional genetic analysis identified two CTLs, CTL4 and CTLMA2 that act as *Plasmodium* agonists, whereby silencing either one resulted in *P. berghei* ookinete melanization in S mosquitoes [71]. These proteins are secreted in the hemolymph and maintained as an obligate disulfide-linked heterodimer and exhibit anti-bacterial activities [72]. In 2006 however, a study by Cohuet *et al.* showed that *P. falciparum* field isolates were not melanized in either *CTL4* or *CTLMA2* knockdown backgrounds [73], suggesting that the melanization response triggered in these CTL kd does not affect *P. falciparum*.

In 2017 however, a study by Simoes *et al.* showed that upon increasing *P. falciparum* infection intensity, prominent ookinete melanization in was recorded in either *CTL4* or *CTLMA2* knockdown *A. gambiae* [74], emphasizing that *P. falciparum* melanization in *CTL4* or *CTLMA2* kd backgrounds is dependent on the intensity of infection. Indeed, in the paper of Couhet *et al.* the infection intensity was low which could explain the absence of *P. falciparum* melanization.

Immunofluorescence localization assays revealed that CTLs are recruited to *Plasmodium berghei* surface in TEP1-dependent manner (Osta and christophides unpublished), suggesting that they may be part of the complement-like response [72].

2. Signal modulation by CLIP-domain serine proteases

a. Structure, function, activation and regulation

In insects, non-self recognition by PRRs triggers series of clip-domain serine protease cascades culminating in activation of several immune effector mechanisms such as coagulation, melanization, Toll activation and complement regulation [75]. CLIPs are specific to invertebrates. They contain one or more N-terminal clip domain and a C-terminal chemotrypsin-like serine protease domain, conferring its catalytic activity. Not all CLIPs are catalytic; those that lack one or more of the three residues (His, Asp, Ser) that form the catalytic triad are non-catalytic [also known as clip-domain containing serine proteinase homologs (cSPHs)] [8].

The clip domain is usually composed of 30-60 amino acid residues linked by three disulfide bonds, and is connected to the serine protease domain via a linker region of variable length. The SP and SPH domains start with a cysteine residue linked to another cysteine by a disulfide bond within the same domain (Figure 3). These proteases are secreted as zymogens into the hemolymph and are activated by cleavage at specific cleavage sites [76, 77]. After cleavage, the clip-domain remains attached to protease domain by a disulfide bond [78]. While the function of the serine protease domain is to activate downstream zymogens or other molecules by proteolytic cleavage, the clip domain function is yet to be defined, however, it is speculated to mediate protein-protein interactions with PRRs, with other clip domain serine proteases or with SPH cofactors [77-79].

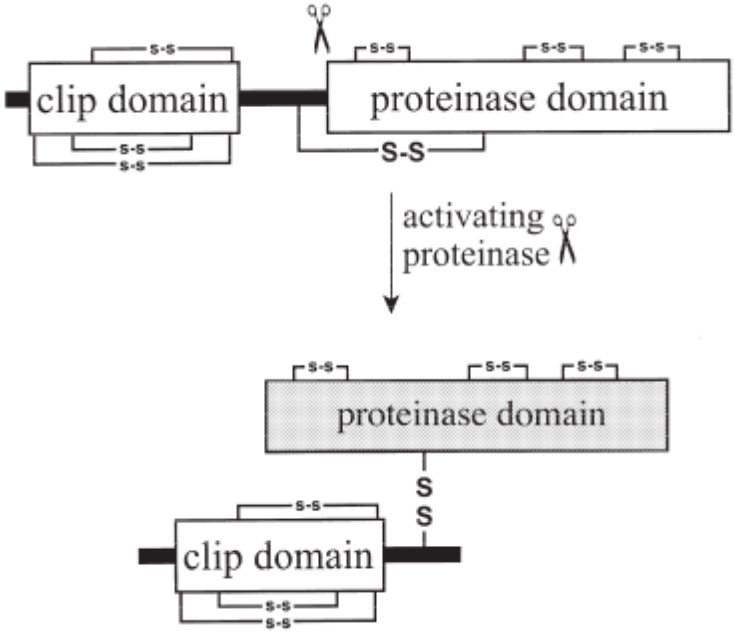


Figure 3. Mechanism of CLIPs activation.
CLIP serine proteases are secreted into the hemolymph as zymogens and undergo proteolytic cleavage in the linker region rendering the protease active. The CLIP domain remains attached to the serine protease domain by disulfide bonds. [78]

Phylogenetic analysis of mosquito CLIPs based on whole sequence alignment lead to their classification into 5 groups A to E; groups A and E include non-catalytic CLIPs, while groups B, C and D are catalytic[8]. A structure-function analysis of *Drosophila* grass cSP and comparative analysis with other cSPs of known function allowed the classification of cSPs into two functional groups: Those which contain a 75-loop protruding from the calcium-binding 70-loop in close proximity to the activation site are considered terminal proteinases that are directly involved in the processing of PPO triggering the melanization response, or processing proSpätzle leading to Toll pathway activation. The remaining cSPs

that lack the 75-loop are penultimate proteinases that are likely to act upstream in the cascade [80].

The clip domain serine protease cascades are tightly regulated by serine protease inhibitors known as Serpins (SRPNs). Serpins regulate several physiological processes in insects including embryonic development, wound clotting and host defense [reviewed in[81, 82]. Serpins bind to the proteases at their active site via an exposed reactive center loop (RCL), resulting in the formation of covalent irreversible serpin-protease complex that is eventually eliminated from the hemolymph [83-85]. Tight regulation of proteases by serpins is important to avoid exaggerated immune responses that might impose a fitness cost upon the host [86, 87]. Indeed, silencing certain *SRPN* genes in *Drosophila* and *A. gambiae* lead to exaggerated melanization which compromised the life span of the insect. In *Drosophila*, *Serpin 27A* mutants exhibit spontaneous tissue melanization in both larvae and adults [88]. Similarly, *SRPN2* kd mosquitoes showed spontaneous pseudotumors in tissues associated with life span reduction, in addition to significant *P.berghei* lysis and melanization [89].

b. The role of clip serine proteases in innate immunity

i. Activation of prophenoloxidase cascade

The rate limiting step of melanization is the activation of prophenol oxidase (PPO) to phenol oxidase (PO) [90]. This step is mediated by terminal CLIPs (also known as

prophenoloxidase activating enzymes, PPAEs). Studies in *Himophilia diomphalia* showed that both catalytic and non-catalytic CLIPs are needed for PPO activation [76] [91] [92]. Piao et al showed that PPAF-I promotes the direct cleavage of PPO into PO. Nevertheless, PO activation does not occur until catalytic PPAF-III cleaves and activates a non-catalytic proPPAF-II that in turn complexes with PO triggering a conformational change simulating its proper cleavage [77].

Studies in *Manduca sexta* showed that upon non-self recognition, an autoactivated modular serine protease HP14 cleaves the cSP proHP21 into active HP21 which in turn cleaves the PPO activating proteinase-2 zymogen (PAP-2) into active PAP-2, the terminal cSP in the cascade that processes PPO into PO [93]. In addition to PAP-2, HP21 was also shown to cleave PAP-3[94]. PAP-1 on the other hand is regulated by a different pathway requiring HP6. HP6 is an apparent orthologue of *Drosophila* persephone which is cleaved upon treatment of *Manduca* plasma with bacteria or curdlan [95]. Two additional cSPHs, SPH1 and SPH2, seem to be required as cofactors for efficient processing and activation of PPO. Interestingly, the precursor forms of SPH1 and SPH2 cannot activate PPO [96, 97] but rather require processing by PAP-3 and PAP-1 (to a lesser extent) to become active[98, 99].

The infection-induced melanization in *Drosophila* requires two CLIPs termed MP1 and MP2 whereby MP2 is thought to act upstream of MP1 in the cascade[100]. Although MP1 is suggested to act as a PPO activating enzyme (PPAE), direct cleavage of PPO has been shown only for MP2 [101]. Spn77Ba was shown to regulate the melanization in the

epithelium of the *Drosophila* respiratory tract by targeting MP1 and MP2. *Spn77Ba* RNAi flies showed excessive melanization of the tracheal system, followed by death of almost all the larvae before reaching the pupal stage. Silencing either *MP1* or *MP2* in *Spn77Ba* RNAi larvae completely suppressed tracheal melanization. These results suggest that the proteinase cascade involving MP1 and MP2 is required for local epithelial melanization, possibly to block microbial entry into the hemolymph[102].

In addition to MP1 and MP2, another CLIP called Hayan was identified as a key activator of PPO in the systemic wound response. *Hayan* RNAi flies exhibited reduced survival rate in response to sterile injury, whereas neither *MP1* nor *MP2* RNAi flies exhibited a similar phenotype [103]. Active recombinant Hayan directly cleaved *Drosophila* PPO1 suggesting that it acts as a PAP in the wound-induced melanization response, despite the fact that Hayan is required for both, wound-induced and microbe-induced hemolymph PO activity [103].

Studies in *Tenebrio molitor* showed that recognition of Lys-type PGN by PGRP-SA/GNBP1 complex leads to the recruitment of ModSp, which autoactivates and cleaves the downstream CLIP SAE (spätzle processing-activating enzyme) which in turn activates spätzle processing enzyme (SPE) [12]. In addition to Toll pathway activation through spätzle processing, SPE was also shown to process PPO and pro-cSPH1 into their active forms thus triggering the reaction [104], suggesting the presence of cross-talk between Toll pathway and melanization response through certain CLIPs.

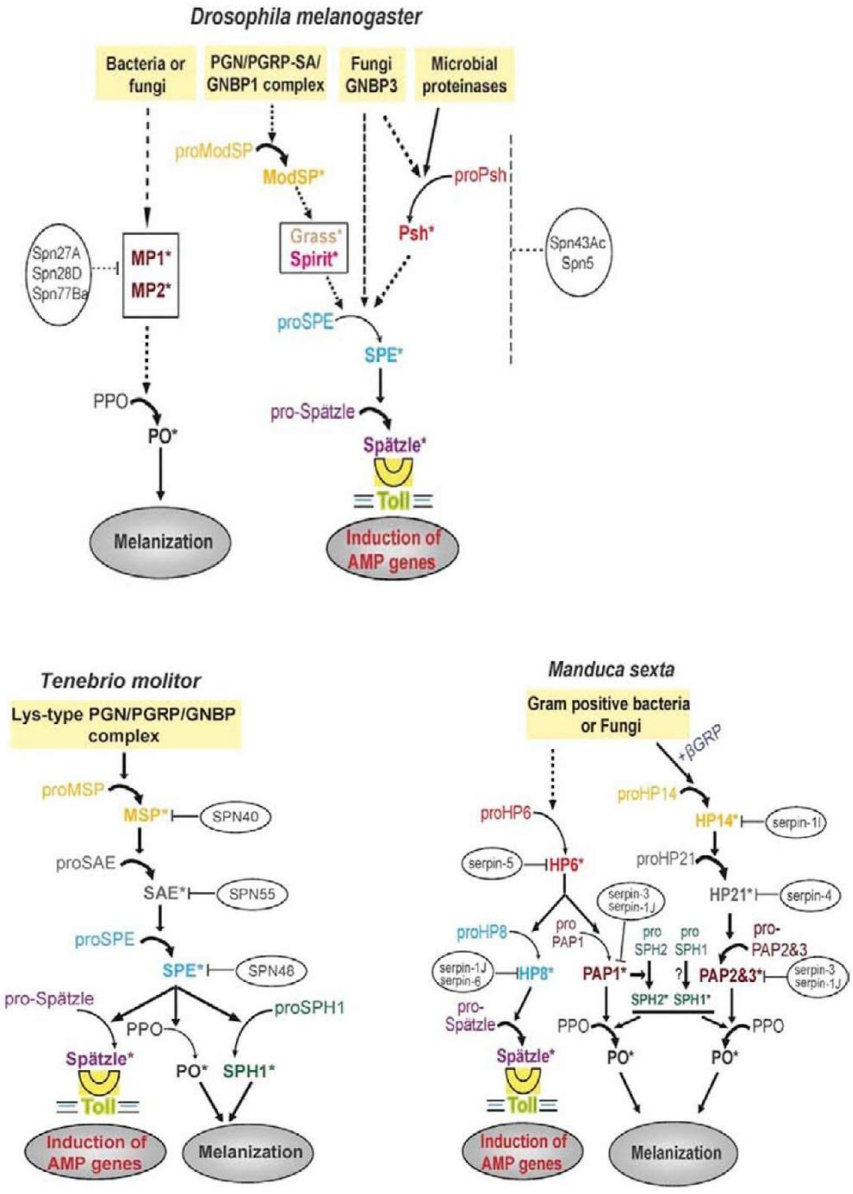


Figure 4. A proposed model of the role of protease cascades in innate immunity of three different insected species.

PAMPs recognition by PRRs promotes autoactivation of a modular serine protease in an unknown mechanism. This modular serine protease then initiates the activation of a network of cSPs and cSPHs. The penultimate proteases in these cascades either promote the cleavage of proPO into PO, culminating in the melanization response, or the cleavage of

proSpätzle into Spätzle that binds to Toll receptor resulting in AMP synthesis. Over-activation of the melanization response or AMP synthesis is tightly regulated by serpins. (Adapted from [105]).

ii. Activation of the Toll pathway

In *Drosophila*, the Toll pathway is involved in development of the dorso-ventral axis in embryos, as well as innate immunity in adults [106, 107]. Two CLIPs, Easter and Snake contribute to the proteolytic cleavage of inactive pro-spätzle into active spätzle, which in turn binds to the Toll receptor and activates the pathway, resulting in proper dorso-ventral polarity during development [106]. *In vitro* studies revealed a direct role for Spätzle processing enzyme (SPE) in cleaving pro-Spätzle in response to fungal and Gram-positive bacterial infections[108, 109]. Kambris *et al*. identified two additional *Drosophila* CLIPs, grass and spirit as important members of the Toll-activating cascade [43]. Similar to *Drosophila* SPE, *Tenebrio molitor* SPE was also shown to activate the Toll signaling pathway [12].

In addition to PRR-dependent signaling, the Toll pathway is also activated in response to danger signals in a persephone-dependent manner. Persephone (Psh), a serine protease that senses exogenous bacterial or fungal proteases, cleaves spätzle leading to Toll pathway activation [44]. Psh contains a pro-domain region that necessitates its proteolytic cleavage in order to become active. Issa N *et al.* showed that Psh can be activated via two possible mechanisms. Pro-Psh can either be proteolytically cleaved into active Psh in response to *B.*

subtilis bacterial infection, or it can be activated in a stepwise manner in response to other bacterial and fungal infections; pro-Psh gets sequentially cleaved in its pro-domain region (also identified as the bait region) in response to bacterial and fungal infections. Exogenous microbial proteases activate cathepsin 26-29-p that further cleaves Psh hence rendering it active[110].Psh is controlled by spn43Ac; Spn43Ac mutant flies exhibit constitutive Toll activation due to Persephone (psh) activation, resulting in an exaggerated immune response [111] [112].

iii. Role of cSPs and cSPHs in *Anopheles gambiae* immunity

CLIPs contribute to many immune responses in *A. gambiae* and play a role in defense against fungi [113], bacteria [52, 114] and Plasmodium [114-116]. *In vivo* genetic studies showed that SPHs have broader functions in immunity than previously thought acting as positive or negative regulators of melanization. For instance, CLIPA8, a positive regulator, is required for the melanization response against fungi [113], bacteria [117] and *Plasmodium* ookinetes [116]. Silencing *CLIPA8* in *A. gambiae* significantly increased its susceptibility to fungal infections compared to controls; hyphal body colony forming units were significantly higher in *CLIPA8* kd mosquitoes compared to controls [113]. Conversely, abolishing PO activity by silencing *CLIPA8* did not affect the survival of *A. gambiae* mosquitoes after infections with either *E. coli* or *S. aureus*. Both bacterial species were cleared from *CLIPA8* kd mosquitoes as efficiently as controls suggesting that

melanization is not critical for antibacterial defense in the mosquito at least against the two tested species [117].

CLIPA2 on the other hand, is a negative regulator of melanization. CLIPA2 indirectly regulates the complement-like response mediated by TEP1, a hallmark factor in mosquito immune response against *Plasmodium* ookinetes, bacteria and fungi [52, 113, 118-120]. TEP1 exists in the hemolymph as TEP1 full form (TEP1F) and processed form called TEP1 cut (TEP1c). It was shown that CLIPA2 negatively regulates melanization indirectly by controlling the intensity of TEP1 activation during systemic infections; *CLIPA2* kd enhanced TEP1-mediated response leading to an exaggerated PO activity in the hemolymph following *E. coli* infections [118]. CLIPA14 is another negative regulator of melanization, whereby *CLIPA14* knockdown (kd) revealed a more potent melanization phenotype in *P. berghei* infections than *CLIPA2* kd. In contrast, the SPH SPCLIP1 was shown to co-localize with TEP1 on ookinete and bacterial surfaces whereby it seems to facilitate the conversion of TEP1-F to TEP1-cut [118].

Catalytic CLIPs are also important positive regulators of melanization. Silencing either *CLIPB4* or *CLIPB17* induced a significant loss in ookinete melanization in different mosquito backgrounds, whereas silencing *CLIPB3* and *CLIPB8* reduced melanization in refractory mosquito strain [116] (figure 5). While the hierarchical order of catalytic CLIP activation remains unknown, biochemical studies on CLIPB9 revealed that it acts mostly downstream to directly cleaves PPO [121]. Also, CLIPB9 partially reversed SRPN2 RNAi

phenotype characterized by extensive tissue melanization suggesting that it is an important player of the melanization reposne [121].

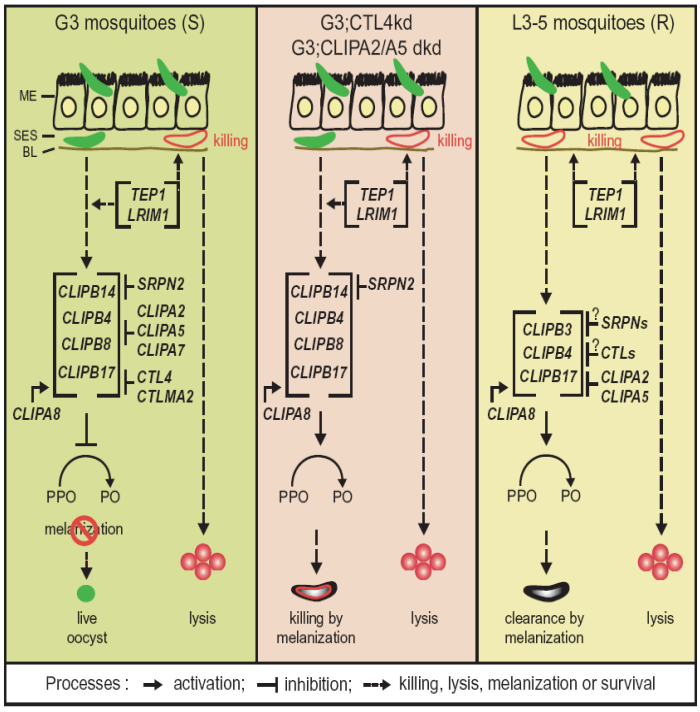


Figure 5. Melanization in different *A. gambiae* genetic backgrounds.
In the susceptible G3 mosquitoes (left panel), around 20% of the invading parasites escape TEP1-mediated killing. Ookinete melanization is prevented due to tight regulation of the melanotic cascade exerted by serpins, CLIPA2 and CTLs. In *CTL4*kd or *CLIPA2/CLIPA5*dkd mosquitoes (middle lane), TEP1-dependent melanization is activated and results in ookinete killing. In L3-5 refractory mosquitoes (right panel), ookinete melanization is constitutively active due to the actions of CLIPA8 and several CLIPBs which positively regulate the melanization cascade. On the other hand, CLIPA2 negatively regulates the melanization cascade [116].

3. Immune signaling pathways in mosquito immunity

The *Drosophila* immune system is governed by two main signaling pathways, Toll and Immune deficiency (Imd). Both pathways regulate AMP gene expression in response to bacterial and fungal infections; Toll pathway is involved mainly against Gram-positive and fungal infections whereas Imd is involved against Gram-negative infections (reviewed in[122]). In flies, Toll pathway activates the NF-κB-like transcription factor DIF (Dorsal-related immunity) in response to infection, and Dorsal during embryonic development; whereas the Imd pathway activates NF- κB-like transcription factor Relish [123, 124] [125].

Comparative genomic analysis showed that several genes downstream of the Toll and Imd pathways in *Drosophila* are conserved in *A. gambiae*. Rel1 and Rel2 are two mosquito NF-κB-like transcription factors orthologous to *Drosophila* Dorsal and Relish, respectively [9] [8]. *Drosophila* DIF and mosquito Rel1 are both regulated by the Toll pathways and are controlled by the negative regulator Cactus. Silencing *cactus* in *A. gambiae* promotes over-activation of the Toll pathway, resulting in complete refractoriness to *P. berghei* [126] [127] and a significant reduction in the number of live *P. falciparum* oocysts [127]. The absence of cactus was associated with elevated levels of anti-parasitic genes such as *TEP1, LRIM1* and *APL1C*, reflecting the important contribution of the Rel1 pathway to mosquito's immunity [126, 128]. The Toll/Rel1 pathway was also implicated in anti-viral [129] [130] and anti-fungal immunity in the dengue vector *A.aegypti* [131] [132].

In *A. gambiae*, Rel2 has two isoforms due to alternative splicing: full length and short length isoforms named Rel2-F and Rel2-S respectively. Rel2-F is involved in defense against *P. berghei* parasties and Gram-positive bacterial infections, whereas Rel2-S is mainly implicated in immunity against Gram-negative bacterial infections [133]. Further studies showed that Rel2 pathway is involved in anti-*Plasmodium* immunity, whereby silencing *Rel2* significantly increased the number of live *P. falciparum* parasites in *A. gambiae* [52, 127, 134]. Silencing *caspar*, the negative regulator of the Rel2 pathway resulted in near refractoriness to *P. falciparum* in three malaria vectors: *A. gambiae*, *A. stephensi*, and *A. albimanus* [127]. A study by Dong *et al.* showed that overexpressing Rel2-S in *Anopheles gambiae* transgenic mosquitoes promoted potent anti-*Plasmodium* activities by enhancing the expression of TEP1, APL1C and LRRD7. These transgenic mosquitoes also exhibited increased resistance to gut bacteria and to systemic infections (*E. coli* and *S. aureus*) highlighting the importance of this pathway in mosquito immunity [135].

In addition to the Rel1 and Rel2 immune signaling pathways, the JAK-STAT pathway also contributes to immune defense against *P. berghei* and *P. falciparum*, by targeting specifically the later oocyst stages of the parasite [136]. *Anopheles gambiae* has two STAT proteins *Ag*STAT-A and *Ag*STAT-B. *Ag*STAT-A has anti-bacterial and anti-parasitic roles since it upregulates Nitric Oxide Synthase (NOS) transcript levels consequently increasing Nitric oxide (NO) levels. It is also responsible for TEP1 transcript upregulation during the

post-invasion phase of the midgut, possibly serving TEP1 replenishment [136]. *Ag*STAT-B regulates *Ag*STAT-A and translocates into the nuclei of fat body cells in response to bacterial infections, thus inducing the activation of several anti-bacterial target genes [137].

In addition to Toll, Imd and JAK-STAT pathways, few studies emphasized the role of JNK pathway as a key mediator of mosquito anti-*Plasmodial* immunity. Suppressing JNK through silencing *Hep, JNK* of *Fos* significantly enhanced *P. berghei* infection. Silencing *Puckered* on the other hand which is a JNK suppressor resulted in opposite effects, hence reflecting JNK involvement in anti-*Plasmodial* immunity. JNK pathway limits *Plasmodium* infection by inducing the expression of both HPX2 and NOX5 (thus potentiating epithelial nitration) or by regulating the expression of TEP1 and FBN9 (two key hemocyte-derived immune effectors) [138]. Ramphul *et al*. showed that *Plasmodium falciparum* parasites that have *Pfs47* are able to evade the mosquito immune system through disrupting the JNK pathway, whereas survival of parasites knocked out for *Pfs47* was compromised. This study showed that *Pfs47* KO parasites invading epithelial cells triggered the activation of JNK pathway that resulted in activation of several caspases such as caspase-S2, which in turn lead to stronger epithelial nitration mediated by HPX2 and NOX5 eventually culminating in parasite lysis [139].

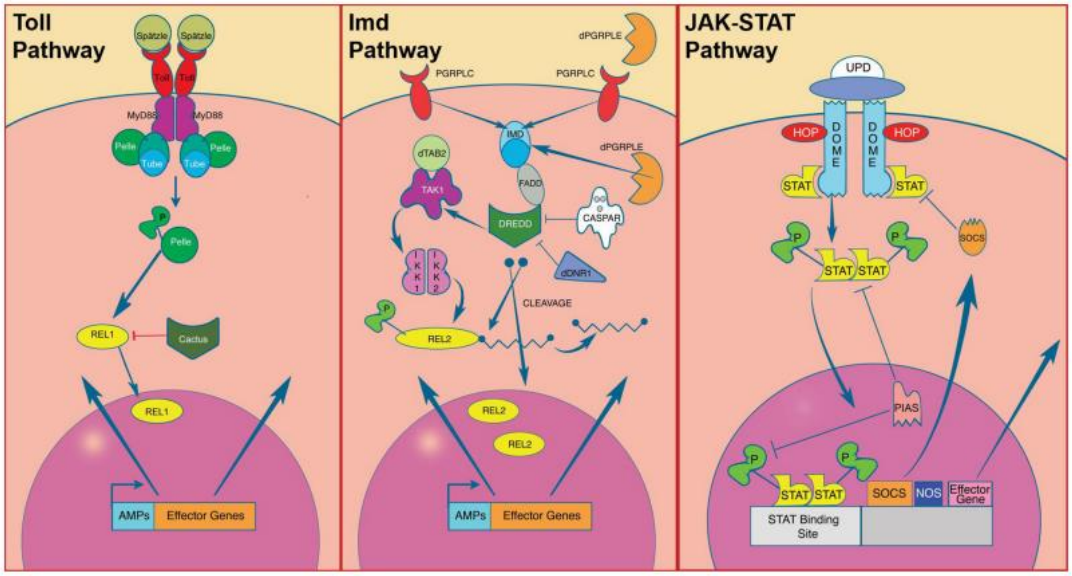


Figure 6. The role of Toll, Imd, and JAK-STAT signaling pathways in immune defense.
Spätzle binds to the Toll transmembrane receptor in response to bacterial or *Plasmodium* recognition. This is followed by a series of molecular events leading to nuclear translocation of Rel1, and transcriptional up-regulation of several immune genes involved in killing (left panel). Imd pathway is activated upon bacterial or *Plamsodium* recognition by PGRP-LC. This recognition initiates a downstream signaling cascade, resulting in the cleavage of Rel2-F into Rel2-S, and its translocation into the nucleus thus promoting transcriptional activation of immune genes involved in microbial defense (middle panel). Upon parasite, bacterial or viral recognition, the cytokine ligand unpaired (UPD) binds to the transmembrane receptor DOME, resulting in JAK-STAT pathway activation, thus promoting the translocation of STAT to the nucleus, followed by transcriptional activation of genes involved in mosquito immunity (right panel) (adapted from [140]).

4. Immune effector mechanisms in mosquitoes

a. Mosquito complement: a hallmark of immune defense

TEP1 is a member of the thioester containing protein family. It is a complement-like protein that structurally resembles C3 complement in vertebrates, however it lacks the

C345C and anaphylatoxin domains [141]. Mammalian C3 activation is triggered upon infection by antigen-antibody complexes (classical pathway), mannose binding lectins (lectin pathway) or spontaneous hydrolysis of C3 (alternative pathway). All pathways activate C3 convertase complexes that culminate in proteolytic cleavage of C3 into C3b (reviewed [142]). C3 cleavage exposes its thioester bond needed for covalent binding to nucleophilic groups on pathogen surfaces triggering phagocytosis or the assembly of a membrane attack complex (MAC) that mediates lysis (figure 7) [143]. In addition to C3b, other small C3 fragments (C3a) promote an inflammatory response and serve as a chemotactic factor that guides phagocytes to the infection site [142]. Similar to C3, TEP1 is also cleaved and has been shown to be the hallmark of mosquito immunity.

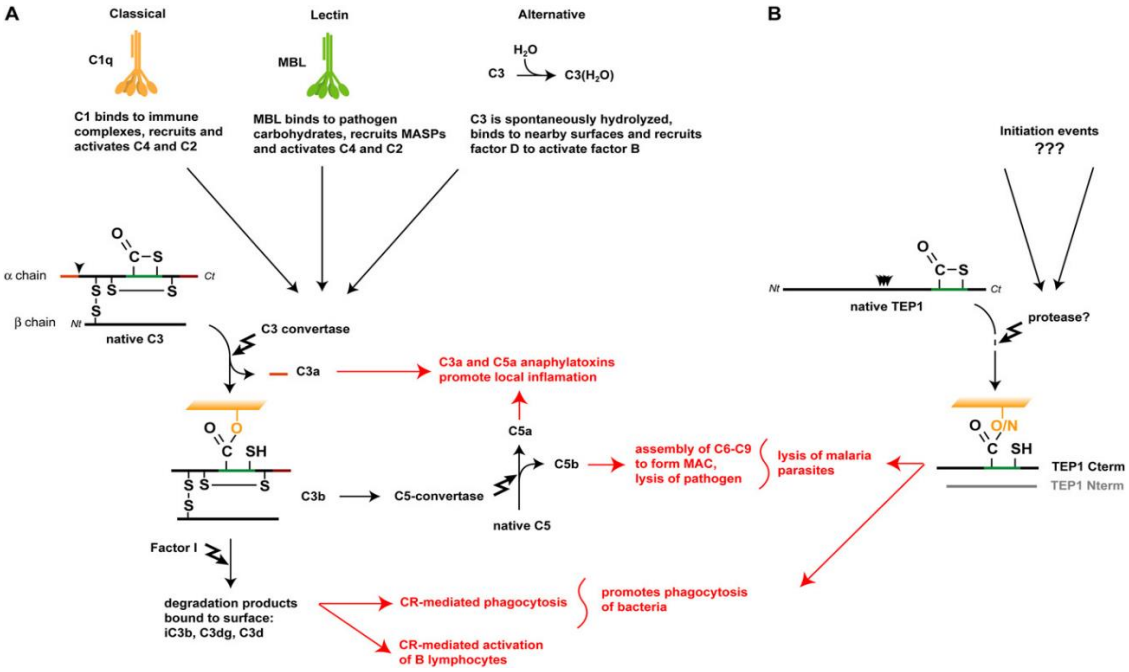


Figure 7. The mammalian complement system and the mosquito complement-like systems.

(A) Schematic representation of the mammalian system. Complement factor C3 is secreted into the blood as a mature two-chain molecule (α and β chains held together by a disulfide bond). The classical, lectin and alternative pathways all lead to C3 activation by C3 convertases. C3 cleavage results in several different effector responses such as inflammatory responses (by C3a fragment), lysis of malaria parasites and phagocytosis of bacteria. (B) A model of the complement-like system of *A. gambiae*. Unlike mammalian C3, TEP1 is processed in the hemolymph into a two-chain molecule (TEP1-N and TEP1-C, non-covalently associated). The proteolytic activation/cleavage of TEP1 remains poorly understood, however, TEP1 activation promotes similar functions to those of C3 such as phagocytosis of bacteria and lysis of malaria parasites. Reused with permission [144].

TEP1 is secreted into the hemolymph by hemocytes as a full length TEP1F form (165 kDa) which is processed into a cleaved form $TEP1_{cut}$ (80 kDa) [120]. $TEP1_{cut}$ is stabilized in the hemolymph by the LRIM1/APL1C complex and deposited on the surface of invading

microbes [145]. Several studies identified TEP1 as a key player in the mosquito immune response including phagocytosis, lysis and melanization (figure 8).

A study done by Levashina *et al.* provided the first evidence for TEP1 role in phagocytosis. Mosquito hemocyte-like cells were less efficient at phagocytizing bacteria incubated in TEP1 depleted medium [120]. Another assay based on time-lapse microscopy further confirmed the role of TEP1 in the phagocytosis of *E. coli* and *S. aureus in vivo* [146]; silencing TEP1 increased the accumulation of live bacterial cells in adult mosquitoes suggesting a significant contribution of TEP1 to bacterial clearance. However, it was not clear whether this increased bacterial accumulation is due to the impaired phagocytic or lytic arm of TEP1. To assess TEP1 role in bacterial lysis, Kamareddine *et al.* developed a GFP release assay based on injecting adult *Anopheles gambiae* mosquitoes with *E. coli*-expressing cytoplasmic GFP. The level of GFP released in the hemolymph was detected by western blot analysis and reflects the extent of bacterial lysis that occurred in the hemolymph. Hemolymph of *TEP1* kd mosquitoes showed a barely detectable GFP signal compared to that of control mosquitoes suggesting that TEP1 is indeed involved in bacterial lysis [147]. Silencing *TEP1* in the refractory *A. gambiae* strain completely abolished *P. berghei* melanization [119], so did co-silencing *TEP1* and *CTL4* in susceptible mosquito strain [71]. Furthermore, *TEP1* kd abolished hemolymph PPO activity in response to *E. coli* infections [115] and inhibited CLIPA8 cleavage upon bacterial infection suggesting that TEP1 is upstream in the melanization response pathway. PPO recruitment to *B. bassiana*

hyphae was also abolished in *TEP1* kd mosquitoes, hence inhibiting melanization [113]. Altogether these results clearly reflect the key roles of mosquito complement in phagocytosis, lysis and melanization.

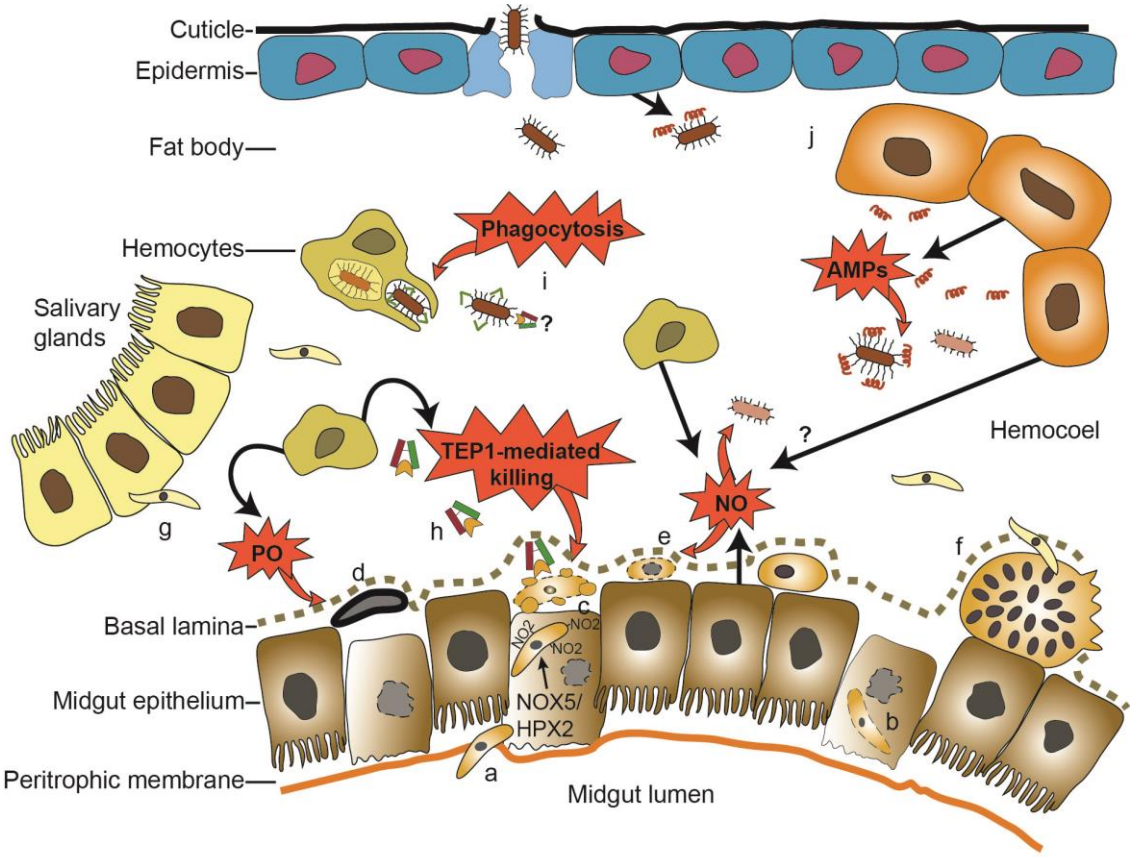


Figure 8. Immune effector mechanisms in *Anopheles gambiae* innate immunity against invading *Plasmodium* parasites.

(a) Ookinetes invade the midgut epithelium 24 hours post infection (b) Few ookinetes are killed within the cytoplasm of the midgut epithelium via unknown mechanisms. (c) Surface molecules on ookinetes get nitrated due to the activity of NOX5/HPX2 as they invade the epithelial cells. This nitration renders the ookinetes susceptible to TEP1-mediated lysis. (d) Ookinete melanization in the mosquito basal labyrinth. (e) Early oocysts are killed by nitric oxide (NO) production by midgut epithelium cells and possibly by fat body. (f) Mature

oocysts become resistant to killing, and rupture almost 14 days post infection releasing sporozoites in the hemolymph. (g) Sporozoites then migrate the hemolymph until they reach the salivary glands approximately 21 days post infection. (h) The TEP1/LRIM1/APL1C complex in the hemolymph mediates several immune responses such as melanization of bacteria and parasites and (i) bacterial phagocytosis. (j) Systemic and local AMP production by fat body cells to help against invading bacteria and possibly parasites (Modified from [5]).

As we mentioned earlier $TEP1_{cut}$ is stabilized by the LRIM1/APL1C complex and dislodged on the surface of the targeted microbe after recognition in an unknown manner (figure 9). $TEP1_{cut}$ is lost from the hemolymph of naïve mosquitoes after silencing either *LRIM1* or *APL1C* [145] [148]. However, there is no clear indication that the complex interacts with microbial surfaces. Several studies showed that silencing either *LRIM1* [71] or APL1C [149] resulted in a similar RNAi phenotype to that of *TEP1* kd [119], characterized by increased numbers of GFP-expressing oocysts in the gut. Furthermore, all three, *TEP1*, *LRIM1* and *APL1C* are required for melanization response triggered against *Plasmodium* ookinetes in certain melanizing genotypes [71] [119, 145]. In addition to TEP1 stabilization by the APL1C/LRIM1 complex, several SPHs, namely SPCLIP1 and CLIPA2 appear to regulate TEP1 consumption during immune responses. SPCLIP1 was shown to co-localize with TEP1 on ookinete and bacterial surfaces where it positively regulates the conversion of TEP1-F to $TEP1_{cut}$ [115]. SPCLIP1 was lost from the hemolymph of naïve mosquitoes upon *LRIM1* silencing similar to *TEP1*, strongly suggesting that SPCLIP1 is a component of the TEP1 pathway. Contrary to SPCLIP1, CLIPA2 is shown to negatively regulate the TEP1-F conversion to $TEP1_{cut}$. Interestingly, LRIM1 kd also triggered CLIPA2

loss from the hemolymph of naïve mosquitoes suggesting that CLIPA2 is also another component of the TEP1 pathway [118].

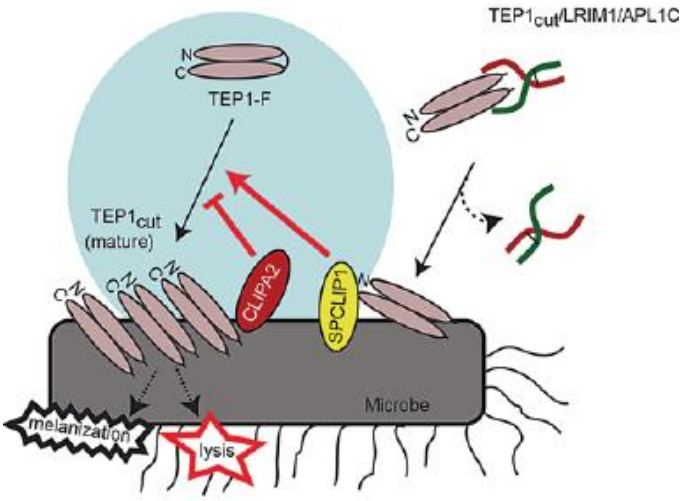


Figure 9. Schematic representation of TEP1 activity regulation.
TEP1c is released from the LRIM1/APL1C complex upon microbial invasion. TEP1c targets microbial surfaces where it triggers a series of immune effector responses such as lysis and melanization. SPCLIP1 appears to positively regulate the conversion of TEP1F to TEP1c whereas CLIPA2 seems to function as a negative regulator controlling the extent of TEP1F processing.

b. <u>The melanization response in insects</u>

Cuticle injury triggers a local melanization response that helps seal the wound to limit microbial entry, whereas microbial invasion of the hemolymph triggers a systemic melanization response. Melanization is characterized by the synthesis of melanin and its cross-linking with molecules on microbial surfaces or in injured areas resulting in the

killing of the invader and hardening of the wound clot [reviewed in [150]]. In addition to its role in immunity, melanization is essential for cuticle sclerotization or tanning that leads to the hardening of the insect exoskeleton by cross-linking the cuticular proteins by quinones generated during that process [151].

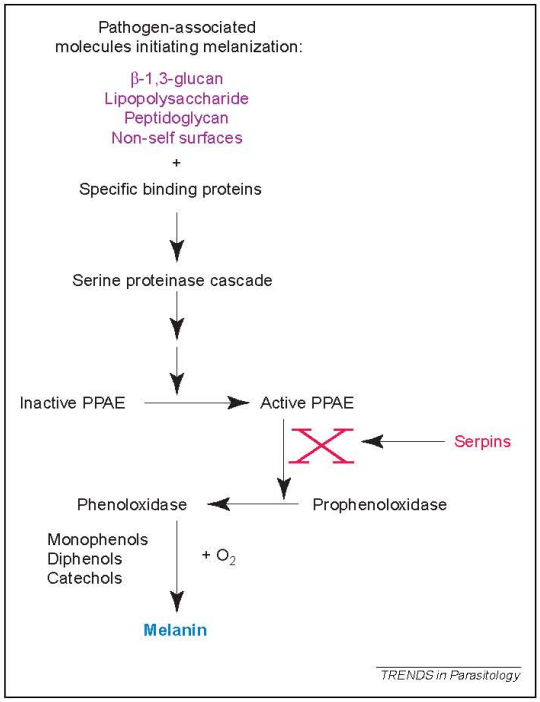


Figure 10. Schematic representation of the melanization activation cascade in insects. Recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) results in activation of serine protease cascades resulting in the proteolytic cleavage of proPO into active PO eventually culminating in melanin production and deposition of the surface of the invading microbes. (Adapted from [90]).

i. The biochemical pathway of melanin biosynthesis in insects

Melanogenesis in insects is initiated by the hydroxylation of phenylalanine by phenylalanine 4-monooxygenase (PAH), to form tyrosine, the rate limiting substrate (figure

11) [152, 153]. PAH is involved in cuticular sclerotization [152, 154] and in the melanization immune response. Challenging mosquitoes with *Dirofilaria immitis* microfilariae [155] or bacteria [156] lead to a significant increase in *PAH* transcript levels. Also, the melanotic encapsulation of microfilariae worms in *A. aegypti* and *Armigeres subulbatus* [157] and of *P. berghei* ookinetes in *A. gambaie* [158] was significantly reduced upon *PAH* knockdown.

Following its synthesis, tyrosine undergoes subsequent oxidation into dihydroxyphenylalanine (Dopa) which gets oxidated into dopaquinone. In the presence of thiol compounds dopaquinone is converted to cysteinyl and glutathionyl conjugates that mediate the synthesis of the cutaneous reddish pigment pheomelanin. Dopaquinone can also undergo spontaneous cyclization into dopachrome, that is decarboxylated by DCE (dopachrome conversion enzyme) generating 5, 6-dihydroxyindole (DHI). DHI undergoes PO-mediated oxidation leading to polymerization of indole quinones, giving rise to eumelanin [reviewed in [159]]. Decarboxylation of dopa by dopa dexarboxylase (DDC) results in the production of DHI-euemlanin as well. In addition to DDC involvement in cuticular sclerotization in several insects [152, 160-162], several studies highlighted a potential role for DDC in immunity. In *Drosophila*, both Gram-positive and Gram-negative bacterial infections and not sterile injury resulted in upregulation of DDC expression throughout the epidermis [163]. In the medfly *Ceratitis capitata*, DDC activity was required for efficient phagocytosis, melanization and nodulation responses after *E. coli*

infections [164].

The immunoprotective functions of melanization are attributed in part to the oxidoreductive properties of melanogenic precursors that engage in various redox reactions to create a biochemically hostile environment to invaders. Euemelanin and quinoides deposit as cross-linking complexes on foreign nucleophilic surfaces to effectively encapsulate, immobilize, and deprive circulating pathogens from nutrients [90, 165].

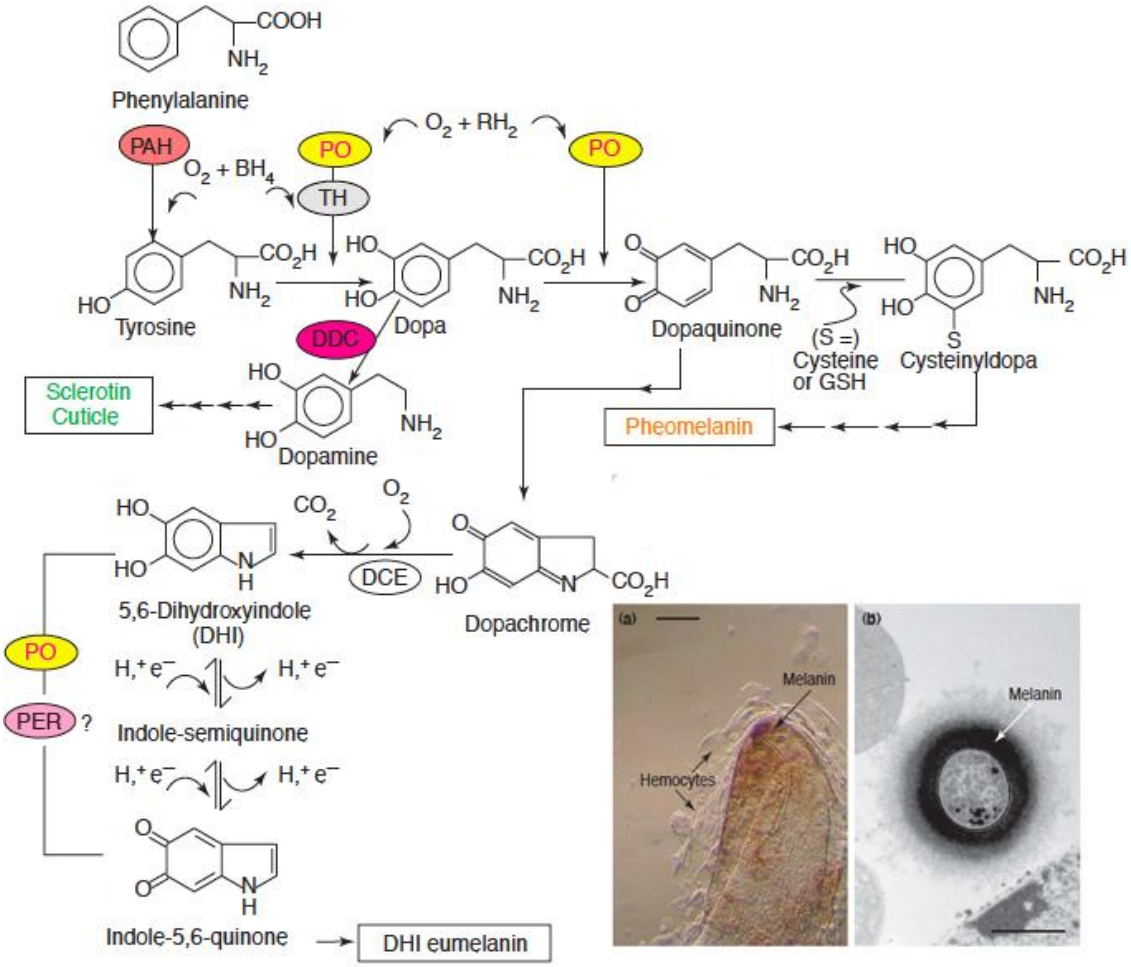


Figure 11. Biochemical pathway of melanin synthesis.
Biochemical reactions in the melanogenesis pathway. Refer to the text. (a) Cell mediated melanization of the parasitoid wasp *Leptopilina boulardi* in the hemolymph of fruit fly larvae. (b) Bacterium *Enterobacter cloacae* gets melanized in the hemolymph of adult *Aedes aegypti* mosquitoes without the involvement of hemocytes [4].

ii. Contribution of melanization to insect immunity

Genetic studies in *Drosophila melanogaster* initially revealed conflicting data on the significance of melanization as an immune defense mechanism. Leclerc *et al.* showed that PPO activating enzyme 1 mutants (PAE1) had similar survival rates to those of wild type flies in response to fungi, Gram-positive and Gram-negative bacteria. PAE1 mutants also harbored similar bacterial colony forming units as wildtypes suggesting that PO activity does not contribute to the fly's tolerance and resistance to infection [166]. Conversely, another study showed that *Drosophila* having a loss of function mutation in gene CG3066 (encoding PAE1) showed variations in the resistance and tolerance against a broad panel of bacteria hence supporting a significant role for melanization in immune defense [167]. More recently, *PPO1*/*PPO2* double mutant flies showed a marked susceptibility to Gram-positive bacteria and fungi. However, the contribution of PO to tolerance and resistance in these flies is markedly different in response to various microorganisms [168]. These different phenotypes observed in the absence of PO activity are most likely due to several factors such as virulence of the bacterial strain and efficiency of bacterial neutralization by other effector mechanisms such as AMPs and phagocytosis. Abolishing hemolymph PO activity by silencing *CLIPA8* in *A. gambiae* did not affect mosquito survival after infections with *E. coli* and *S. aureus* [117]. These bacteria were as efficiently cleared from *CLIPA8* kd mosquitoes as from controls suggesting that melanization is not important for anti-bacterial defense. However, since *E. coli* and *S.*

aureus are not natural pathogens for the mosquito, these results should be interpreted cautiously and a larger panel of preferably natural pathogens for mosquitoes (or other natively insect pathogens) should be used while readdressing the importance of melanization in mosquito immunity. On the other hand, silencing *CLIPA8* in *A. gambiae* revealed compromised mosquito survival and resistance to *B. bassiana* fungal infections indicating that melanization contributes significantly to anti-fungal defense [113].

In *A. gambiae*, the melanization response to *P. berghei* is also controlled by CLIPA8 [116], in addition to the complement-like protein TEP1 [119] and two leucine-rich immune proteins, LRIM1 [71] and APL1C [128, 149]. The TEP1/LRIM1/APL1C complex is required for ookinete lysis in the basal labyrinth of the midgut epithelium [71, 119, 149], as well as for the melanotic encapsulation of ookinetes in refractory mosquito genotypes [71, 119]. Nevertheless, wildtype laboratory and field caught *A. gambiae* mosquitoes rarely melanize malaria parasites [169] indicating that this response is dispensable for anti-*Plasmodium* defense [117].

In addition to bacteria and fungi, viruses also seem to trigger melanization. Semliki Forest virus (SFV) triggered the PO activity in a medium containing U4.4 *Ae. albopictus* cells [170]. In the crustacean *P. mondon* (shrimp), co-silencing the only two PPO genes by RNAi increased the shrimp's mortality to infections with the white spot syndrome virus [171]. Even though these results reflect a role for melanization (through the role of PO

activity) in anti-viral defense, the mechanism by which melanization kills the viral particles remains to be addressed.

c. Hemocyte-mediated defense

Hemocytes are central to both the cellular and humoral arms of the mosquito immune system. Unlike the highly specialized immune cells in mammalian systems, only three populations of hemocytes have been described in mosquitoes: granulocytes, oenocytoids and prohemocytes [172]. Granulocytes comprise 90 % of the circulating hemocytes, while oenocytoids and prohemocytes constitute the remaining 10 % combined [172]. Around 95 % of hemocytes are phagocytic [173]. Hemocyte phagocytosis is important in sequestration of bacteria and yeast [174] [175] [176] [177]. In response to infection, circulating hemocytes migrate toward the periostal regions (immune tissue flanking the heart) and aggregate with the sessile hemocytes where they rapidly phagocytose bacteria and malaria parasites after they enter the hemocoel [178]. A recent study in *A. gambiae* revealed a role for cytoplasmic actin in phagocytosis. Following bacterial systemic infections, Actin5c binds to bacteria and facilitates their uptake by granulocytes [179].

In addition to their role in phagocytosis, hemocytes also contribute to melanization and lytic responses. Mosquito hemocytes produce several factors such as dopachrome converting enzyme (DCE), serine proteases, serine protease inhibitors, C-type lectins and TEP1, all of which are essential in the melanization response [180-185]. Hemocytes

contribute to pathogen lysis through the production of TEP1 and other lytic factors such as antimicrobial peptides, reactive oxygen and nitrogen species [180-185].

The contribution of hemocytes to anti-*Plasmodium* immunity is under-explored. A "two phase" immune response model was proposed after Gupta *et al.* observed that the number of mature oocysts is significantly less than the number of early oocysts [136]. Parasites that are not melanized or lysed by TEP1 in the "early phase" response are confronted by a "late phase" response mounted by the mosquito hemocytes [136, 186]. Smith *et al.* showed that upon *P. berghei* infection, mosquitoes harbored significantly more circulating oenocytoids and granulocytes, however upon *LL3* (*LITAF*-like 3, transcription factor) silencing, the number of circulating hemocytes was unchanged compared to control mosquitoes. This result suggests that hemocytes respond to ookinete invasion through the action of LL3 [186]. Recently, a study identified a hemocyte differentiation factor (HDF), consisting mainly of a lipoxin/lipocalin complex, that is constitutively released in the hemolymph upon *Plasmodium* challenge. HDF release involves an increase in Evokin expression (a lipid carrier of the lipocalin family), and in its ability to convert arachidonic acid into lipoxins. This continuous release of lipoxins in the hemolymph causes a significant increase in granulocyte numbers, hence, a more potent immune response against *Plasmodium* [187]. Hemocytes then detect the epithelial nitration of the basal side of the midgut caused by ookinete invasion, and come in contact with the basal surface of the midgut. Hemocytes undergo apoptosis releasing hemocyte-derived microvesicles (HdMv), which promotes

TEP1 binding to the ookinete surface in a yet unidentified mechanism. Several lines of evidence corroborated this proposed model, HdMv are present in 33% of *Plasmodium*-invaded cells but are absent in noninvaded cells from the same midguts; TEP1 binding to the ookinete surface was decreased due to disruption of HdMv [188].

Hemocytes are clearly an essential component of the mosquito immune system. However, several points remain to be addressed in the future including the role of sessile hemocytes, how mosquito hemocytes recognize entities as invaders and which signaling pathways control hemocyte function.

d. Immune defenses mediated by the gut epithelium

i. Epithelial nitration

Ookinetes invading midgut epithelial cells promote the expression of nitric oxide synthase (NOS), heme peroxidase 2 (HPX2) and hydrogen peroxide generating enzyme NADPH oxidase 5 (NOX5). These three enzymes catalyze protein nitration in ookinete infected midgut [189] [190] [191]. Invaded epithelial cells show high HPX2 and NOX5 levels compared to weak undetected levels in uninvaded cells. Co-silencing *HPX2* and *NOX5* increased the intensity of *Plasmodium* infection as did silencing either one alone, indicating that they mediate the same anti-plasmodial response. Silencing *HPX2* significantly reduced the number of TEP1-labeled parasites. Moreover, co-silencing *HPX2* and *TEP1* increased *Plasmodium* infection similarly to *TEP1* silencing. These results

suggest that the nitration response mediated by HPX2/NOX5 is required for TEP1 effective lysis of ookinetes [191]. This orchestrated response initiated by epithelial nitration and followed by complement system activation is controlled by the JNK pathway. Garver *et al.* showed that JNK pathway promotes HPX2 and NOX5 expression in addition to up-regulating *TEP1* and *FBN9* [138]. It was proposed that rapidly invading ookinetes may be able to evade nitration and complement mediated killing [191].

ii. The actin hood and defense against ookinetes

Invasion of midgut epithelium by ookinetes is a critical step of the parasite life cycle. Damage inflicted on epithelial cells by ookinetes migrating intracellularly induces cytoplasmic protrusions. Invaded epithelial cells surround the ookinetes forming an actin-based hood [192]. Among the genes that were upregulated in response to ookinete invasion were genes encoding for proteins implicated in actin-cytoskeleton dynamics such as WASP (Wiskott-Aldrich syndrome protein). Silencing *WASP* resulted in a significant increase in both *P. berghei* and *P. falciparum* infections [193] [194]. WASP depletion by RNAi also resulted in concomitant decrease in hood formation. Furthermore, actin hood and TEP1 were frequently associated with the same parasite however, the co-localization of these two proteins was never observed [195]. All these data suggest that the "ookinete hood" functions among epithelial defense responses.

iii. Peritrophic matrix as an immune barrier

Ingestion of blood by mosquitoes triggers the formation of a peritrophic matric (PM), which is a physical barrier between the midgut lumen and epithelial cells. The PM is an acellular structure composed of chitin, proteins and glycoproteins (reviewed in [196]). The mosquito PM is considered an important barrier against *Plasmodium* parasite infections. Both *P. berghei* and *P. falciparum* mutants lacking the chitinase gene *CHT1* were markedly unable to form oocysts in infected mosquitoes [197, 198], highly suggesting the importance of the PM as a barrier against parasites. Recently, a study by Zhang *et al.* showed that *Plasmodium* parasites exploit FREP1 as a critical anchor in the PM, hence facilitating *Plasmodium* invasion of the mosquito midgut. Silencing *FREP1* resulted in significant reduction in oocyst numbers, as did the addition of anti-FREP1 antibody. These results suggest that physical interaction between *Plasmodium* and PM protein FREP1 is critical for midgut invasion [55]. Rodgers *et al.* showed that the PM plays a critical role in resistance to *Enterobacteriaceae* through reducing the extent of its growth and preventing it from inducing a systemic infection. Genes encoding chitin-binding domain and enzymes involved in chitin synthesis pathway were upregulated after bacterial infection, while these genes were down-regulated following antibiotic treatment [199]. Furthermore, disrupting the PM lead to bacterial translocation from the midgut to the hemocoel, seeding a systemic infection [199]. Taken together, these studies reflect the importance of the PM in immunity against invading pathogens, be it bacteria or *Plasmodium*.

Situated between the PM and the midgut epithelium is another physical barrier, the mucin dityrosine network. HPX15 and DUOX catalyze protein cross-linking in the mucin layer forming a dityrosine network. *Plasmodium* infection was reduced upon silencing either HPX15 or DUOX due to increased priming of the immune system by the gut microbiota [200].

e. Production of anti-microbial peptides

In dipterans, fat body produces and secretes large amounts of AMPs into the hemolymph in response to microbial infections [201]. Some AMPs have a broad spectrum of activity against bacteria and fungi and in some cases parasites and enveloped viruses [202]. AMPs are typically cationic ranging between 12-50 amino acids. AMPs are broadly classified into four classes; amphipathic α-helical peptides deprived of cysteine residues, β-pleated peptides containing disulfide bridges, peptides rich in proline, glycine, histidine, arginine and tryptophan residues, and circular antimicrobial peptides [203]. Due to their cationic nature, AMPs generally interact with negatively-charged pathogen membranes promoting their disruption [204]. However, AMPs may have other modes of action, such as inhibiting protein synthesis, inducing ROS production and manipulating microbial homeostasis [205, 206].

There are seven identified AMP families in *Drosophila* so far: Drosocin, Attacin, Diptericin, Cecropin, Defensins, Metchnikowin and Drosomycin. Cecropins for instance,

are described as anti-Gram-negative peptides, whereas Defensins are anti-gram-positive peptides, while Metchnikown and Drosomycin are anti-fungal peptides. AMP production in *Drosophila* is tightly regulated by both the Toll and pathways [207].

In *A. gambiae*, only four AMP families have been identified: Cecropins, Defensins, Diptericin and Gambicin [9] [8]. *Anopheles gambiae* AMPs are active against a broad range of microbes. CEC1 and GAM both have anti-bacterial and anti-*Plasmodium* activities [208] [209] [52] [210]. DEF1 on the other hand, is active against some filamentous fungi and Gram-positive bacteria [211].

C. Metallocarboxypeptidases

Metallocarboxypeptidases (MCPs) are peptide hydrolyzing enzymes that cleave C-terminal residues from their substrates. MCPs have functions such as recovery of dietary amino acids, tissue organogenesis, neurohormone and cytokine maturation and other physiological processes [212]. Structurally, MCPs can be divided into two groups, cowrins and funnelins. Cowrins comprise enzymes related to neurolysin and angiotensin-converting enzyme. Funnelins, on the other hand, comprise mammalian and insect proteins with carboxypeptidase activities. Funnelins catalytic domains are globular in shape with a funnel-like opening on the top, which is why these carboxypeptidases were named "funnelins". Their catalytic domains are around 300 residues, and are composed of eight-stranded β-sheet flanked by a total of eight helices [213]. The catalytic domain of all MCPs

displays a α/β hydrolase fold which harbors the catalytic Zn^{2+} ion. The active site clefts are shallow retaining the ability to bind the C-terminal ends of substrate proteins upon minimal contact, rendering these enzymes able to cleave a plethora of folded proteins [213].

1. Metallocarboxypeptidases in mammals

Funnelin MCPs are divided into four subfamilies. Digestive carboxypeptidases such as CPA, CPB and CPU/TAFI which belong to the M14A subfamily. M14B subfamily which encompasses regulatory carboxypeptidases such as carboxypeptidase D (CPD), CPE, CPM and CPN. The remaining two subfamilies include bacterial peptidoglycan hydrolyzing enzymes (M14C) and cytosolic carboxypeptidases (M14D) [214].

MCPs belonging to M14A subfamily (Type A/B MCPs) are synthesized as zymogens having a pro-domain that physically covers the active site, and upon its proteolytic cleavage the active site becomes exposed. Type A MCPs recognize specifically C-terminal aliphatic or aromatic amino acid residues, whereas type B MCPs recognize C-terminal lysines or arginines [214].

Certain MCPs have been extensively studied in humans, mainly carboxypeptidase N (CPN) and thrombin-activatable fibrinolysis inhibitor (TAFI). CPN is expressed in the liver and secreted into the bloodstream as a 280 kDa tetramer. Structurally it is composed of two heterodimers, each composed of a small catalytic subunit CPN1 (55 kDa) and a larger regulatory subunit CPN2 (83 kDa) [215]. Hydrolysis of the regulatory subunit exposes the catalytic subunit which upon hydrolysis becomes active [216]. The main substrates for

CPN were shown to be complement anaphylatoxins C3a, C4a and C5a. CPN cleaves the C-terminal arginine of C3a, C4a and C5a into C3a-desArg, C4a-desArg and C5a-desArg respectively. This hydrolysis reduces anaphylatoxins activity by 10 to 100 folds hence inhibiting prolonged inflammation [217]. Other substrates for CPN include bradykinin which is involved in acute phase inflammatory response [218] [219], and creatine kinase MM [220]. Thrombin-activatable fibrinolysis inhibitor (TAFI) is another well studied carboxpeptidase. TAFI is secreted into the plasma as a zymogen that gets activated upon cleavage similar to CPN. Several activators of TAFI have been identified such as thrombin, which is relatively a weak activator of TAFI, however, in the presence of both thrombin and thrombomodulin, TAFI activation increases 3 folds [221]. Another TAFI activator is plasmin which was shown to activate TAFI 8 folds more than thrombin alone [222]. After activation, TAFIa (activated form of TAFI) cleaves C-terminal lysine residues from fibrin decreasing plasminogen activation, which consequently down regulates plasmin formation and culminates in fibrinolysis inhibition [223] [224]. TAFIa was also shown to cleave C-terminal lysine residues from anaphylatoxins C3a and C5a thus inhibiting prolonged inflammation [225]. However, contrary to CPN which is relatively stable, TAFI is rapidly deactivated. Hence it may be considered a local anti-inflammatory molecule at sites of tissue injury [212].

TAFI and CPN are not the only MCPs to be studied. CPA3 for example, was shown to be necessary for mast cell maturation [226]. CPA4 on the other hand, is thought to play a role

in prostate cancer, although much of its functions remain to be addressed [227, 228]. CPD, another MCP, was shown to digest arginine from substrates leading to enhanced nitric oxide production in the mouse macrophage-like cell line RAW 264.7. These elevated nitric oxide levels were in turn shown to inhibit apoptosis in MCF7 breast cancer cells [229-231]. *CPE* KO mice developed multiple endocrine disorders such as obesity, diabetes and neurological deficits [232-234].

2. Metallocarboxypeptidases in mosquitoes

Mosquito carboxypeptidases are still under-explored compared to those in mammals, however, several studies have focused mainly on the expression levels of carboxypeptidases in response to blood feeding and infections with *Plasmodium*. A study in 2017 showed that *carboxypeptidase A* (*CPA)* in *Anopheles stephensi* is significantly overexpressed 14 hours after a blood meal compared to non-fed control mosquitoes. Moreover, adding CPA antiserum to *P. berghei* infected blood reduced the infection rate up to 16% compared to 81% in the control group [235]. Additionally, Isoe *et al.* molecularly characterized 18 carboxypeptidase genes, 11 of which belonged to the carboxypeptidase A family, whereas and the rest belonged to the carboxypeptidase B family. Most of these carboxypeptidase genes were shown to be differentially overexpressed upon 24-36 hours post blood feeding, hence suggesting a possible role for carboxypeptidases in digestion [236]. A study in *Anopheles gambiae* by Lavazec *et al.* identified two overexpressed *carboxypeptidase B* genes (*CPB1* and *CPB2*) in response to *P. falciparum* or *P. berghei*

infected blood meal. Addition of CPB1 antibodies to a *P. falciparum* or *P. berghei* infected blood meals significantly decreased parasite development compared to control mosquitoes [237].

Since antibodies against certain carboxypeptidases seem to significantly inhibit *Plamsodium* transmission, studies on mosquito carboxypeptidases seem to be focused on its promising use as targets for *Plasmodium falciparum* transmission-blocking vaccines (TBVs). However, their role in insect immunity has not been addressed yet, as such, the second objective of this project is to investigate the role of two hemolymph carboxypeptidases in mosquito immune responses to bacteria and *Plasmodium*, and eventually unravel the molecular basis of their functional interaction with the mosquito immune proteins specifically TEP1 and the SPHs involved in the melanization response.

CHAPTER II
AIMS OF THE PROJECT

Mosquitoes transmit a wide range of diseases such as dengue fever, yellow fever and malaria. *Anopheles gambiae* mosquitoes are the main vector for malaria in Africa. The interaction and interplay between the mosquito's immune system and malaria parasites contribute significantly to the host's vectorial capacity. The hallmark of *A. gambiae* immune system is the complement-like protein TEP1 [119]. TEP1 controls melanization by regulating the activation cleavage of CLIP domain serine protease homologues (cSPHs) and possibly catalytic serine proteases cSPs. cSPHs are mainly involved in the regulation of PPO activation in several insect species [76, 96, 117]. Studies in *A. gambiae* revealed that they exhibit multilayered control of the melanization response that includes also TEP1[238]. Hence, cSPHs exhibit complex regulatory roles, however, their mechanism of action and their main role in immunity remain to be thoroughly investigated. Hence, the **first specific aim** of my thesis is to understand and dissect the role of CLIPA14, a serine protease homolog, in mosquito immune responses to bacterial and *Plasmodium* infections.

CLIPA14 was initially identified in our lab as a protein that co-immunoprecipitated with CTL4-CTLMA2 complex (Osta MA, unpublished) and later with CLIPA2, a negative regulator of melanization, which prompted the characterization of its role in the melanization response.

In **specific aim 1**, I investigated the contribution of CLIPA14 to mosquito immunity by studying its role in defense against infections with bacteria and with *P. berghei* (model rodent malaria parasite). I specifically scored the impact of silencing CLIPA14 on mosquito resistance and tolerance to bacterial infection and on resistance to malaria parasties. I also investigated the effect of co-silencing *CLIPA14* and *CLIPA2* on the level of the melanization response to *P. berghei* parasites in order to understand whether these two negative regulators act on the same step or regulate distinct steps of the response. At the molecular level, I further characterized CLIPA14 involvement in the melanization response by investigating the impact of *CIPA14* kd on hemolymph PO activity after septic infections. Since all known cSPs and cSPHs are secreted as zymogens and require cleavage for their activation, I studied whether CLIPA14 follows the same rule, by investigating its activation cleavage following bacterial infections and identifying the immunity genes required for its cleavage. Specifically, I studied the effect of knocking down *TEP1* and other key cSPHs such as CLIPA8, CLIPA2 and CLIPA28 on CLIPA14 cleavage.

So far, all known cSPHs with role in immunity require TEP1 for their cleavage. Based on this fact, we adopted a high throughput degradomic approach to identify novel CLIPs whose cleavage is TEP1-dependent. This approach aims to identify differentially cleaved proteins specifically CLIPs, in hemolymph of *E. coli*-infected *TEP1* kd compared to *LacZ* kd controls. Using this approach, we identified several cSPHs with already proven roles in immunity as well as novel ones, in addition to two carboxypeptidases, CP1 and

CP2 which appeared among the top 10 TEP1-dependent cleaved substrates. To this date, there is no evidence that carboxypeptidases are involved in insect immune responses which prompted the characterization of these genes in the context of mosquito immunity, specifically their interplay with TEP1. Hence, in **specific aim 2** of my thesis, I performed an in depth functional and molecular analysis of carboxypeptidases 1 and 2, by investigating the contribution of both CP1 and CP2 to mosquito tolerance and resistance against Gram-positive and Gram-negative bacterial infections. I also studied the impact of *CP1* kd and *CP2* kd on parasite development in susceptible and melanizing backgrounds (*CTL4* kd mosquitoes [71]). Since CP1 and CP2 cleavage is TEP1-dependent, I investigated whether knocking down either *CP1* or *CP2* would affect TEP1 localization to parasite surfaces. Additionally, I also studied whether *CP1* and *CP2* contribute to the regulation the melanization response by measuring the impact of their knockdown on hemolymph PO activity and on the cleavage of key cSPHs. Finally, I expressed the recombinant forms of CP1 and CP2 in order to raise antibodies against them that would allow their future characterization at the biochemical level.

PREFACE TO CHAPTER III

The following chapter is composed of two parts, with each part representing results from one independent study. The first study characterizes the role of CLIPA14 in the mosquito immune response to bacteria and *Plasmodium*. This first study is already published in *Journal of Biological Chemistry* ([238]) and the accepted manuscript was reformatted to match the desired style of the dissertation. This study also includes novel unpublished data regarding CLIPA14 hierarchical position in the cSPHs network that were added as an independent section just after the manuscript. In the second study, I included all the data relevant to the characterization of carboxypeptidases in mosquito immunity against bacterial and malaria parasite infections.

CHAPTER III

RESULTS

A. CLIPA14 a novel serine protease homolog that modulates the intensity of mosquito immune attack against malaria parasites

1. Abstract

CLIP domain serine protease homologues (cSPHs) act as positive and negative regulators of *A. gambiae* immune responses mediated by the complement-like protein TEP1 against malaria parasites and microbial infections. We have previously shown that the SPH CLIPA2 is a negative regulator of the TEP1-mediated response whereby its knockdown (kd) enhanced mosquito resistance to infections with fungi, bacteria and *Plasmodium* parasites. Here, we identify CLIPA14 as a novel negative regulator of mosquito immunity. CLIPA14 is a hemolymph protein that is rapidly cleaved following a systemic infection. *CLIPA14* kd mosquitoes elicited a potent melanization response against *Plasmodium* ookinetes and exhibited significantly increased resistance to *Plasmodium* infections as well as to systemic and oral bacterial infections. The enzyme phenoloxidase that initiates melanin biosynthesis exhibited a dramatic increase in activity in the hemolymph of *CLIPA14* kd mosquitoes in response to systemic bacterial infections. Ookinete melanization and hemolymph phenoloxidase activity were further increased after co-silencing *CLIPA14* and *CLIPA2*, suggesting that these two SPHs act in concert to control the melanization response. Interestingly, *CLIPA14* RNAi phenotypes and its infection-

induced cleavage were abolished in a TEP1 loss of function background. Our results suggest that a complex network of SPHs functions downstream of TEP1 to regulate its effector functions in particular melanization.

2. Introduction

Clip domain serine proteases (CLIPs) are key components of insect immune responses leading to melanization and antimicrobial peptide synthesis through the Toll pathway. CLIPs are specific to invertebrates and form large gene families in insect genomes [8]. Their function has been particularly studied in the context of the melanization response which, in insects, plays important roles in several physiological processes including cuticle sclerotization or tanning [239], hardening of wound clots [150] and resistance to microbial infections [113, 167, 168, 240]. There is also convincing evidence for an antiviral role of melanization [170, 171]. The infection-induced melanotic response is initiated when pattern recognition receptors (PRRs) bind to microbial cell components triggering the activation of a cascade of serine proteases, constituted mostly by CLIPs, which culminates in the limited proteolytic cleavage of the zymogen prophenoloxidase (PPO) into active phenoloxidase (PO), the rate limiting enzyme in melanin biosynthetic pathways.

The serine protease cascades acting upstream of PPO are complex and finely regulated to control the spatial, temporal and intensity of PPO activation [reviewed in [241, 242]]. The initiator protease in these cascades is a modular serine protease (ModSp)

composed of a complex assortment of domains that allow multiple interactions with upstream PRRs and downstream proteases [243-245]. The serine proteases acting downstream of ModSp are clip domain serine proteases (CLIPs). Among these are non-catalytic CLIPs (also called clip domain serine protease homologs, SPHs) which lack one or more of the three residues (His, Asp, Ser) that form the protease catalytic triad. Both catalytic CLIPs and SPHs are secreted as precursor proteins and require cleavage at a specific site between the clip and protease domain to become active. The role of SPHs as inferred from studies in other insects seems to be confined to the terminal step of PPO cleavage [reviewed in [241]]. SPHs act as cofactors for terminal CLIPs in the cascade, called prophenoloxidase activating proteases (PAPs), for the efficient cleavage and activation of PPO [76, 246, 247]. PPOs cleaved by PAPs in the absence of SPH cofactors showed no activity *in vitro* even when the SPH was added later to the active PO [104, 246, 248], indicating that SPHs are required for correct cleavage of PPOs.

Studies in the malaria vector *Anopheles gambiae* revealed that SPHs have a broader role in the regulation of immune responses. A systematic functional genetic screen by RNA interference (RNAi) identified several SPHs (CLIPA8, CLIPA2, CLIPA5 and CLIPA7) to be involved in the melanization of the rodent malaria parasite *Plasmodium berghei* whilst invading the mosquito midgut epithelium [116]. CLIPA8 acts as a positive regulator of the melanization response triggered against bacterial [117] and fungal infections [113], as well as against infections with *P. berghei* in certain mosquito melanotic backgrounds [116].

CLIPA8 is cleaved following bacterial challenge, and this cleavage is controlled by the thioester-containing protein 1 (TEP1) [115], a homolog of the mammalian C3 complement factor that mediates key effector functions in mosquito immune responses including microbial lysis, phagocytosis and melanization [119, 120, 146, 249, 250]. The knockdown of either *TEP1* or *CLIPA8* abolished hemolymph PO activity in response to bacterial infections [113, 115] indicating a tight control by TEP1 over the melanization response.

The RNAi phenotypes of CLIPA2, CLIPA5 and CLIPA7 suggested a negative regulatory role for these SPHs in the melanization response to *P. berghei* [116]. Recently, CLIPA2 was shown to regulate melanization indirectly by controlling TEP1 activity during systemic infections; *CLIPA2* kd enhanced TEP1 activity leading to an exaggerated PO activity in the hemolymph following *E. coli* infections [118, 147]. CLIPA2 is thought to negatively regulate the conversion of full-length TEP1 (TEP1-F) to the processed form ($TEP1_{cut}$), which was shown to be the active form of TEP1 that is stabilized by the two leucine-rich immune proteins APL1C and LRIM1 [145, 148]. A more recent study identified the SPH SPCLIP1 as a major positive regulator of TEP1, whereby the localization of TEP1 and SPCLIP1 to *Plasmodium* ookinetes was shown to be mutually dependent [115].

Here, we show that a novel SPH termed CLIPA14 acts as a major negative regulator of the mosquito melanization response acting downstream of TEP1 and SPCLIP1. We have previously shown that CLIPA14 co-immunoprecipitates with CLIPA2 from mosquito

hemolymph extracts [147]. RNAi-mediated silencing of *CLIPA14* in adult, female *A. gambiae* mosquitoes triggered melanization of most *P. berghei* ookinetes invading their midgut in a TEP1-dependent manner. These mosquitoes exhibited an unusually high hemolymph PO activity following bacterial systemic infections in addition to strong resistance to systemic and oral bacterial infections. We also show that the melanization of ookinetes and hemolymph PO activity were significantly enhanced when *CLIPA14* and *CLIPA2* were co-silenced, suggesting that they act in concert to regulate the TEP1-mediated melanization response. Our results reveal a new level of complexity in SPH function in mosquito immunity and provide further evidence for their key role in regulating the mosquito complement-like response.

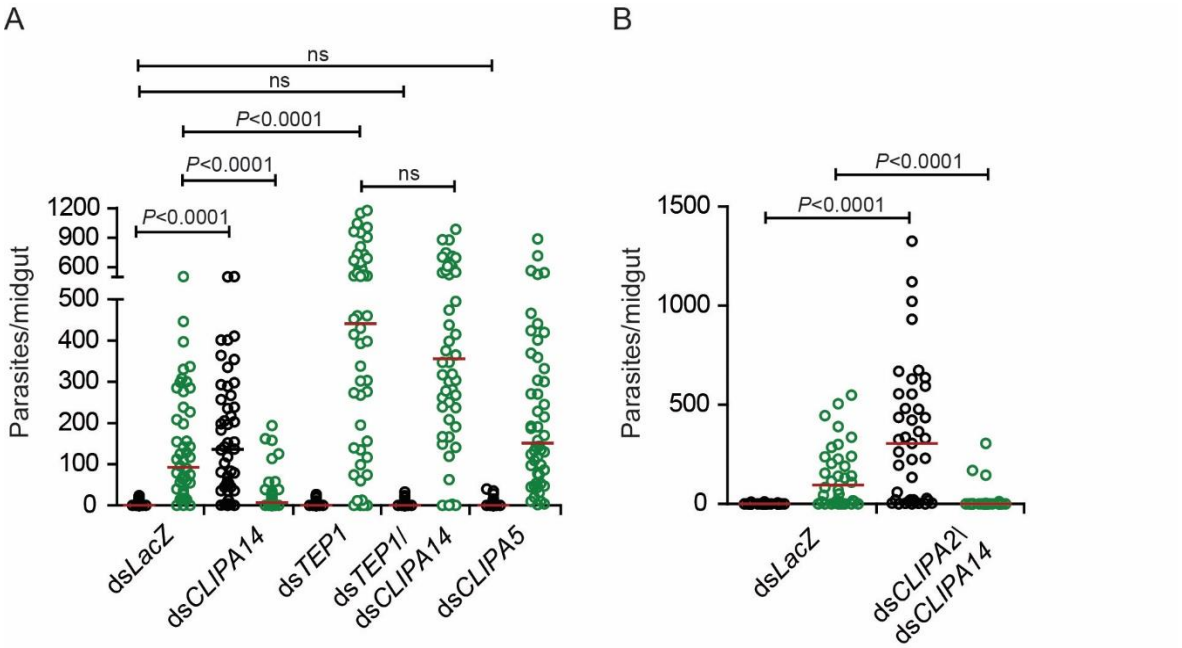
3. Results

a. CLIPA14 regulates Plasmodium melanization in a TEP1-dependent manner

We have previously identified CLIPA14 among the list of proteins that co-immunoprecipitated with CLIPA2 in hemolymph extracts of *B. bassiana* infected mosquitoes [147]. To address the function of CLIPA14 in mosquito immunity, we first silenced this gene in adult female *A. gambiae* mosquitoes by RNAi and scored the effect on the survival of *P. berghei* oocysts at day 7 after ingestion of an infectious blood meal. Interestingly, *CLIP14* kd triggered a potent melanization response against ookinetes resulting in the melanotic encapsulation of the majority (86%) of these parasite stages

(Figure 12A); in 26% of dissected mosquitoes, midguts contained only melanized ookinetes with no single live oocyst detected. *LacZ* kd controls revealed a background level of melanization as expected for the G3 strain. This *CLIPA14* RNAi phenotype is stronger than that observed for *CLIPA2* [116, 118] and similar to that previously reported for *CTL4* kd mosquitoes [71]. Parasite melanization was abolished when *CLIPA14* and *TEP1* were co-silenced, and oocyst counts were similar to those in *TEP1* single kd mosquitoes, indicating that the enhanced immunity against parasites is TEP1-dependent. This result confirms the central role of TEP1 in initiating the melanization response as reported previously in different mosquito genetic backgrounds [118, 119, 251].

CLIPA2 kd mosquitoes also melanize a significant number of ookinetes in a TEP1-dependent manner [118]. The RNAi phenotypes of CLIPA2 and CLIPA14 suggest that they play non-redundant roles in fine tuning the melanization response. Hence, we asked whether a higher level of parasite melanization can be achieved if both genes are co-silenced. Interestingly, most *CLIPA14*/*CLIPA2* dkd mosquitoes were completely refractory to *P. berghei* (Figure 12B); the infection prevalence (i.e. percentage of mosquitoes carrying live parasites) was 26% as compared to 72% and 76% for *CLIPA14* (Figure 12A) and *CLIPA2* [118] single knockdowns, respectively. These results indicate that CLIPA2 and CLIPA14 exhibit additive roles in the mosquito melanization response.



Gene kd	n	% mel	prevalence (%)
LacZ	50	2.3	96
CLIPA14	50	86	72
TEP1	50	0.5	92
TEP1/CLIPA14	50	1.0	94
CLIPA5	50	1.6	100

Gene kd	n	% mel	prevalence (%)
LacZ	36	0.78	86
CLIPA2/ CLIPA14	41	95.5	26

Figure 12. *CLIPA14* kd triggers a potent TEP1-dependent melanotic response against malaria parasites.
(A) and (B) Scatter plots of live GFP-expressing *P. berghei* oocysts (green circles) and dead melanized ookinetes (black circles) scored in the midguts of the indicated mosquito genotypes seven days post-infection. Red lines indicate median parasite numbers. Statistical analysis was performed using the Mann-Whitney test and *P*-values less than 0.05 were considered significant. Data were pooled from three independent biological experiments.

Phylogenetic analysis of *A. gambiae* CLIPAs revealed that CLIPA5 is the closest paralogue to CLIPA14 [8]. However, when we aligned the protein sequences of CLIPA5 and CLIPA14 (AGAP011788) available in VectorBase (www.vectorbase.org) using the

MUSCLE alignment tool we found that CLIPA14 sequence is partial and is missing approximately 146 aa from the C-terminus of its protease-like domain (Figure 13). We reconstructed the full length *CLIPA14* cDNA using the GENSCAN Web Server at MIT [genes.mit.edu/GENSCAN.html [252]] to determine coding sequences downstream (3' flanking) of the CLIPA14 cDNA available in VectorBase. We designed primers based on the newly predicted sequence, amplified full-length CLIPA14 from *A. gambiae* G3 cDNA and cloned it into pIEx-10 expression plasmid. The sequence of the cloned amplicon matched the predicted sequence we reconstructed using GENSCAN, indicating that CLIPA14 has a gene structure typical of a clip-domain containing SPH. However, we found that this gene segment of CLIPA14 that was missing from the annotation contained a contiguous sequence of 53 nucleotides that showed 100% complementarity to a region of the dsRNA we used previously to silence CLIPA5 (Figure 14) in the context of a large genetic screen of *A. gambiae* CLIPs [116]; It is worth noting that, at that time, CLIPA14 was absent from the initial list of *A. gambiae* annotated genes based on which the CLIP gene screen was performed [9]. This raised the possibility that CLIPA5 RNAi phenotype characterized by increased ookinete melanization [116], could be due to cross-silencing CLIPA14. To address this issue, we re-silenced CLIPA5 with a new gene-specific dsRNA complementary to the region situated between the clip domain and the beginning of the protease domain; this region shows significant sequence diversification in CLIPA14 and CLIPA5, which exhibit their highest sequence similarity in their protease domains

especially in the last 241 aa (Figure 13). Following infection by *P. berghei*, *CLIPA5* kd mosquitoes exhibited a basal level of ookinete melanization similar to that in *LacZ* kd controls (Figure 12A), indicating that CLIPA5 does not regulate melanization and its previous RNAi phenotype is due to cross-silencing CLIPA14. Indeed, western blot analysis revealed that CLIPA5 dsRNA used in our previous genetic screen [116] silenced *CLIPA14* with a similar efficiency as ds*CLIPA14* did (Figure S3A). Despite the significant sequence similarity between CLIPA14 and CLIPA5, CLIPA14 antibody does not cross-react with CLIPA5 since CLIPA14 signal in *CLIPA14*/*CLIPA5* double kd (dkd) was similar to that in *CLIPA14* single kd mosquitoes (Figure 15B).

Figure 13. Alignment of the protein sequences of CLIPA5 and CLIPA14.
Full-length CLIPA14 (CLIPA14-full) of our *A. gambiae* G3 strain was aligned with CLIPA5 (AGAP011787) and CLIPA14 (AGAP011788) sequences available in VectorBase using MUSCLE sequence alignment tool. Note that the CLIPA14 protein sequence in VectorBase (AGAP011788) is missing the last 146 aa (residues 283-429) of the C-terminal domain.

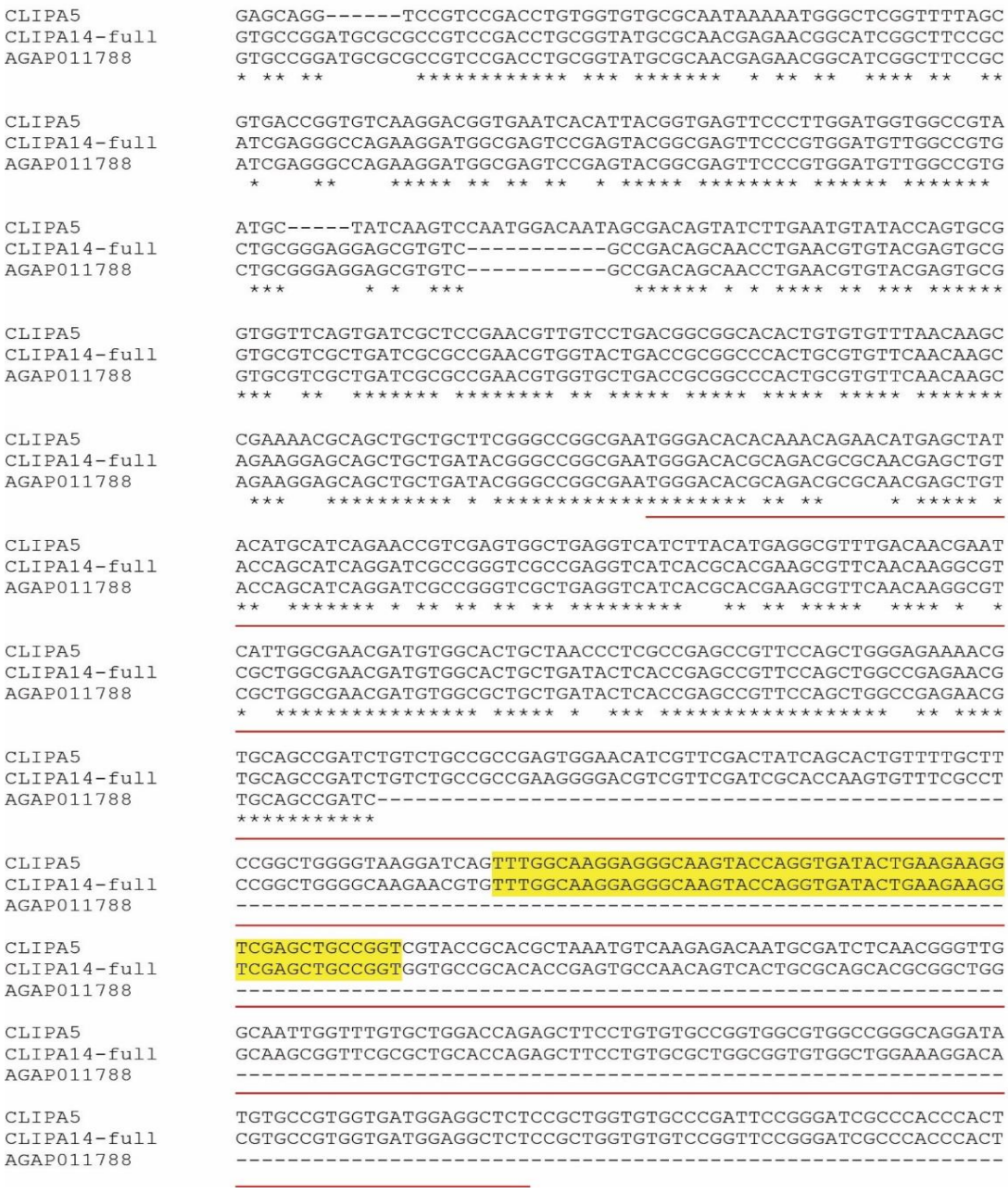


Figure 14. Alignment of the coding sequences of CLIPA5 and CLIPA14 corresponding to the protease-like domain.

The coding sequence (cds) corresponding to the protease-like domain of full-length CLIPA14 (CLIPA14-full) cloned from the G3 strain was aligned with the respective

VectorBase sequences of CLIPA5 (AGAP011787) and CLIPA14 (AGAP011788) using MUSCLE sequence alignment tool. The sequence complementary to the dsRNA that was previously used to silence CLIPA5 [116] is underlined in red. Highlighted in yellow is a contiguous sequence of 53 nucleotides within the ds*CLIPA5* complementary region that shares 100% identity with its corresponding sequence in *CLIPA14* cds.

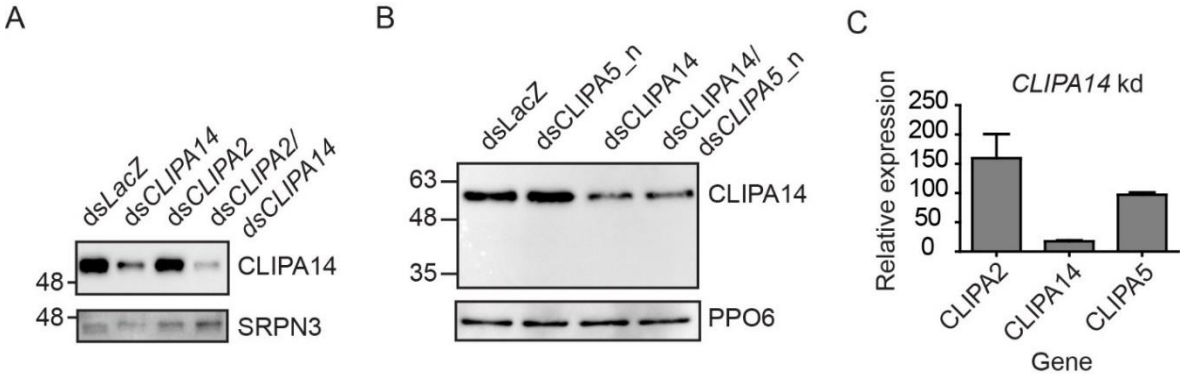


Figure 15. Absence of cross-silencing between the different CLIPAs.
(A) Western blot showing the absence of cross-silencing between *CLIPA2* and *CLIPA14* in hemolymph extracts of mosquitoes treated with the indicated dsRNAs. The blot was probed with SRPN3 antibody to confirm equal loading. (B) Western blot showing the absence of cross-interaction between CLIPA14 antibody and CLIPA5. (C) Quantitative RT-PCR showing the relative expression of *CLIPA2, CLIPA14* and *CLIPA5* genes in *CLIPA14* kd mosquitoes.

b. CLIPA14 kd mosquitoes are resistant to bacterial infections

The potent melanotic response elicited by *CLIPA14* kd mosquitoes against *Plasmodium* ookinetes does not necessarily indicate that these mosquitoes are similarly resistant to infections with other classes of microoganisms. This is supported by the RNAi phenotypes previously reported for the *A. gambiae* CTL4 gene; While *CTL4* kd mosquitoes exhibited a similar level of resistance to *Plasmodium* ookinetes as *CLIPA14* kds [71] they were more susceptible to systemic *E. coli* infections [72]. To address the role of CLIPA14 in anti-microbial defense, *CLIPA14* kd mosquitoes were challenged with bacteria either by

direct injection into the hemocoel or through the oral route. *LacZ* kd mosquitoes were used as controls. The results showed that *CLIPA14* kd mosquitoes harbored significantly less *E. coli* (Figure 16D) and *S. aureus* (Figure 16E) in their tissues at 48 hrs after bacterial injections as compared to controls. Interestingly, while the survival rates of *E. coli*-infected *CLIPA14* kd mosquitoes were similar to controls (Figure 16A), *S. aureus*-infected *CLIPA14* kd mosquitoes showed compromised survival compared to controls (Figure 16B) despite harboring less bacteria. Indeed, two criteria are known to influence the host survival to infections: reducing the microbial burden in tissues or tolerating the damage triggered by the immune response and the microbe [253, 254]. Hence, the compromised survival of *CLIPA14* kd mosquitoes to *S. aureus* but not to *E. coli* infections could be due to an increased magnitude of the immune response in the presence of the former bacteria, possibly leading to tissue pathology and host toxicity.

The direct injection of bacteria into the hemocoel has been widely used as a practical approach to trigger systemic infections in mosquitoes and other insects, despite that this route of infection is unlikely to be common in natural habitats due to the rigidity of the external cuticle. So we examined whether *CLIPA14* kd mosquitoes are also able to clear oral infections with *Serratia marcescens* bacteria that are commonly present within the gut flora of field caught mosquitoes [255-257] and known to invade the insect gut epithelium reaching into the hemocoel [258]. Silencing *CLIPA14* resulted in significantly lower numbers of *S. marcescens* in the hemocoel compared to *LacZ* kd controls (Figure 16F),

indicating that CLIPA14 modulates the mosquito immune response to oral bacterial infections. Like *E. coli*, *S. marcescens* infections did not compromise the survival of *CLIPA14* kd mosquitoes (Figure 16C), suggesting that Gram-negative bacterial infections may not influence host tolerance in this genetic background.

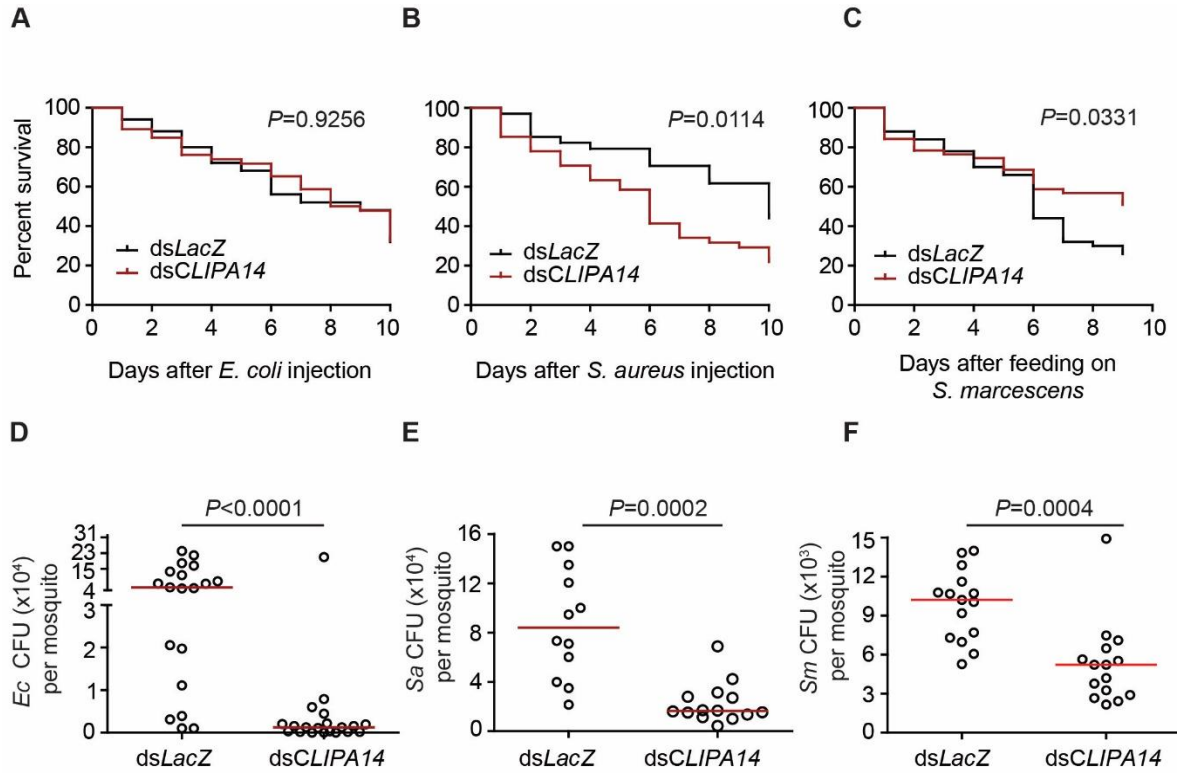


Figure 16. *CLIPA14* kd increases resistance to systemic and oral bacterial infections. *CLIPA14* kd mosquitoes are resistant to bacterial infections. (A-C) mosquito survival assays following injection of (A) *E. coli* (OD_{600nm} = 0.4) and (B) *S. aureus* (OD_{600nm} = 0.4) into mosquito hemocoel, or (C) after oral infection with *S. marcescens*. One representative experiment is shown from at least three independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. (D-F) Bacterial proliferation assays conducted on mosquitoes injected with (D) *E. coli* (OD_{600nm} = 0.4), (E) *S. aureus* (OD_{600nm} = 0.4) or (F) fed on sugar pads containing *S. marcescens* (OD_{600nm} = 0.1). Batches of 8 (D-E) whole mosquitoes or (F) mosquito carcasses (excluding midgut) were grinded in LB medium at 48 hrs after infection and colony forming units (CFU) were scored on LB

plates supplemented with the appropriate antibiotic. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (red lines) were considered significant if $P < 0.05$. Data shown are from three independent biological experiments.

The enhanced bacterial clearance in *CLIPA14* kd mosquitoes prompted us to address the contribution of TEP1 which is known to play an important role in antibacterial defense [146, 147]. To address this point *E. coli* CFUs in *LacZ* (control), *CLIPA14* and *TEP1* single kd were compared to those in *CLIPA14*/*TEP1* dkd mosquitoes at 48 hrs after bacteria injection into the hemocoel. As expected, *TEP1* kd triggered increased proliferation of *E. coli* compared to the control group (Figure 17). Interestingly, *E. coli* CFUs in the dkd group were similar to those in the *TEP1* single kd clearly indicating that rapid bacterial clearance in *CLIPA14* kd mosquitoes is TEP1-dependent.

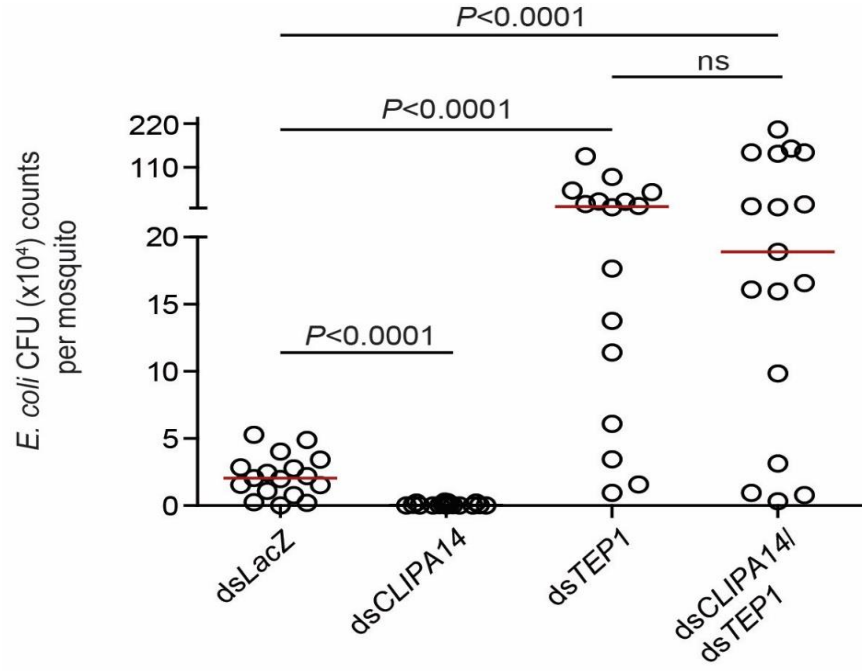


Figure 17. The resistance of *CLIPA14* kd mosquitoes to bacterial infections is TEP1-dependent.
Bacterial CFU were scored in the indicated mosquito genotypes at 48 hrs after injection with *E. coli* (OD_{600nm} = 0.4). For each genotype, batches of 8 whole mosquitoes were grinded in LB medium and CFU were scored on LB plates supplemented with ampicillin. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (red lines) were considered significant if $P < 0.05$. Data shown are from three independent biological experiments.

c. CLIPA14 is cleaved in response to bacterial systemic infections

SPHs require proteolytic cleavage by catalytic CLIPs to become functional despite their lack of catalytic activity. This has been shown in several insect SPHs, including SPH1 and SPH2 of *Manduca sexta* [96, 99], PPAFII of *Holotrichia diomphalia* [76] and CLIPA8 of *A. gamb*iae [117]. CLIPA14 does not seem to be an exception in this regard, since

injecting mosquitoes with *S. aureus* triggered its rapid cleavage in the hemolymph (Figures 18 and 19A). CLIPA14 was also cleaved after *E. coli* injections but sometimes at a weaker level (data not shown), which prompted the use of *S. aureus* in these assays. The apparent molecular weights of full-length CLIPA14 (CLIPA14-F) and the cleaved C-terminal domain (CLIPA14-C) are 59 and 39 kDa, respectively. We were not able to detect the N-terminal fragment containing the clip-domain possibly because it is weakly antigenic compared to the larger C-terminal protease-like domain hence generating an undetectable chemiluminescent signal. Of note, even CLIPA14-C band was always weak and required high exposures (signal saturation) for clear detection, suggesting that either a small fraction of CLIPA14-F is cleaved or the cleaved product is quickly sequestered possibly on microbial surfaces. *CLIPA14* kd strongly reduced CLIPA14 hemolymph levels by approximately 85% (Figure 19B); this reduction is not apparent in Figure 18 as it is over-exposed. Interestingly, silencing either TEP1 or its positive regulator SPCLIP1 completely abolished CLIPA14 cleavage (Figures 18 and 19A), indicating that CLIPA14 is tightly controlled by the TEP1 pathway.

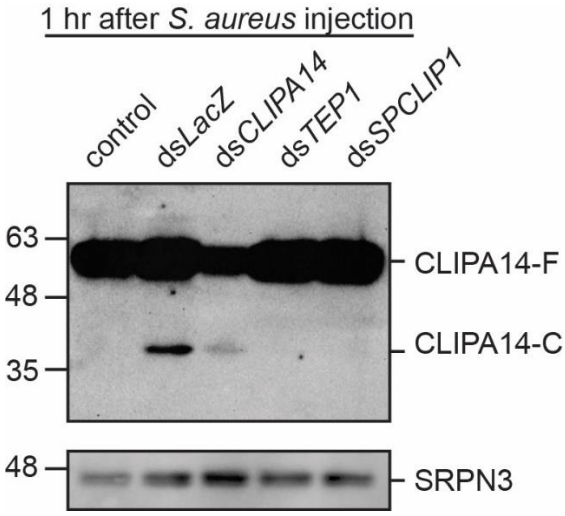


Figure 18. TEP1 and SPCLIP1 control the infection-induced cleavage of CLIPA14. Western blot showing CLIPA14 cleavage in the indicated mosquito genotypes. Hemolymph was extracted from mosquitoes at 1 hr after injection with *S. aureus* (OD_{600}=0.8). Protein quantification was performed using the Bradford protein assay and 1.2 μg of hemolymph proteins were loaded per lane. The membrane was probed with antibodies against CLIPA14 and SRPN3 (as loading control). Shown is a high exposure image (saturated) in order to detect the cleaved C-terminal protease-like domain of CLIPA14 (CLIPA14-C). The image is representative of three independent biological experiments. CLIPA14-F, full-length non-cleaved form.

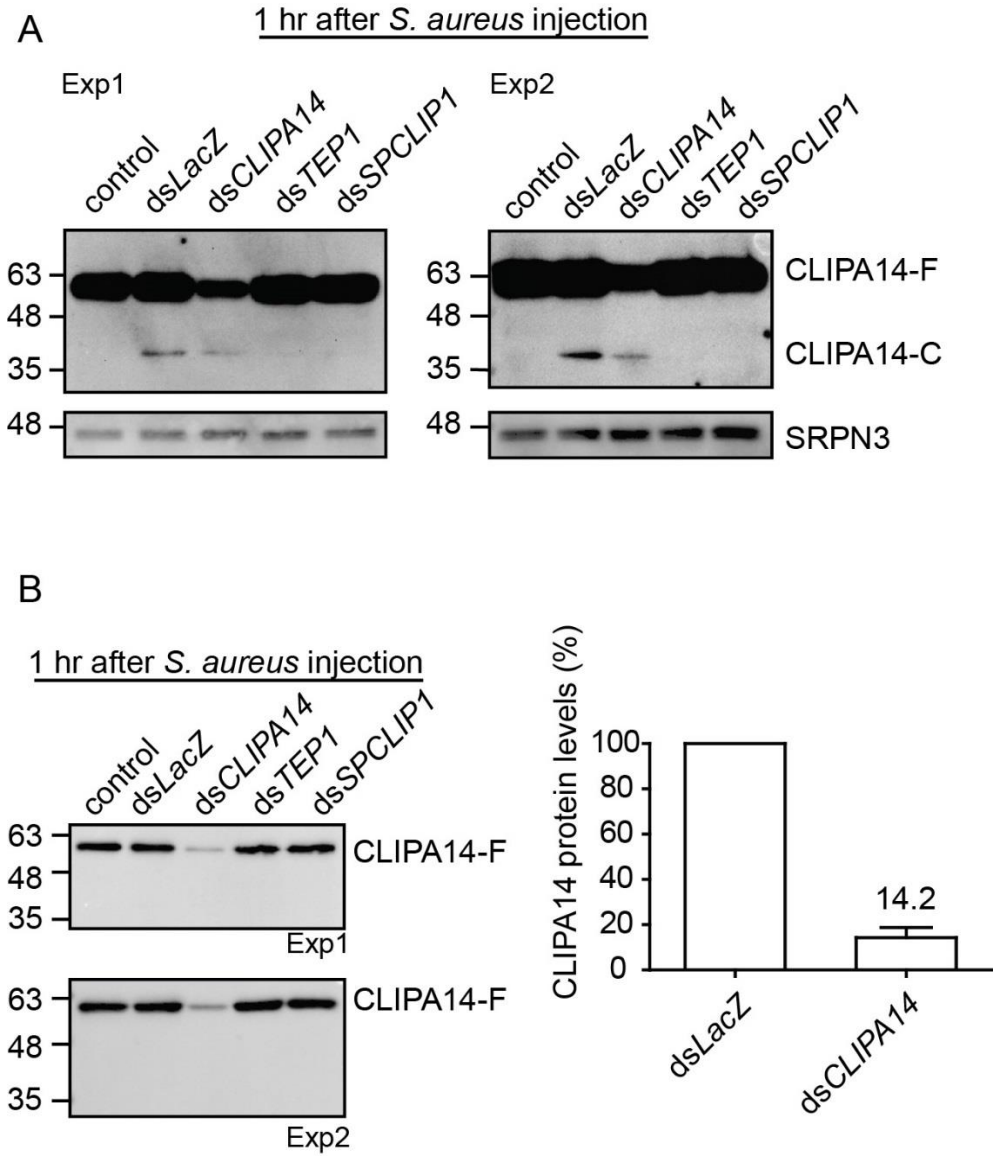


Figure 19. The infection-induced cleavage of CLIPA14 is dependent on TEP1 and SPCLIP1.

(A) Western blots from independent biological experiments showing CLIPA14 cleavage in the indicated mosquito genotypes. Hemolymph was extracted from mosquitoes at 1 hr after injection with *S. aureus* (OD_{600}=0.8). Protein quantification was performed using the Bradford protein assay and 1.2 μg of hemolymph proteins were loaded per lane. The control group was not infected with *S. aureus*. The membranes were probed with antibodies

against CLIPA14 and SRPN3 (as loading control). Shown are high exposure images (saturated) in order to detect CLIPA14-C cleaved form. (B) Low exposures (unsaturated) of the blots shown in (A). Note that at low exposures the cleaved form of CLIPA14 cannot be detected. CLIPA14 protein levels in ds*CLIPA14* and ds*LacZ* mosquitoes were quantified from the two blots using ImageJ and presented in the bar graph.

d. CLIPA14 controls the level of hemolymph PPO activation during systemic infections

The fact that *CLIPA14* kd triggered a potent melanotic response against *Plasmodium* ookinetes prompted us to ask whether these mosquitoes elicit an enhanced PO activity against systemic bacterial infections. To address this point, *CLIPA14* and *LacZ* kd mosquitoes were injected with a suspension of live *E. coli* (OD_{600}=0.8) and hemolymph was extracted 3 hrs later to measure PO activity. Our data revealed an approximately 4.5 times higher PO activity in *CLIPA14* compared to that in the infected *LacZ* kd control (Figure 20A), indicating indeed the presence of an enhanced melanotic response. *CLIPA2* kd mosquitoes were also shown previously to exhibit enhanced hemolymph PO activity following *E. coli* infections [118]. Hence, we compared the hemolymph PO activities in both genotypes to determine which of these SPHs is a more potent regulator of the systemic melanization response. While both *CLIPA2* and *CLIPA14* kd mosquitoes exhibited a higher PO activity than *LacZ* kd control, this activity was significantly higher (2-fold; *P*=0.0107) in *CLIPA14* relative to *CLIPA2* kds (Figure 20B), which correlates well with the increased parasite melanization observed in the former genotype. Interestingly, the PO activity in *CLIPA14*/*CLIPA2* dkd was even higher than that in *CLIPA14* single kd, revealing an

additive effect when both genes were co-silenced. Altogether, our results suggest that CLIPA2 and CLIPA14 act concertedly to regulate the TEP1-mediated immune response leading to melanization.

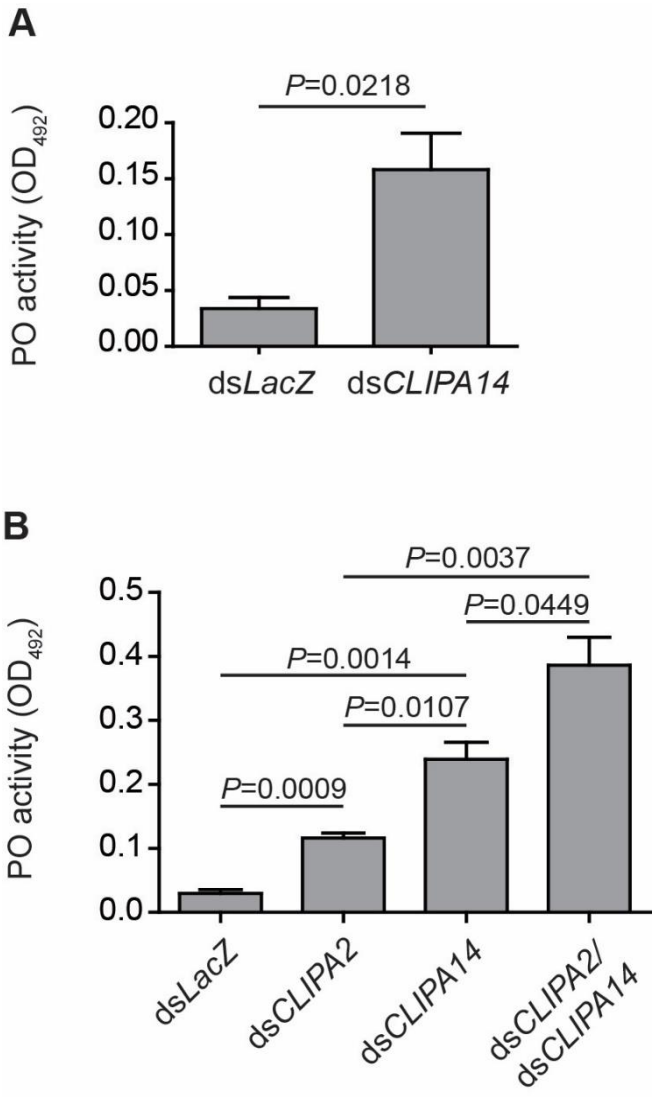


Figure 20. CLIPA14 regulates the intensity of hemolymph PPO activation.
Phenoloxidase (PO) enzymatic activity [detected as absorbance at OD_{492}, after conversion of L-3,4-dihydroxyphenylalanine (L-DOPA)] was measured in hemolymph extracted from ds*LacZ* (control), ds*CLIPA2*, *dsCLIPA14* and ds*CLIPA2*/ds*CLIPA14* mosquitoes at 3 hrs post-injection of live *E. coli* (OD_{600}=0.8). The graphs show PO activity measured at 30 min after addition of L-DOPA. Means were calculated from three independent biological experiments. Error bars represent standard deviation of the mean. Statistical analysis was performed using the Student's t-test and differences were considered to be significant if $P < 0.05$.

e. CLIPA14 steady state protein levels are not influenced by TEP1

$TEP1_{cut}$ is stabilized in the mosquito hemolymph by LRIM1 and APL1C, and silencing either *LRIM1* or *APL1C* in naïve mosquitoes triggers the loss of $TEP1_{cut}$ from the hemolymph [145, 148]. We have previously shown that in *LRIM1* kd naïve mosquitoes SPCLIP1 [115] and CLIPA2 [118] are lost from the hemolymph concomitant with the loss of $TEP1_{cut}$ suggesting that the steady state hemolymph levels of these proteins are directly dependent on $TEP1_{cut}$. To determine whether a similar correlation exists between CLIPA14 and TEP1, naive mosquitoes were injected with dsRNAs corresponding to *LacZ* (control), *CLIPA14*, *TEP1* and *LRIM1*, and hemolymph was extracted 48 hrs later for western blot analysis. As previously reported, *LRIM1* kd triggered the loss of $TEP1_{cut}$ and SPCLIP1 from the hemolymph; however, no effect was observed on CLIPA14, suggesting that CLIPA14 steady state levels are not regulated by $TEP1_{cut}$ (Figure 21).

To further investigate the nature of the relationship between CLIPA14 and TEP1 we asked whether CLIPA14 follows TEP1 to bacterial surfaces using the previously described *E. coli* bioparticle surface extraction assay [115, 118]. Using this assay we had previously shown that CLIPA2 [118] and SPCLIP1 [115] are recruited to bioparticle surfaces in a TEP1-dependent manner, suggesting that SPHs may act locally to modulate immune responses on microbial surfaces. As shown in Figure 22, a small fraction of CLIPA14 did bind to bioparticle surfaces in control (ds*LacZ*) mosquitoes; however, *TEP1* kd did not abolish this binding suggesting that TEP1 does not mediate CLIPA14 localization to

bacterial surfaces. It is worth noting that compared to CLIPA14, SPCLIP1 and CLIPA2 recruitment to bioparticles was more pronounced [115, 118] probably reflecting their strong dependency on $TEP1_{cut}$.

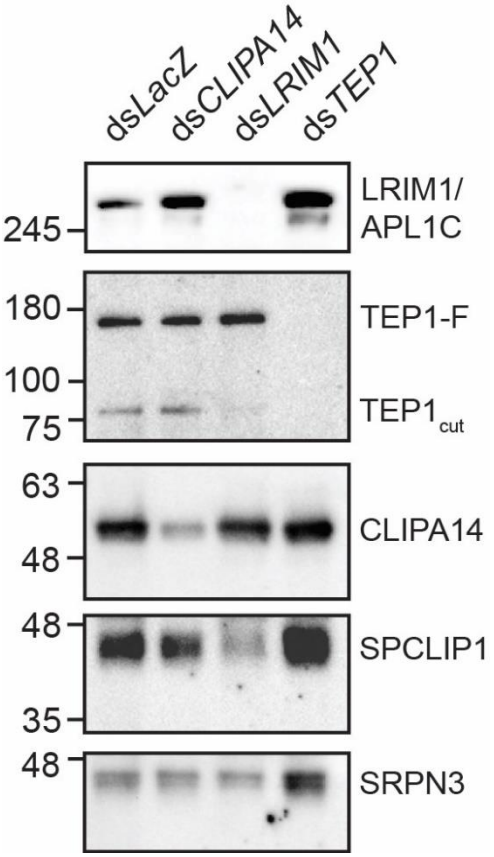


Figure 21. CLIPA14 steady state levels are not influenced by TEP1.
Western blot analysis of hemolymph extracts collected two days after injecting naive mosquitoes with the indicated dsRNAs. The membrane was probed with antibodies against CLIPA14, TEP1, LRIM1, SPCLIP1 and SRPN3 (as loading control). Protein quantification was performed using the Bradford protein assay and 0.9 μg of hemolymph proteins were loaded per lane. The image is representative of two independent biological experiments.

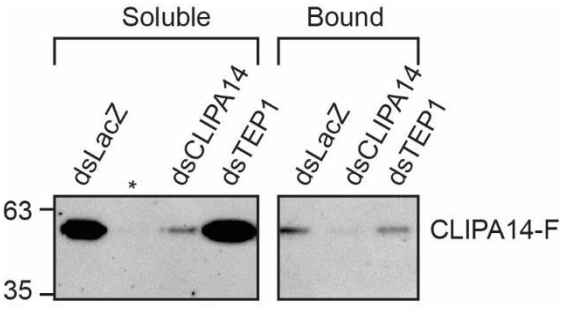


Figure 22. CLIPA14 localizes to *E. coli* bioparticles in a TEP1-independent manner. Hemolymph containing *E. coli* bioparticles was extracted from the indicated mosquito genotypes 15 min after bioparticle inoculation into the hemocoel. Bioparticles were pelleted by centrifugation, and the soluble fractions collected. Bacterial pellets were washed with PBS and bound proteins were extracted with protein loading buffer. Shown is a western blot analysis of the soluble and bound fractions. The image is representative of two independent experiments.

4. Discussion

The roles of clip domain containing SPHs characterized in insects [76, 246, 247] and crustaceans [259] have been almost exclusively restricted to the positive regulation of the PPO cascade, where they seem to act as cofactors for the proper cleavage and activation of PPO by PAPs. While the mosquito CLIPA8 [117] is thought to fall within this functional category of SPHs, CLIPA2 [118] negatively regulates PPO activation indirectly by fine tuning the level of activation of TEP1 in a yet undefined mechanism [52, 113, 119, 146]. Here, we identify CLIPA14 as a novel key negative regulator of TEP1-mediated immune responses. *CLIPA14* and *CLIPA2* exhibit distinct RNAi phenotypes but also distinct relationships with TEP1. First, more parasites are melanized in *CLIPA14* (86%) compared to *CLIPA2* (56%, [118]) kd mosquitoes. Second, while *CLIPA2* kd mosquitoes exhibited

significantly enhanced tolerance to *E. coli* infections [118], *CLIPA14* kd mosquitoes showed basal level tolerance to *E. coli* but reduced tolerance to *S. aureus* infections. Third, the hemolymph PO activity in *CLIPA14* was two-fold higher than that in *CLIPA2* kd mosquitoes after *E. coli* infections. However, silencing both genes exhibited an additive effect on PO activity, indicating that CLIPA2 and CLIPA14 cooperate to control the melanization response. In addition to differences in their RNAi phenotypes, CLIPA2 [118] and CLIPA14 steady state levels in the hemolymph showed distinct control by $TEP1_{cut}$. Additionally, CLIPA14 localization to bacterial surfaces does not seem to require TEP1 in contrast to that of CLIPA2 which is strictly TEP1-dependent [118]. This indicates that CLIPA14 may be recruited to bacterial surfaces by a yet unknown PRR or it may exhibit intrinsic ability to interact with microbial surfaces. In fact, some SPHs were reported to interact intrinsically with bacterial cell wall components [260, 261]. Since CLIPA14 binding pattern to bioparticles was weaker than that previously reported for CLIPA2 [118] and SPCLIP1 [115], we could not detect whether the cleaved CLIPA14-C is also bound to the bioparticles since this form is weakly detected in hemolymph extracts relative to the non-cleaved CLIPA14-F as shown in Figure 4. *In vitro* binding assays whereby full-length and artificially cleaved recombinant CLIPA14 are incubated with bacteria will be required to provide a definitive answer as to the intrinsic ability of CLIPA14 to recognize bacterial cell wall components.

CLIPA14 infection-induced cleavage is clearly dependent on TEP1 and its positive regulator SPCLIP1, which further supports our previous observation that SPCLIP1 acts upstream in the TEP1 pathway whereby it seems to regulate the amount of active TEP1 that deposits on microbial surfaces [115]. The enhanced killing of *P. berghei* ookinetes and rapid clearance of *E. coli* in *CLIPA14* kd mosquitoes were clearly dependent on TEP1 function, suggesting that *CLIPA14* kd is triggering a potent TEP1-mediated response. How and at what level is CLIPA14 negatively regulating the TEP1 response remain to be elucidated. We have previously shown that *CLIPA2* kd enhanced TEP1-F consumption during systemic infections indicating that it is an upstream negative regulator of TEP1, however TEP1-F dynamics in *CLIPA14* kd mosquitoes did not provide convincing evidence to support a similar role for CLIPA14 (data not shown), suggesting that CLIPA14 may be acting downstream of CLIPA2.

Three SPHs have been involved so far in regulating mosquito immune responses; SPCLIP1 acts a positive regulator while CLIPA2 and CLIPA14 act as negative regulators. The common feature among all three is that their functions are tightly linked to TEP1. It is intriguing that SPHs, despite being non-catalytic, have so far produced the most pronounced RNAi phenotypes with respect to *Plasmodium* ookinete survival among the larger mosquito clip-domain family which includes several catalytic members of the CLIPB and CLIPC subgroups. For instance, while CLIPA8 [116] and SPCLIP1 [115] kds completely abolished ookinete melanization in melanotic mosquito genotypes, the kd of

several CLIPB genes showed only partial rescue [116]. This suggests that SPHs play unique roles in mosquito immunity while catalytic CLIPs may exhibit partial functional redundancy. The exact functions of these SPHs remain unknown but their RNAi phenotypes suggest a multilayered regulation of the mosquito melanization response, unlike other insect SPHs whose role has been so far restricted to inducing the proper activation cleavage of PPO [76, 96, 104]. It is tempting to speculate that SPHs may dictate the substrate specificity for certain catalytic CLIPs not only with respect to PPO but also to other downstream CLIPs in the cascade. The future characterization of the infection-induced cleavage patterns of catalytic CLIPs *in vivo* will be required to determine whether SPHs do act upstream of certain catalytic CLIPs. This will also facilitate the design of *in vitro* reconstitution assays that would gauge the effect of SPHs on the enzymatic activities and target specificities of candidate catalytic CLIPs. On the other hand the nature of the enzymes that cleave CLIPA14 and other mosquito SPHs remain unknown. By analogy to biochemical studies in other insects we speculate that they belong to the catalytic CLIPB subgroup. Systematic RNAi screens of catalytic CLIPs are currently being conducted in our lab to identify candidate CLIPs required for the cleavage of CLIPA14 and other SPHs that exhibit clear infection-induced cleavage patterns such as CLIPA8.

Collectively, our results reveal so far an unprecedented complexity in the function of insect SPHs that extends beyond their commonly ascribed role as regulators of PPO

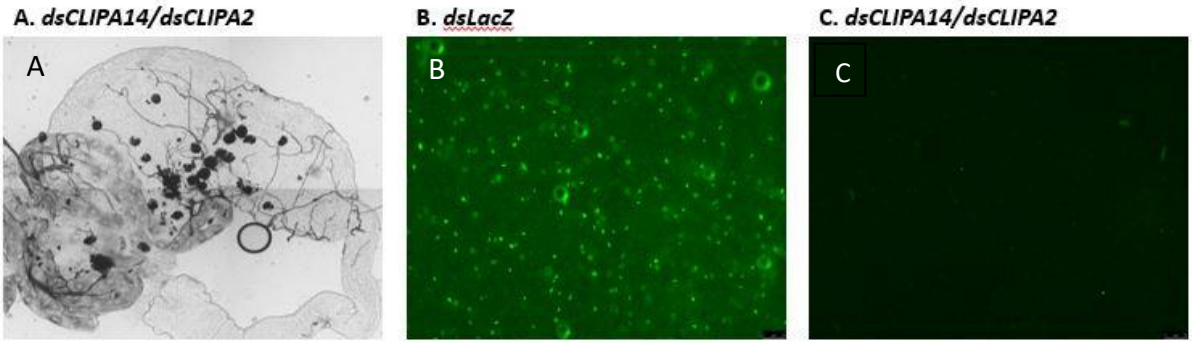
activation cleavage. Future biochemical and biophysical studies will be required to highlight the exact molecular functions of these SPHs in CLIP cascades.

5. Novel insights into CLIPA14 function

a. CLIPA14/CLIPA2 dkd highly reduces Plasmodium berghei sporozoite counts

We showed that *CLIPA14/CLIPA2* dkd results in an excessive *P. berghei* ookinete melanization accompanied with a significant decrease in live oocyst counts [238]. Similarly, we noticed that this double knockdown phenotype also causes exaggerated late stage oocyst melanization (figure 23), hence we opted to study the effect of *CLIPA14/CLIPA2* silencing on sporozoite counts in order to determine whether the late sage melanization of apparently ruptured oocysts may also influence sprozoite survival. To that purpose, we co-silenced *CLIPA14* and *CLIPA2* three days before *P. berghei* infection and 11 days post infection to ensure continuous silencing of the indicated genes. Live GFP-expressing sporozoites in whole mosquito lysates were counted 21 days post infection using a hemocytometer. Preliminary data showed that *CLIPA14/CLIPA2* dkd mosquitoes harbor extremely lower number of live sporozoites compared to *LacZ* kd mosquitoes. These results need to be further corroborated, however they may suggest a possible role for the mealnization response triggered in *CLIPA14/CLIPA2* dkd mosquitoes in eliminating a number of sporozoites at a later stage. Another plausible explanation is that the low number of sporozoites in *CLIPA14/CLIPA2* dkd is due to the small number of ookinetes that

survive to the oocyst stage. In future experiments, *CLIPA14/CLIPA2* dkd will be performed only after *P. berghei* infections to determine whether silencing these genes affect sporozoite survival.



D.

	Samples	*dsLacZ*	*dsCLIPA14*/dsCLIPA2
Sporozoite/mosquito count	Sample 1	30250	0
	Sample 2	93750	2750

Figure 23. *CLIPA14/CLIPA2* dkd causes extensive oocyst melanization and significantly decreases live sporozoite counts.
Mosquito midguts were dissected 17 days post *P. berghei* infection, fixed, mounted on slides and scored for late stage oocyst melanization. (A) Extensive oocyst melanization in *dsCLIPA14/CLIPA2* midguts 17 days post infection. (B-C) Mosquito thoraces were dissected from (B) *LacZ* and (C) *CLIPA14/CLIPA2* kd mosquitoes, 21 days after GFP-expressing *P. berghei* infection, grinded in sterile PBSx1 in batches of 8 osquitoes/sample. Sporozoites were visualized and counted using an upright fluorescent microscope and a hemocytometer. (D) Table showing the numbers of sporozoites in *LacZ* and *CLIPA14/CLIPA2* dkd mosquitoes. 8 to 10 mosquitoes were used in each sample to generate these data.

b. CLIPA14 is the most downstream in the cSPH network

The melanization response in *A. gambiae* is governed by a network of cSP and cSPH in addition to other proteins such as SRPNs and TEPs. Coordinated interaction between members of this network especially between cSPHs and cSPs regulate the level of PO activity in response to infection. Biochemical data from our lab point to a hierarchical activation of cSPHs downstream of TEP1 (El Moussawi L, unpublished). To determine the position of CLIPA14 in this cSPH module, we opted to study CLIPA14 cleavage pattern in *SPCLIP1*, *CLIPA28*, *CLIPA8* and *CLIPA2* kd mosquitoes. Interestingly, CLIPA14 celavage pattern was ablolished in *TEP1*, *SPCLIP1*, *CLIPA28* and *CLIPA8* knockdown mosquitoes (figure 24). These results clearly suggest that CLIPA14 is so far the most downstream in the cSPH module, involved in regulation of the melanization response. We have previously shown that *CLIPA14/CLIPA2* dkd results in extensive *P. berghei* ookinete melanization, in addition to higher hemolymph PO activity compared to control groups which suggest that CLIPA14 and CLIPA2 have an additive effect on the melanization response. Interestingly CLIPA14 cleavage was significantly enhanced in *CLIPA2* kd mosquitoes suggesting that CLIPA14 may be acting downstream of CLIPA2.

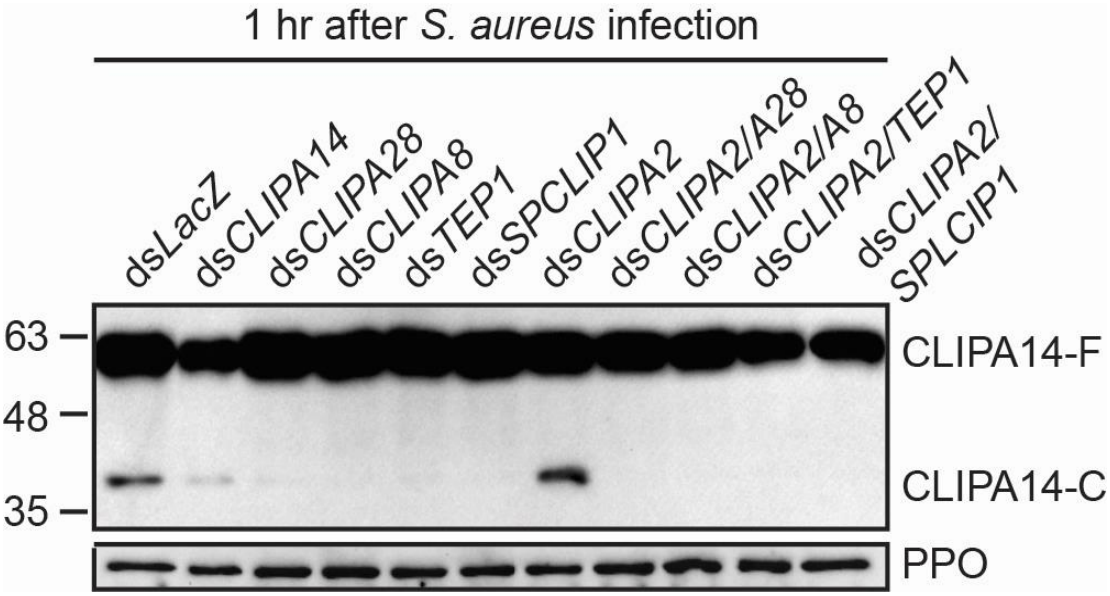


Figure 24. TEP1 and several cSPHs control the infection-induced cleavage of CLIPA14.

Western blot showing CLIPA14 cleavage in the indicated mosquito genotypes. Hemolymph was extracted from mosquitoes at 1 hr after injection with *S. aureus* (OD_{600}=0.8). The membrane was probed with antibodies against CLIPA14 and PPO (as loading control). Shown is a high exposure image (saturated) in order to detect the cleaved C-terminal protease-like domain of CLIPA14 (CLIPA14-C). The image is representative of two independent biological experiments. CLIPA14-F, full-length non-cleaved form.

B. Two novel carboxypeptidases CP1 and CP2 modulate the intensity of mosquito immune response against malaria parasites

1. Abstract

Carboxypeptidases are a family of peptide hydrolyzing enzymes that cleave C-terminal residues from their substrates. Carboxypeptidases are characterized with a broad range of functions ranging from recovery of dietary amino acids, tissue organogenesis to digestion and immunity. In mammals, certain carboxypeptidases have been extensively studied in the context of coagulation and inflammatory responses, mainly thrombin-activatable fibrinolysis inhibitor (TAFI) and Carboxypeptidase N (CPN) [216, 221]. In mosquitoes, however, studies have focused mainly on carboxypeptidases in the context of digestion [237]. Here, we identified two carboxypeptidases, CP1 and CP2 using a degradomics approach which aimed to identify differentially cleaved proteins in the hemolymph of *E. coli*-infected *TEP1* kd mosquitoes as compared to *LacZ* kd controls. Interestingly, we showed that CP1 and CP2 contribute to anti-*Plasmodium* immunity in *Anopheles gambiae*; their single knockdown triggers an increase in the numbers of live parasites and a reduction in TEP1 localization to ookinete surface. The mechanism by which these carboxypeptidases confer immunity and their interplay with mosquito key players such as TEP1 and CLIP network remain to be elucidated.

2. Results

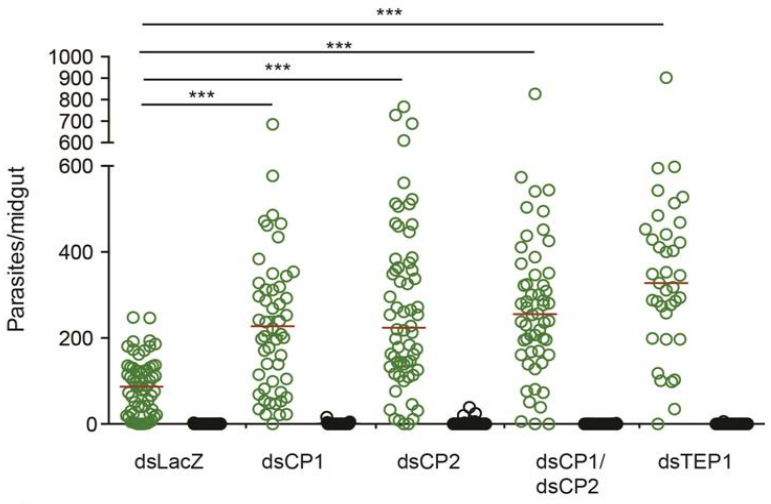
a. CP1 and CP2 are required for anti-*Plasmodium* defense

CP1 and CP2 were discovered in our degradomics approach as differentially cleaved enzymes in the hemolymph of *E. coli*-infected *TEP1* kd mosquitoes relative to *LacZ* controls. To determine whether they play a role in anti-plasmodium defense, we injected *LacZ*, *CP1*, *CP2*, *TEP1* and *CP1/CP2* (mix) dsRNA in adult female *A. gambiae* and scored the numbers of live *P. berghei* oocysts in midguts dissected 7 days after an infectious blood meal. Interestingly both *CP1* and *CP2* kd showed significant increase in live oocyst counts as compared to *LacZ* kd control, a phenotype similar to that observed for *TEP1* kd (Figure 25). The RNAi phenotype of CP1 and CP2 as well as their TEP1-dependent cleavage suggest that a functional link might exist between them and TEP1.

b. CP1 and CP2 knockdowns reduce the recruitment of TEP1 to ookinete surfaces

To investigate whether a functional interaction exists between CP1, CP2 and TEP1, we studied the effect of silencing these carboxypeptidases on TEP1 recruitment to ookinete surfaces. To do so, midguts were dissected from *LacZ*, *CP1*, *CP2* and *TEP1* kd as well as *CP1/CP2* dkd mosquitoes at 22 hours after infection with *P. berghei*, and immunostained with TEP1 antibody. The numbers of ookinetes positive for GFP (GFP^+), TEP1 (TEP^+), both GFP and TEP1 (GFP^+/TEP^+) were scored using an upright fluorescence microscope. Our results revealed that the total percentage of TEP1 positive parasites ($TEP1^+$) decreased

significantly from 50.16 % in *LacZ* kd mosquitoes to 2.98% and 9.97% in *CP1* and *CP2* kd mosquitoes, respectively. Concomitantly, the percentage of GFP+ ookinetes (i.e. live parasites) increased significantly from 47.63 % in *LacZ* kd mosquitoes to 96.26 % and 87 % in *CP1* and *CP2* kd mosquitoes respectively. These results show that both CP1 and CP2 knockdowns significantly reduce TEP1 binding to ookinete surfaces at early time points of midgut invasion (table 1), suggesting that they play a regulatory role in mosquito complement activation.



Gene kd	n	% melanization	Prevalence
LacZ	59	1.7	94.9
CP1	51	9.8	98
CP2	62	14.5	96.8
TEP1	37	0	97.3
CP1/CP2	54	1.85	96.3

Figure 25. CP1 and CP2 RNAi phenotypes are similar to TEP1 with respect to oocyst development.
Scatter plots of live GFP-expressing *P. berghei* oocysts (green circles) and dead melanized ookinetes (black circles) scored in the midguts of the indicated mosquito genotypes seven days post-infection. Red lines indicate median parasite numbers. Statistical analysis for the parasite distribution was performed using the Mann-Whitney test and *P*-values less than 0.05 were considered significant. Data were pooled from four independent biological experiments. Statistical analysis for prevalence was performed using the Chi-Square test followed by Fischer's exact test and *P*-values less than 0.05 were considered significant

Table 1. Table showing the numbers of GFP$^+$, TEP$^+$, GFP$^+$/TEP$^+$ ookinete in *LacZ, CP1, CP2* and *CP1/CP2* dkd mosquitoes.
Midguts of the indicated mosquito genotypes were dissected at 22 hours after *P. berghei* infection, immunostained for TEP1, and ookinetes positive for GFP, TEP1 or both were scored using an upright fluorescence micrscope. (Around 2000 ookinetes were counted per kd)

Gene kd	GFP$^+$	TEP$^+$	GFP$^+$/TEP$^+$
LacZ	47.63	50.16	2.19
CP1	96.26	2.98	0.74
CP2	87.68	9.97	2.34
CP1/CP2	91.5	8.49	0

c. CP1 and CP2 do not contribute to mosquito tolerance in response to bacterial infection

The contribution of CP1 and CP2 to anti-*Plasmodium* immunity prompted us to study the effect of their silencing on mosquito tolerance and resistance to Gram-negative and Gram-positive bacterial infections. Silencing *CP1*, *CP2*, or *CP1/CP2* simultaneously

did not alter neither resistance nor tolerance to *E. coli* infections compared to *LacZ* controls. Similarly, mosquito survival was not compromised in *CP1, CP2,* and *CP1/CP2* dkd in response to *S. aureus* systemic infections (Figure 26).

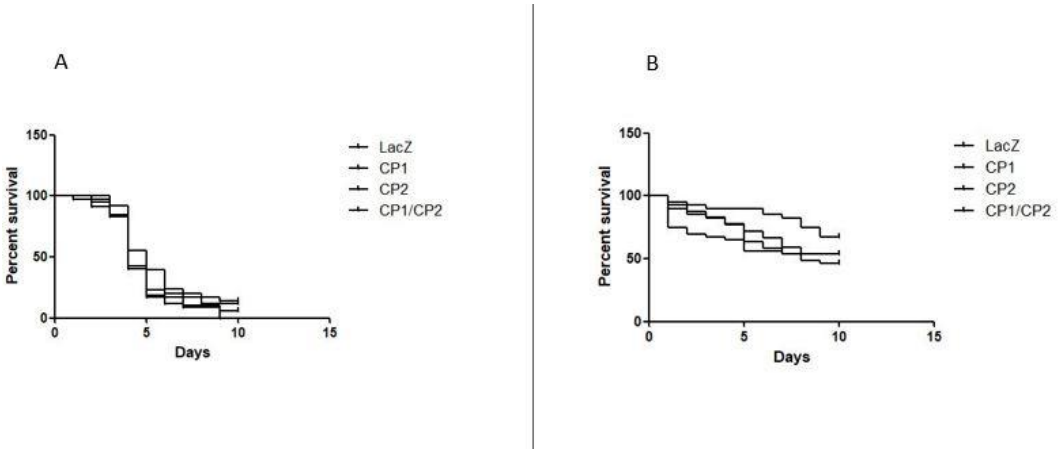


Figure 26. CP1 and CP2 do not contribute to mosquito tolerance against *E. coli* and *S. aureus* bacterial infections.
(A-B) mosquito survival assays following injection of (A) *E. coli* (OD600nm = 0.4) and (B) *S. aureus* (OD600nm = 0.4) into mosquito hemocoel. One representative experiment is shown from at least three independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Data shown are from three independent biological experiments.

CHAPTER IV
GENERAL DISCUSSION

Melanization is a potent immune response unique to arthropods. It is characterized by the deposition of a melanin coat at the site of injury to strengthen the clot resulting in wound healing, or on the surfaces of invading pathogens leading to their intoxication and asphyxiation. The key enzyme in melanin biosynthesis is PO which is produced as PPO zymogen that is converted to active PO by a terminal clip domain serine proteinase (CLIP). Despite the extensive studies on the melanization response in insects (reviewed in [4, 238, 241]), a comprehensive understanding of the molecular interactions that control PPO activation is still lacking. This is especially due to the complexity of the serine protease cascades that control PPO activation, which are mainly composed of CLIPs, and their fine regulation by serpins and cSPHs (reviewed in [238, 241]). In the mosquito *Anopheles gambiae*, an additional layer of control over PPO activation exists through the functional interactions between the melanization and complement-like response mediated by the hemolymph protein TEP1. Several studies pointed to a role for TEP1 in controlling the melanization response. First, TEP1 is required for the melanization of *P. berghei* ookinetes in the refractory L3-5 [119] and in *CTL4* kd susceptible mosquitoes [251]. *TEP1* kd also abolished PO localization to *Beauveria bassiana* hyphae in the mosquito hemocoel [113] as well as blocked PPO activation in the hemolymph in response to *E. coli* systemic infections [115]. At the molecular level, *TEP1* kd abolished the cleavage of CLIPA8, an essential

positive regulator of PPO activation, in response to systemic bacterial infections [117]) as well as the cleavage of CLIPA14 as described in this thesis, suggesting that it acts upstream of the enzymatic cascade that controls PPO activation. At the same time certain cSPHs were shown to control the intensity of the TEP1-mediated response either negatively (CLIPA2) or positively (SPCLIP1). *CLIPA2* kd enhanced TEP1 activity leading to an exaggerated PO activity in the hemolymph following *E. coli* systemic infections [113], while *SPCLIP1* kd significantly reduced TEP1 localization to ookinetes hence compromising the lytic and melanization responses [115]. This upstream position of SPCLIP1 was also confirmed by results obtained herein whereby its kd abolished the cleavage of CLIPA14 after bacterial systemic infections. Altogether, these results indicate that the mosquito melanization response is controlled by complex molecular interactions between TEPs, CLIPs, cSPHs, SRPNs and PPOs and possibly yet unidentified PRRs. This complexity infers that regulatory switches in this response occur at multiple levels possibly to tailor the effector response output (measured as PO activity) to the dose of the invader, in order to control fitness cost and avoid unnecessary energy expenditure by the host on an exaggerated immune response. Indeed, the kd of key regulatory SRPNs, SRPN27A [112, 262] and SRPN2 [263] in *Drosophila* and *A. gambiae*, respectively, triggered an exaggerated melanization response leading to tissue melanization and compromised host survival. Also *CLIPA2* kd in *A. gambiae* was associated with increased PO activity paralleled by a high reproductive cost characterized by reduced egg laying [118].

The functional interactions between TEP1 and mosquito cSPHs are especially notable. In this study, we identified CLIPA14 as a key negative regulator of TEP1-mediated immune responses, mainly melanization. *CLIPA14* kd resulted in the potent melanization of *P. berghei* ookinetes (86%) in a TEP1-dependent manner. Additionally, CLIPA14 infection-induced cleavage was abolished in *TEP1* kd. This and the previous observation that TEP1 controls CLIPA8 cleavage [115] clearly suggest that TEP1 is an upstream regulator of the cSPH network involved in melanization. This TEP1 control over the cSPH network suggests that melanization may be an effector arm of mosquito complement. It remains unclear whether TEP1 controls the activation cleavage of catalytic CLIPs also since the cleavage patterns of these remain to be characterized. TEP1 seems to launch an effector response rather than act as a PRR, as inferred from several studies [119, 147, 148, 191, 264]. The nature of the PRRs that act upstream of TEP1 and that might also exhibit control over the activation cleavage of cSPHs and cSPs remain to be determined. However, several potential candidates include those PRRs whose knockdown gave similar RNAi phenotypes as TEP1 with respect to ookinete survival, such as LRRD7 [52], Dscam [52, 265], members of GNBP family including GNBPA2, GNBPB3 and GNBPB4 [49] and members of the FREP gene family including in particular FBN9, FBN8 and FBN39 [52, 54]. Despite the fact that GNBPB4 [49], Dscam [265] and FBN9 [53] bind to the surface of *Plasmodium* ookinetes, it is still not clear whether their recruitment to these surfaces is required for TEP1 binding. Future studies that focus on assessing the effects of gene kd of

these PRRs on the cleavage patterns of cSPHs and cSPs should provide insight into the complexity of the upstream molecular events that control the activation of CLIP cascades during melanization.

CLIPA14 seems to act most downstream in the cSPH network controlling melanization since, as shown in this thesis, its cleavage requires SPCLIP1, CLIPA8 and a newly identified cSPH in our lab called CLIPA28 (Osta MA, unpublished). In other insect models in which the melanization response has been relatively well studied such as *Manduca sexta* [96] [98] [99] [266] and *Tenebrio molitor* [267] [268], cSPHs were shown to act at the terminal step in the cascade controlling the proper activation cleavage of PPO. The presence of such a complex hierarchical cSPH network in *A. gambiae* indicate that they may exhibit a multilayered control on the cascade possibly controlling the cleavage of cSPs in addition to PPO. The fact that *CLIPA14* kd triggers an increase in hemolymph PO activity after bacterial infections suggests that it negatively regulates the activation of a cSP in the clip cascade. The identity of this cSP remains unknown, however we are currently using a degradomic approach to identify those cSPs that are cleaved in the hemolymph in response to systemic infections with fungi, Gram-negative and Gram-negative bacteria. Once specific candidates have been identified then their cleavage patterns in response to silencing CLIPA14 as well as other key cSPHs will be studied in order to identify specific cSPH-cSP associations. Our working hypothesis is that negative regulators such as CLIPA14 and CLIPA2 may exhibit their effects by interfering with positive regulatory

cSPHs or by interacting with the target cSP itself modulating its activation cleavage. This will be tested in the future using *in vitro* reconstitution experiments whereby specific combinations of recombinant forms of candidate cSPHs and cSPs, with engineered cleavage sites that permit their artificial activation using commercial enzymes, will be incubated in the presence of a chromogenic substrate that can be cleaved by cSP in order to score the efficiency of cSP activation. The cleavage patterns of cSPHs and cSPs in these experiments can also be monitored using quantitative western blot analysis.

The rapid clearance of *E. coli* bacteria in *CLIPA14* kd mosquitoes was clearly dependent on TEP1, suggesting that *CLIPA14* kd is triggering a potent TEP1-mediated response. Whether this rapid clearance is due to enhanced bacterial opsonization, melanization or increased bacterial lysis remains unclear. We have shown that *CLIPA14* kd enhances PO activity and PO is required for bacterial clearance in *Drosophila* [168]. However, this does not exclude a role for enhanced lysis and/or phagocytosis. Employing the GFP release assay established in our lab [147] may help address whether increased lysis is occurring in the hemolymph of *CLIPA14* kd mosquitoes. We have not tested herein whether the clearance of Gram-positive bacteria such as *S. aureus* is also TEP1-dependent. While this might hold true it is worth noting that clip cascades also control activation of the Toll pathway which plays an important anti-bacterial role especially against Gram-positive bacteria [43]. The is also evidence of crosstalk between the toll pathway and the melanization response. In *Drosophila,* Toll gain-of-function mutants exhibit sponatenous

melantic tumor formation [269] [270] [271]. Furthermore, *Drosophila* Toll seems to regulate the expression of factors that activate PPO [88]. Similarly in the mosquitoes *A. gambiae* and *Ae. aegypti* silencing *Cactus*, the negative regulator of the Toll pathway, triggered and enhanced melanization response [126] [272]). In *A. gambiae* Toll was also shown to regulate the expression of several cSPs [127]. Altogether, these studies inform that interfering with the clip cascades upstream of Toll may also modulate the hemolymph PO activity. However, the absence of a clear read out (i.e marker gene expression) for the activation of the Toll pathway in *A. gambiae* makes it difficult to address the potential functional interactions between candidate cSPHs and Toll pathway activation.

Our preliminary results showed that in addition to ookinete melanization *CLIPA14/CLIPA2* dkd triggerd the melanization of late stage oocysts that have already ruptured and resulted in a significant decrease in sporozoite counts compared to control groups. The low sporozoite numbers in *CLIPA14/CLIPA2* dkd mosquitoes is either due to the small number of ookinetes that survive to the oocyst stage or due to collateral damage to sporozoites as oocysts become melanized during rupture. In these experiments, to ensure long term gene silencing, *dsCLIPA14* and *dsCLIPA2* RNAi were injected at two time intervals; 3 days before *P. berghei* infection and 11 days after infection. In future experiments, *CLIPA14/CLIPA2* dkd will be performed only after *P. berghei* infections to determine whether the enhanced melanotic response in these mosquitoes affects the survival of sporozoites. Kwon *et al.* provided several pieces of evidence that late phase

immune responses against mature oocysts is TEP1-independent and is mainly carried by hemocytes [273]. Whether this late immune response triggered by *CLIPA14/CLIPA2* dkd is TEP1-dependent remains to be elucidated.

The fact that TEP1 controls the cleavage of several cSPHs prompted us to adopt a high throughput degradomic approach to identify cSPHs and cSP whose cleavage is controlled by TEP1. Interestingly, among the differentially cleaved proteins in the hemolymph of *E. coli*-infected *TEP1* kd mosquitoes relative to *LacZ* kd controls, were two carboxypeptidases (termed CP1 and CP2). Carboxypeptidases have pro-domains that need to be cleaved for the enzyme to become active (reviewed in [212]), which explains why they were captured in our degradomic approach. The single kd of either *CP1* or *CP2* triggered an increase in the numbers of live parasites, an RNAi phenotype similar to that of *TEP1*. Interestingly, *CP1* and *CP2* kd also reduced TEP1 localization to ookinete surfaces, suggesting that they are part of the complement response in the mosquito. Interestingly, preliminary data indicates that the knockdown of these CPs does not affect mosquito resistance nor tolerance to bacterial infections. Their RNAi phenotypes seem so far to be mainly associated with anti-*Plasmodium* defense. The mechanism of action of these carboxypeptidases and their interplay with key immunity players such as TEP1 and the clip network remain to be elucidated. Also, according to our knowledge this is the first evidence for an immune role for carboxypeptidases in insects, hence analogous studies in insects are absent. However, in humans there is evidence that CPN removes C-terminal arginine

residues from its substrates, which is directly used by nitric oxide synthase (NOS) to produce nitric oxide (NO) [215]. Interestingly, in *A. gambiae*, NOS along with HPX2 and NOX5 catalyze protein nitration in ookinete-infected midgut epithelial cells, and this nitration seems to be prerequisite for parasite attack by TEP1 [189] [190] [274]. We hypothesize that in response to *P. berghei* infection, CP1 and CP2 might release C-terminal arginine residues from their substrate protein(s), leading to increased NO levels, which along with HPX2/NOX5 result in strong epithelial nitration reactions, and subsequent killing of parasites by TEP1. This is supported by the fact that the kd of *CP1* or *CP2* reduced TEP1-localication to *P. berghei* surface, similar to *HPX2* kd [274]. In order to address whether CP1 and CP2 are acting in the same pathway of HPX2 and NOX5, *P. berghei* infection intensity will be monitored in mosquitoes injected with the following dsRNA combinations; *CP1*/*HPX2*, *CP1*/*NOX5*, *CP2*/*HPX2* and *CP2*/*N*OX5. If the RNAi phenotypes in the double kds are similar to those in the single kds it would suggest that the CPs are likely part of the anti-*Plasmodial* response triggered by HPX2 and NOX5. It is also imperative to compare the levels of free arginine in the hemolymph of *CP1* and *CP2* kd mosquitoes with those in *LacZ* kd as controls by GC-MS to determine whether indeed they are involved in releasing arginines from target proteins. Currently antibodies are being produced against the recombinant forms of CP1 and CP2 in order to characterize their cleavage profiles in response to systemic bacterial infections, validate the requirement of TEP1 for their cleavage, and assess whether their cleavage is also regulated by key cSPHs

and *vice versa*. Another important question pertains to the identity of the enzyme that cleaves CP1 and CP2 and how it is activated by mosquito complement.

In summary the work presented herein identified a novel key cSPH in the melanization response and highlighted the complex nature of the clip network that regulates melanization. . Extensive biochemical and biophysical studies will need to follow in order to unravel the exact molecular interactions that control the proper activation of clips in these networks and eventually that of PPO. Our data also added a novel complexity to the mosquito complement response which in addition to TEPs, LRIMs, cSPs, cSPHs and PPOs, it also involves members of the
carboxypeptidase family. In mammals the complement system is known to be complex involving more than 30 proteins. Despite being primitive in nature the complement system in mosquitoes does not promise to be less complex than its mammalian counterpart and according to the data in hand it seems to coordinate not only humoral defenses in the hemolymph but also cellular responses, a functional dichotomy that constitutes an evolutionary conserved strategy of complement.

CHAPTER V
MATERIALS AND METHODS

A. Ethics statement

This study was carried according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, USA). Animal protocol was approved by the Institutional Animal Care and Use committee IACUC of the American University of Beirut (permit number 16-03-369). The IACUC functions in compliance with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (USA), and adopts the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

B. Materials

1. Anopheles gambiae mosquito strain

Anopheles gambiae G3 strain was used in all experiments. *Anopheles gambiae* were reared at 27°C, 80% humidity with a 12 hour day-night life cycle [275]. Larvae were maintained on tropical fish food, whereas adult mosquitoes were fed 10% sucrose solution. For egg production, mosquitoes were fed on blood of anesthetized adult mice. Anesthesia was performed by intraperitoneal injection of mice with a 100 µl solution containing ketamine (4.2 mg) and xylazine (0.3 mg).

2. Bacterial and P. berghei parasite strains

a. Bacterial strains

Ampicillin resistant, GFP-expressing *E. coli* (a kind gift from J.J. Ewbank), tetracycline resistant *S.aureus* (a kind gift from P. Bulet) and gentamycin resistant *Serratia marcescens* (DB11 strain) were either cultured in LB medium (Luria-Bertani broth) or plated on LB agar plates. *E. coli, S. aureus* and *S. marcescens* cultures were supplemented with ampicillin (100 µg/ml), tetracycline (50 µg/ml) and gentamycin (50 µg/ml) respectively.

b. Parasite strain

GFP-expressing *P. berghei* parasite strain (CON_{GFP}) [276] was passaged in mice. Percentage of parasitemia in mice was determined after preparing blood smears and staining them with 1% Giemsa solution. Mosquitoes were fed on anesthetized mice having around 4-5 % parasitemia. All *P. berghei* infections were carried at 20 °C with a 12-hour day-night cycle.

3. Eukaryotic cell line

Sf9 cells (Invitrogen) were grown and maintained in Sf-900 II SFM (Gibco), according to manufacturer's protocol, and supplemented with penicillin (50 U/μl) and streptomycin (50 μg/μl)

C. Mosquito bioassays and quantifications of microbial proliferation

1. Mosquito survival and proliferation assays after bacterial infections

GFP-expressing ampicillin-resistant *E. coli*, tetracycline-resistant *S. aureus* and Ds-Red-expressing gentamycin-resistant *Serratia marcescens* were grown overnight at 37 °C in Luria Broth medium containing ampicillin, tetracycline and gentamycin respectively. Bacterial cultures were pelleted by centrifugation and washed with 1xPBS three times. The pellets were then resuspended with 1xPBS and adjusted to O.D. $_{600nm}$ of 0.4 for *E. coli* and *S. aureus* and O.D. $_{600nm}$ of 0.1 for *S. marcescens*. Female mosquitoes that were silenced for *LacZ*, *CLIPA14*, *CP1*, *CP2* and *CP1/CP2* were either injected with the prepared *E. coli* and *S. aureus* cultures or fed with sugar solution containing *S. marcescens* culture 3 days post silencing. Concerning the latter route of infection, mosquitoes were allowed to feed on sugar pads containing *S. marcescens* and a red food colorant for two days, after which non-fed mosquitoes (lacking red color in their abdomens) were sorted out under a stereoscope while the rest were returned back to the cups, provided with sugar pads that did not contain *Serratia*. A batch of at least forty adult female mosquitoes was infected per genotype.

Mosquito survival rates were scored on a daily basis over nine days. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the Log-rank test. Survival assays were repeated at least three times. A P-value < 0.05 was considered significant.

Regarding bacterial proliferation assays, *LacZ, TEP1, CLIPA14, CP1, CP2, CLIPA14/TEP1* and *CP1/CP2* dkd mosquitoes were injected with 69 nl of prepared *E. coli* or *S. aureus* suspensions (O.D. $_{600nm}$ of 0.4) three days post silencing. Two days post infection, 5 batches of 8 mosquitoes each per genotype were grinded in 500 µl 1xPBS on ice. Lysates were plated in serial dilutions on ampicillin or tetracycline – containing LB agar plates and incubated at 37 °C to score for the *E. coli* and *S. aureus* bacterial CFUs respectively. Regarding *Serratia* proliferation assay and to eliminate bias from gut resident *Serratia* that are subject to distinct immune control, we opted to measure the CFU levels of *Serratia* in the hemocoel rather than in the whole mosquito. To that end, each *Serratia* infected mosquito was dissected in a 30 µl drop of sterile PBS, the gut was discarded. The remaining carcass in addition to the PBS drop were transferred to a 1.5 ml Eppendorf tube. Carcasses were grinded in batches of 8, and lysates were plated in serial dilutions on agar plates containing gentamycin to score for *Serratia* CFUs. All bacterial CFU assays were performed three times and statistical significance was calculated using the Mann-Whitney test. Medians were considered significantly different if P < 0.05.

2. P. berghei infection assay

Mosquitoes silenced for *LacZ, CLIPA14, TEP1, CLIPA14/TEP1, CLIPA5* and *CLIPA14/CLIPA2* were fed on an anesthetized BALB/c mouse infected with *P. berghei* (4-5% parasitemia) 3 days post silencing. These mosquitoes were maintained in an incubator with a 12-hour day/night cycle, at 20 °C. Mosquitoes were anesthetized on CO_2 24-48 hours post infection, and non-blood fed mosquitoes were removed whereas blood fed mosquitoes were returned to the incubator until they were dissected. Mosquito midguts were dissected 7-9 days post infection, fixed in 4% paraformaldehyde for 50-60 minutes, washed three times with 1xPBS, and mounted in ProLong® Gold antifade reagent (Invitrogen). The number of live oocysts (GFP positive) and melanized ookinetes (dark brown crescent shaped particles) per midgut were scored using upright fluorescent microscope Leica DM6 B. Three independent biological experiments were done. The Mann-Whitney test was used to calculate statistical significance and medians were considered significantly different if $P < 0.05$.

Regarding *P. berghei* sporozoite count assay, mosquitoes were silenced for *LacZ, TEP1* and *CLIPA14/CLIPA2*. *CLIPA14/CLIPA2 dsRNA* was injected twice, 3 days before *P. berghei* infection and 11 days post infection, to ensure adequate silencing of both genes. Batches of 8 Mosquitoes were dissected just below the thorax in a way to retain the salivary glands, and were grinded in 200 µl 1xPBS. The lysates were then centrifuged at 1000g in order to remove any debris, and 10 µl of the lysates were mounted on hemocytometer

followed by counting and visualizing GFP-expressing sporozoites using the fluorescent microscope Leica DM6 B. The Mann-Whitney test was used to calculate statistical significance and medians were considered significantly different if $P < 0.05$.

3. Bioparticle surface extraction assay

Female mosquitoes that were silenced for *LacZ, TEP1* and *CLIPA14* were injected with a 20mg/ml of bacterial bioparticle suspension of pHrodo labeled *E. coli* K-12 bacterial strain (Invitrogen) resuspended in 1xPBS. Mosquito hemolymph from 60 mosquitoes for each genotype was collected into 60 µl of 15 mM Tris (pH 8.0) containing 1xEDTA-free protease inhibitor cocktail (Roche) 15 min after bacterial injection. The unbound soluble fraction was collected after pelleting the bacteria by centrifugation for 5 minutes at 6000 g at 4 °C. The bacterial pellet was washed with 400 µL of collection buffer and the bound fraction was extracted with 25-30 µL of 1xSDS-PAGE sample buffer. Bound and unbound fractions were analyzed by western blotting.

D. Molecular Biology

1. Gene silencing by RNA interference

Genes of interest were silenced in adult female mosquitoes by microinjection of double stranded RNA (dsRNA) intrathoracically as previously described [277]. For dsRNA production, gene specific DNA amplicons flanked by T7 promoter sequences were

produced by PCR amplification of cDNA or plasmids containing full length cDNA sequence, using T7-tagged primers (table 2). Illustra GFX PCR DNA, Gel Band Purification Kit (GE Healthcare) was used to purify the PCR amplicons, according to the manufacturer's instructions. These purified PCR amplicons were used as DNA templates for TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific), according to the manufacturer's protocol. After DNase I treatment, dsRNAs were purified using phenol:chloroform, precipitated using isopropanol and resuspended in nuclease free water at a concentration of 3.5 µg/µl. Efficiency of gene silencing was confirmed either by immunoblotting when antibodies were available, or by semi-quantitative real-time PCR.

Table 2. T7 flanked primers used for dsRNA production.

Primer	**Primer sequence (5'-3') - The T7 promoter sequence is underlined.**
LacZ-F	TAATACGACTCACTATAGGGAGAATCCGACGGGTTGTTACT
LacZ-R	TAATACGACTCACTATAGGGCACCACGCTCATCGATAATTT
TEP1-F	TAATACGACTCACTATAGGGTTTGTGGGCCTTAAAGCGCTG
TEP1-R	TAATACGACTCACTATAGGGACCACGTAACCGCTCGGTAAG
LRIM1-F	TAATACGACTCACTATAGGGAATATCTATCTCGCGAACAATAA
LRIM1-R	TAATACGACTCACTATAGGGAATATCTATCTCGCGAACAATAA
CLIPA2-F	TAATACGACTCACTATAGGGATCCTAACAACGGCACACTGTGTGA

CLIPA2-R	TAATACGACTCACTATAGGGTCCTGATCGCCATGATTGGTGGTGCT
SPCLIP1-F	TAATACGACTCACTATAGGGGTCACCGAACACGTCCAAC
SPCLIP1-R	TAATACGACTCACTATAGGGCTGCATGGCCCTACGTCTA
CLIPA14-F	TAATACGACTCACTATAGGGCGGCATCATCGACATCCGTGTC
CLIPA14-R	TAATACGACTCACTATAGGGGTTGCTGTCGGCGACACGCTCCT
CLIPA5-F new	TAATACGA CTCACTATAGGGATTCGAGTTAATGCTGAACCTGA
CLIPA5-R new	TAATACGA CTCACTATAGGGTGTCCATTGGACTTGATAGCATT
CLIPA5-F old	TAATACGACTCACTATAGGGTGGGACACACAAACAGAACATGAG
CLIPA5-R old	TAATACGACTCACTATAGGGAGAGCCTCCATCACCACGGCACAT
CP1-F	TAATACGACTCACTATAGGGGCCAGCAAGGTCGCGGACTT
CP1-R	TAATACGACTCACTATAGGGTATGCCACCCTCGACCACGA
CP2-F	TAATACGACTCACTATAGGGGCCGAACCAAAACCTCACGAT
CP2-R	TAATACGACTCACTATAGGGATACAGCGACCGATTCTCGT

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

For RNA extraction, around 15 mosquitoes per genotype were stored in 300 µl TRIzol Reagent (Invitrogen). Nucleic acids were extracted with chloroform and treated with DNase I to remove any DNA products. Total RNA was further extracted with phenol:chloroform and precipitated with 0.7 volumes of isopropanol. RNA pellets were resuspended with nuclease free water. For cDNA synthesis, 1 µg of RNA was used in reverse using iScriptTM cDNA Synthesis kit (BioRad) according to the manufacturer's protocol (reagents and volumes used in reverse transcription are listed in table 3). Incubation conditions for reverse transcription is detailed in the table below. The produced cDNA templates were used in real-time PCR. SYBR® Green Quantitative RT-PCR Kit was used to perform the real-time PCR reactions. The prepared cDNA samples were diluted 20x, and were used for the real-time PCR reaction (detailed in the table 4). Components for the real-time PCR were loaded into a 96-well plate, sealed properly, centrifuged at 1000g, 4°C, for 3mins, and placed in a CFx96 Systems light cycler machine (initial denaturation step: 95^{o}C for 3mins followed by a denaturation step: 95^{o}C for 10s repeated 39 cycles, then by an annealing step: 60^{o}C for 30s).

The ribosomal S7 gene was used as an endogenous control gene to normalize the relative mRNA expression level of each tested gene. The qRT-PCR Ct values were used to calculate the relative variation in the mRNA levels.

Table 3. Materials used in reverse transcription.

Component	**Volume/reaction**
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
Nuclease free water	Up to 20 µl
Total RNA	Equivalent to 1 1µg
Total	**20 µl**

Table 4. Real-time PCR components.

Component	**Volume/reaction**
cDNA	5 µl
SYBR Green *Taq* ReadyMix	12.5 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
Nuclease free water	2.5 µl
Total	**25 µl**

Table 5. Real-time primer sequences.

Primers	Primer sequence (5'-3')
CLIPA14-rt-F	GGCACTGCTGATACTCACCG
CLIPA14-rt-R	CGGAGGCGAAACACTTGGTG
CLIPA2-rt-F	GATACTACCTGCACGGGTTGGT
CLIPA2-rt-R	CAGTATAAGGTATCTGCTTCTGATGGC
CLIPA5-rt-F	TCCGTGCTAGATAGTCCTCCAC-3
CLIPA5-rt-R	CCGGTCACGCTAAAACCGAG-3'

3. Generation of pIEx10-CLIPA14^{HIS}, pIEx10-CP1^{HIS} and pIEx10-CP2^{HIS} plasmids

The entire *CLIPA14*, *CP1* and *CP2* open reading frames lacking the endogenous signal peptide were cloned into the *pIEx10* insect cell expression plasmid (Novagen) incorporating a 10xHIS-tag C-terminal. Cloning was done using ligase-independent cloning (LIC) kit (Novagen) according to the manufacturer's protocol.

Primers used in LIC cloning:

CLIPA14-LIC-F: GACGACGACAAGATGCAGGATACGCTCGACGACCTC

CLIPA14-LIC-R: GAGGAGAAGCCCGGTTTCGGCGTGTAGACATAGTCCCG

CP1-LIC-F: GACGACGACAAGATGGGTGCTGCCGCCGTGGATGGA

CP1-LIC-F: GAGGAGAAGCCCGGTTTGCTATCATAGTAACCGAGGC

CP2-LIC-F: GACGACGACAAGATGGATCCGGCCCGGTACGACCACT

CP1-LIC-R: GAGGAGAAGCCCGGTTTCAGTTCCTGCCGCTCACCGTC

The underlined sequences are extensions to allow ligase-independent cloning [145].

E. Cell Biology

Generation of stable cell lines expressing *CLIPA14*, *CP1* and *CP2*

Sf9 cells were seeded in a 6-well plate (approximately 1 x 10^6 cells/well). Using Cellfectin II transfection reagent (Invitrogen), Sf9 cells were co-transfected with 2 µg of the previously cloned plasmids (*pIEx10-CLIPA14*^{HIS}, *pIEx10-CP1*^{HIS} and *pIEx10-CP2*^{HIS}*)* and 0.2 µg of *pIE1-neo*, according to the manufacturer's protocol. Cells were gently detached 24 hours post transfection and diluted in Sf-900 medium supplemented with 10% FBS, 50U/µl penicillin and streptomycin. The cells were then seeded at different densities ($2.5x10^4$, and $1.25x10^4$ cells/ml) in a 6-well plate (3 ml/well). Stably secreting cells were selected over a span of two weeks by the addition of 1mg/ml G-418 antibiotic in the serum containing medium. Medium was changed every five days. After the two-week selection period, the selective pressure was dropped to 0.25mg/ml of G-418 antibiotic. Stable cell lines were always in the presence of 0.25 mg/ml of G-418 antibiotics and were regularly checked for confluence.

F. Antibody production

1. Generation of antisera against CLIPA14, CP1 and CP2

CLIPA14^{HIS}, *CP1^{HIS}* and *CP2^{HIS}* proteins were purified using Talon beads (Clonetech). Talon beads were washed 3 times in 1xPBS and resuspended in 1xPBS (1v:1v ratio). The beads were then incubated with the conditioned media on a rotator/shaker at 4ºC overnight. The beads-condition media mixture was then washed 4-5 times with 1xPBS containing 10mM imidazole (pH 7.4). Bound proteins were then eluted with 1xPBS containing 300mM imidazole pH 8.0. Purified *CLIPA14^{HIS}*, *CP1^{HIS}* and *CP2^{HIS}* proteins were quantified by Coomassie staining of SDS-PAGE gels to make sure the proteins did not undergo any degradation.

Purified recombinant CLIPA14^{HIS} and CP1^{HIS} and CP2 ^{HIS} were used to immunize mice using the TiterMax Gold Adjuvant (Sigma), according to the manufacturer's protocol.

G. Protein biochemistry

1. Mosquito hemolymph extraction

Mosquito hemolymph was extracted from mosquitoes (after clipping the mosquito proboscis) into 1x non-reducing Laemmli Sample Buffer (BioRad), or into 1xPBS containing EDTA-free protease inhibitor cocktail (Roche) when protein quantification was required.

2. Western blot assays

Protein samples were separated on a 10% SDS-PAGE and then wet-transferred to Immuno-Blot PVDF membrane (BioRad). To prevent non-specific antibody binding, PVDF membranes containing transferred samples were blocked in 5% skimmed milk prepared in 1xPBS containing 0.05% Tween 20 for 1 hour at room temperature. Membranes were then incubated with primary antibody overnight at 4 ºC. Membranes were then washed 3x with PBST (1xPBS containing 0.05% tween20), probed with horse radish peroxidase-conjugated α-mouse (1:6000) or α-rabbit secondary antibodies (1:12000) for 1hour at room temperature, and washed again 3x with PBS-T (10min each wash). Bands were revealed by incubating the membranes in ClarityTM Western ECL Substrate (BioRad) for 30s-1min, followed by membrane revelation using Gel Doc XR+ system (Biorad).

Table 6. Different primary antibodies used in western blot analysis.

Antibody	**Type**	**Species**	**Dilution**	**Incubation**
α-TEP1*	Polyclonal	Rabbit	1:1000	O.N. at 4ºC
α-CLIPA2*	Polyclonal	Rabbit	1:1000	O.N. at 4ºC
α-CLIPA14	Polyclonal	Rabbit	1:3000	O.N. at 4ºC
α-CLIPA5	Polyclonal	Rabbit	1:2000	O.N. at 4ºC

α-LRIM1	Polyclonal	Rabbit	1:2000	O.N. at 4°C
α-SPCLIP1*	Polyclonal*	Rabbit	1:2000	O.N. at 4°C
α-PPO6	Polyclonal	Rabbit	1:2000	O.N. at 4°C
α-SRPN3	Polyclonal	Rabbit	1:1000	O.N. at 4°C

* Affinity-purified antibody

3. Phenoloxidase enzymatic assay

Mosquitoes were silenced for *LacZ*, *CLIPA14*, *CLIPA2/CLIPA14* by RNAi. Mosquitoes were then injected with *E. coli* bacteria ($O.D._{600nm}$ of 0.8) three days post gene silencing. Hemolymph was extracted 3 hours post bacterial injection in ice-cold 1xPBS containing EDTA-free protease inhibitor cocktail (Roche). When needed, protein quantification was done using Bradford assay (Fermantas). The PO enzymatic assay was performed 3 h after mosquito injection with *E. coli* strain OP-50 (OD_{600}=0.8) using approximately 5-9 μg of mosquito hemolymph per reaction as described previously [117].The absorbance at 492 nm was measured 30 minutes after incubation with L-DOPA (Sigma) using a ThermoScientific Multiskan EX ELISA machine (Thermo Fisher Scientific).

4. TEP1-dependent protein degradomic approach

We adopted a high throughput proteomic approach to identify novel cSPHs whose cleavage is dependent on TEP1. Mosquitoes silenced for *LacZ* and *TEP1* (600 mosquitoes per genotype) were infected with *E. coli* three days post silencing and hemolymph was extracted in 1xPBS containing EDTA-free protease inhibitor cocktail (Roche) 1 hour after infection. Protein samples were run on a SDS-PAGE and stained by Coomassie. The gel was cut between 25 kDa and 48 kDa and gel slices were used to determine differentially abundant proteins between *dsLacZ* (control) and *dsTEP1* using LC-MS followed by label free quantification by Progenesis.

H. Immunohistochemistry and microscopy

To determine the interaction of immune proteins on *P. berghei* surface, mosquitoes were fed on an anesthetized *P. berghei* infected mouse (5% parasitemia) three days post silencing *LacZ*, *CP1*, *CP2* and *CP1*/*CP2*. Midguts were then dissected 21-22 hours post infection, and cleared from blood. Midguts were fixed in 4% formaldehyde for 50 minutes, washed 3 time in 1xPBS containing 0.05% TritonX-100 (PBT), and blocked with 1xPBS containing 1% BSA and 0.05% TritonX-100 for 1 hour at room temperature. Midguts were then incubated with anti-TEP1 primary antibody in blocking buffer (1:350 dilution) overnight at 4 ^{o}C. After incubation, midguts were washed 3 times with 1x PBT and then incubated with Alexa-546 conjugated α-rabbit secondary antibody diluted 1:800 in blocking buffer for 1 hour at room temperature. Secondary antibody was removed and

midguts were washed 3 times with 1xPBT and nuclei were stained with Hoechst (1:10000) for 5 minutes. Midguts were then mounted in in ProLong® Gold antifade reagent (Invitrogen). Upright fluorescent microscope Leica DM6 B was used to collect fluorescent images.

CHAPTER VI

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