Functional Interaction between Apolipophorins and Complement Regulate the Mosquito Immune Response to Systemic Infections

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Abstract
The complement-like protein thioester-containing protein 1 (TEP1) is the hallmark effector molecule against Plasmodium ookinete in the malaria vector Anopheles gambiae. We have previously shown that the knockdown of the noncatalytic clip domain serine protease CLIPA2 increased TEP1-mediated killing rendering mosquitoes more resistant to Plasmodium, bacterial and fungal infections. Here, CLIPA2 coimmunoprecipitation from the hemolymph of Beauveria bassiana-infected mosquitoes followed by mass spectrometry and functional genetic analysis led to the identification of the Apolipophorin-II/I gene, encoding the two lipid carrier proteins Apo-I and II, as a novel negative regulator of TEP1-mediated immune response during mosquito systemic infections. Apo-II/I exhibits a similar RNAi phenotype as CLIPA2 in mosquito bioassays characterized by increased resistance to B. bassiana and Escherichia coli infections. We provide evidence that this enhanced resistance to systemic infections is TEP1 dependent. Interestingly, silencing Apo-II/I but not CLIPA2 upregulated the expression of TEP1 following systemic infections with E. coli and B. bassiana in a c-Jun N-terminal kinase pathway-dependent manner. Our results suggest that mosquito Apo-II/I plays an important immune regulatory role during systemic infections and provide novel insight into the functional interplay between lipid metabolism and immune gene regulation.

Introduction
Mosquitoes, like other invertebrates, lack adaptive immunity and depend solely on their innate immune system to defend themselves against microbial infections. The recognition of pathogen-associated molecular patterns by mosquito pattern recognition receptors triggers the downstream activation of several immune effector mechanisms that clear the infecting agent. In the malaria vector Anopheles gambiae, the complement-like attack mediated by the thioester-containing protein 1 (TEP1) was shown to be a hallmark effector response against Plasmodium ookinete invading the midgut epithelium [1] and against systemic infections with bacteria [2] and fungi [3]. TEP1 is produced by hemocytes and binds to the surface
of ookinetes as they emerge from midgut epithelial cells into the basal labyrinth of the gut epithelium, triggering their death presumably through lysis. It is also required for the melanization of ookinetes in certain refractory mosquito genotypes [1]. TEP1 also binds to the surfaces of bacteria [4] and fungal hyphae [3], triggering their phagocytosis and melanization, respectively. More recently, it was shown that TEP1 is expressed in the male testes where it plays an essential role in the removal of defective apoptotic sperm cells during spermatogenesis, a process crucial for male fertility [5].

Due to its key role in mosquito immunity, TEP1 is tightly regulated at the transcriptional and activation levels. The basal levels of TEP1 were initially shown to be controlled by the Rel1 and Rel2 signaling pathways [6]. Enhancing TEP1 expression by silencing the negative regulators of these pathways increased mosquito resistance to Plasmodium infections [6, 7]. A more recent report revealed a significant role for the c-Jun N-terminal kinase (JNK) pathway in controlling the basal expression levels of TEP1 in hemocytes [8], suggesting that multiple transcription factors act in concert to control TEP1 promoter activity. At the activation level, full-length TEP1 is cleaved in the hemolymph by an unknown protease into active TEP1 cut, which is stabilized by a complex of two leucine-rich immune proteins, APLIC and LRIM1 [9, 10]. The APLIC/LRIM1 complex controls the precocious activation of TEP1 in the hemolymph and is required for its binding to Plasmodium ookinetes. It was shown later that nitration of ookinetes by HPX2 (heme peroxidase 2) and NOX5 (NADPH oxidase 5) during the traversal of midgut epithelial cells directs TEP1 to their surfaces [11]. HPX2 is also required for TEP1 binding to apoptotic sperm cells in mosquito males [5], suggesting that nitration is probably a prerequisite for TEP1 binding to all its target cells. Following activation, TEP1 accumulation on microbial surfaces is also controlled by two noncatalytic clip domain serine proteases (CLIPs), SPCLIP1 and CLIPA2, which act as positive and negative regulators of that process, respectively, suggesting the presence of a convergent-like activity analogous to that in mammalian complement. Silencing LRIM1 in naive mosquitoes triggered the loss of TEP1 cut [9, 10], SPCLIP1 [12] and CLIPA2 [13] from the hemolymph, demonstrating the tight association of these CLIPs with TEP1 activity.

CLIPA2 has a particular RNAi phenotype characterized by increased mosquito resistance to infections with Plasmodium, bacteria and fungi, in addition to a reduction in egg laying. Here, using CLIPA2 communoprecipitation (coIP) followed by mass spectrometry (MS) and functional genetic analysis, we identified apolipophorins (Apo)-I and II as novel regulators of mosquito complement during systemic infections with bacteria and fungi. Apo-II/I silencing increased mosquito resistance to bacterial and fungal infections in a TEP1-dependent manner. It was previously reported that Apo-II/I knockdown (kd) mosquitoes exhibit increased resistance to Plasmodium infections in a manner also dependent on TEP1 [14], suggesting that Apo-I and II play a broad regulatory role in mosquito immunity. We provide evidence that silencing Apo-II/I upregulated the expression of TEP1 following systemic infections with E. coli and B. bassiana in a JNK pathway-dependent manner. Our data highlight a novel functional link between lipid carrier proteins, complement and JNK signaling in regulating mosquito immune responses during systemic infections.

Materials and Methods

Ethics Statement

This study was carried in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, USA). The Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut approved the animal protocol (permit number 15-05-335). 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.

A. gambiae Rearing, Bacterial and Fungal Strains

All experiments were performed with A. gambiae G3 strain. Briefly, A. gambiae mosquitoes were maintained at 27 °C and 80% humidity with a 12-hour day-night cycle. Larvae were reared on tropical fish food. Adult mosquitoes were maintained on 10% sucrose and given mice blood for egg production. Wild-type B. bassiana strain 80.2 (a kind gift from D. Ferrandon) was cultured on potato dextrose agar plates at 25 °C and 80% humidity. The harvesting of conidia (spores) for mosquito infections was performed as previously described [3]. Freshly prepared spores were used for all experiments. The bacterial species used in this study include ampicillin-resistant Escherichia coli OP-50 (a kind gift from J.J. Ewbank) and tetracycline-resistant Staphylococcus aureus (a kind gift from P. Bulet). Both strains were cultured in Luria-Bertani (LB) broth overnight, washed with phosphate-buffered saline (PBS) and resuspended in PBS to an optical density at 600 nm (OD600 nm) of 0.4 for all mosquito infections, unless otherwise indicated.

Gene Silencing by RNA Interference

Double-stranded RNAs (dsRNA) for LacZ (control), TEP1, CLIPA2, Apo-II/I, Apo-III and Jun were synthesized using the T7-primers previously described [1, 8, 10, 13, 15], respectively. DsRNA for fondue was produced using the following pairs of T7-tagged primers (T7-tag underlined): T7-fondue-F, 5′-TAATAC-
GACTCACA'TATAGGTTGCCAGCTTCTGCACAACAG'T-3' and T7-londu-R, 5'-TTATATGACTACTATAGGGGCTATA-
TTGAACGTTCGGG  TT-3'.  T7-tagged fondue amplicons were 414 bp in size. In vitro transcription reactions to produce the dsRNAs were performed using the Transcript Aid T7 High-Yield Transcription Kit (Fermentas) according to the manufacturer’s instructions. In vivo gene silencing by RNA interference (RNAi) was performed as previously reported [16]. The efficiency of gene silencing by RNAi was quantified by Western blot for TEP1, CLIPA2 and apo-II for which antibodies are available [10, 15], and by real-time PCR in whole sugar-fed mosquitoes for Rel1, Rel2, Fon-
due and apo-III at 3–4 days after dsRNA injection. Jun silencing was quantified in perfused hemocytes since they are the source of TEP1 in the hemolymph and due to the fact that Jun showed poor silencing (approx. 20%) in whole dsJun mosquitoes, suggesting that certain tissues expressing Jun may not be accessible to the dsRNA. Hemocyte perfusion was performed by injecting individual mosquitoes in the thorax with 2–3 μl of an anticoagulant solution containing 60% Schneider medium and 40% citrate buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citric acid, pH 4.5) and collecting the drop that emerges from a small cut made in the abdomen. Hemocytes were collected by centrifugation then mixed with TRIzol for total RNA extraction, as described below. The A. gambiae ribosomal S7 gene was used as an internal control for normalization. Gene silencing efficiencies are shown in the on-
line supplementary table S2 (for all online suppl. material, see www.karger.com/doi/10.1159/000443883).

RNA Extraction and Real-Time PCR
Total RNA was isolated from whole mosquitoes or hemocytes at the indicated time points using TRIzol reagent (Invitrogen) ac-
cording to the supplier’s instructions, and contaminant genomic DNA was removed by DNase I treatment. First-strand cDNA was produced from 1 μg of total RNA using the iScript cDNA synthesis DNA was removed by DNase I treatment. First-strand cDNA was

Survival Assays and Bacterial CFU Counts
Mosquito survival following bacterial infections was performed as previously described [17]. Briefly, mosquitoes were injected with a suspension of E. coli strain OP-50 (OD_{600} = 0.4) and S. au-
reus (OD_{600} = 0.4) in PBS. A batch of at least 50 adult female A. gambiae mosquitoes was injected per genotype. Mosquito survival rates were scored on a daily basis over 8 days. The Kaplan-Meier survival test was used to calculate the percent survival. The statisti-
cal significance of the observed differences was calculated using the log-rank test. Survival assays were repeated at least three times. Mosquito challenges with B. bassiana were performed by spraying mosquitoes with a suspension of 2 × 10^6 spores/ml in 0.05% Tween-80, as previously described [3]. The spores used for mos-
quito challenges were freshly prepared from B. bassiana 3- to 4-week-old cultures grown on potato dextrose agar. The survival tests and statistics were as described above. Experiments were re-
peated at least three times using different batches of mosquitoes and fungal spores. Concerning the bacterial proliferation assay, the different mosquito genotypes were injected with 69 nl of E. coli (OP-50 strain) or S. aureus suspension in PBS at an OD_{600 nm} of 0.4. Bacterial CFU counts were performed exactly as previously described [13]. Experiments were repeated two to three times and statistical significance was calculated using the Mann-Whitney test. Means were considered significantly different at p < 0.05.

Western Blot Analysis
Adult female mosquitoes were injected with 69 nl of an E. coli suspension at an OD_{600} of 0.4 or sprayed with a B. bassiana suspension of 2 × 10^8 spores/ml in 0.05% Tween-80. At the indicated time points, hemolymph was collected by proboscis clipping directly into ice-chilled PBS containing a cocktail of protease inhibitors (Roche). Hemolymph protein quantification was performed using the Bradford protein assay (Fermentas) to ensure equal loading on the gel for all samples of a given experiment. Proteins were resolved by nonreducing SDS-PAGE and transferred to immunoblot PVDF membrane using wet transfer (Bio-Rad). Blots were incubated with mouse αCLIPA2 [13], rabbit αTEP1 [18], mouse αapo-II [15] and rabbit αPPO6 [19] polyclonal antibodies at dilutions 1/1,000, 1/1,000, 1/100 and 1/2,000, respectively. Anti-mouse and anti-rab-
bit IgG-horseradish peroxidase-conjugated secondary antibodies (Promega) were used at 1/6,000 and 1/12,000, respectively.

The bioparticle surface extraction assay was performed exactly as previously described [12]. Proteins in the soluble and bound fractions were resolved by nonreducing SDS-PAGE and Western blot was performed as described above.

Concerning the green fluorescent protein (GFP) release assay, dsRNA-treated mosquitoes were injected with GFP-expressing E. coli (OP-50) at an OD_{600} of 0.8 and hemolymph was extracted at 3 h postinjection. Hemolymph extracts were centrifuged at 4,000 g for 5 min to eliminate cells and live bacteria. The protein concentration in supernatants was determined by the Bradford protein assay. Western blots were performed as described above. Rabbit αGFP polyclonal antibody (Abcam) was used at a dilution of 1/500.

Production of Rabbit CLIPA2 Antibody and Affinity Purification
Recombinant His-tagged CLIPA2 (CLIPA2^{His}) was purified from conditioned media of S9 cells stably expressing CLIPA2 as previously described [13]. The purified protein was used to generate a rabbit polyclonal antibody (Eurogentec). CLIPA2 antibody was affinity purified over an AminoLink column (Pierce) containing covalently bound CLIPA2^{His} according to the manufacturer’s protocol.

colP and MS
colP reactions were performed with the Pierce crosslink Magnetic IP/co-IP kit according to the manufacturer’s protocol (Ther-
mo Scientific). For small-scale colP, hemolymph was extracted from approximately 100 mosquitoes at 48 h after natural infection with B. bassiana, as described above. Hemolymph extracts were centrifuged at 4,000 g for 5 min to remove mosquito and fungal cells. The cleared hemolymph extract was split equally into two samples; one sample was supplemented with 30 μl of a 1:1 slurry of PBS-containing agarose beads cross-linked with 4 μg of purified CLIPA2 antibody, and the other with 30 μl of a 1:1 slurry of PBS-containing control agarose beads. Following overnight incubation at 4°C on a rotating wheel, the beads were collected using the mag-
Apo-II/I Proteins, Novel Negative Regulators of Mosquito Immune Responses to Systemic Infections

So far TEP1 is known to be regulated by two classes of proteins: the leucine-rich immune proteins LRIM1 and APL1C form a heterodimeric complex that binds to and stabilizes TEP1_{cut}, the active form of TEP1, in the mosquito hemolymph [9, 10], while the two noncatalytic CLIPs, SPCLIP1 [12] and CLIPA2 [13], positively and negatively regulate TEP1_{cut} accumulation on microbial surfaces, respectively. In order to provide insight into the functional interaction between CLIPA2 and TEP1, and with the aim of identifying novel complement regulators with similar roles to CLIPA2, we set out to determine the identity of proteins that coIP with CLIPA2 from the hemolymph of B. bassiana-infected mosquitoes using MS. The strategy of coupling coIP with MS was used as a gene-decouverer approach in order to obtain an unbiased, broad view of the interactions of CLIPA2 with hemolymph proteins. There are two important reasons behind choosing B. bassiana as the main model infectious agent in this assay and throughout: first, it follows a natural infection route following mosquito spraying with spores [3], eliminating the wounding effect generated with bacterial infections; second, B. bassiana infections were shown to trigger a potent immune response in the mosquito involving TEP1 [3]. At first, a small-scale CLIPA2 coIP was performed from hemolymph collected from 100 mosquitoes at 48 h after natural infection with B. bassiana (the time when the infection is well established [3]). This was done to test the efficiency of the affinity-purified CLIPA2 antibody cross-linked to magnetic beads in immunoprecipitating CLIPA2, and determine whether we could detect interactions with certain components of the mosquito complement pathway for which antibodies are available. Beads lacking antibody and naive mosquitoes served as controls. The results revealed that αCLIPA2 almost depleted CLIPA2 from the hemolymph of naive mosquitoes while no interactions were detected with control beads, highlighting the specificity of the interaction (fig. 1). When the same experiment was repeated with hemolymph collected from the same number of mosquitoes infected with B. bassiana, CLIPA2 was not completely immunoprecipitated using the same volume of αCLIPA2 beads, suggesting that infection most likely increases CLIPA2 expression. Reprobing the membrane with available antibodies revealed that TEP1_{cut} coimmunoprecipitated with CLIPA2 under infected and noninfected conditions. SPCLIP1 was also detected in both conditions but not the CTL4/CTLMA2 heterodimer, which is as abundant in the hemolymph, indicating that the observed interactions are specific. None of the proteins we probed for were bound to control beads lacking antibody. Our data suggest that CLIPA2 interacts with components of the complement pathway, supporting its previously reported role in the regulation of TEP1-mediated immunity [13].

The efficiency and specificity of αCLIPA2 as well as the specific interactions observed between CLIPA2, TEP1 and SPCLIP1 in our small-scale IP assays prompted us to identify the broad spectrum of hemolymph proteins that may interact with CLIPA2. To this aim, CLIPA2 was coIP from hemolymph collected from 900 mosquitoes and followed by the identification of eluant proteins using a highly sensitive nanoLC MS/MS analysis. The list of identified proteins (online suppl. table S1) included four members of the CLIPA family (CLIPA1, CLIPA6, CLIPA7 and CLIPA14), but none of these are known to exhibit an RNAi phenotype similar to CLIPA2 [20; Osta, pers. commun.]. Other proteins in the list included members of the TEP, LRIM, fibrinogen-related proteins, scavenger receptor (SCRASP1) and prophenoloxidase fami-
In order to determine whether TEP1 is also required for the enhanced immune response to systemic infections in these mosquitoes we performed an epistasis genetic analysis by RNAi. Interestingly, silencing TEP1 simultaneously with either Apo-II/I or CLIPA2 compromised mosquito survival to B. bassiana infections as the single TEP1 kd did (fig. 3a).

To address whether TEP1 is also required for the increased resistance of dsApo-II/I mosquitos to systemic E. coli infections, we designed an assay that could test for a TEP1 role and at the same time provide some insight into the mechanism by which TEP1 mediates the clearance of E. coli in these mosquito genotypes. The assay is based on measuring the amount of

Enhanced Systemic Immune Response in dsApo-II/I Mosquitoes Is TEP1 Dependent

It was previously reported that TEP1 is required for the increased killing of Plasmodium oocinates in the midgut epithelium of dsApo-II/I mosquitoes [14]. To address whether TEP1 is also required for the enhanced immune response to systemic infections in these mosquitoes we performed an epistasis genetic analysis by RNAi. Interestingly, silencing TEP1 simultaneously with either Apo-II/I or CLIPA2 compromised mosquito survival to B. bassiana infections as the single TEP1 kd did (fig. 3a).

To address whether TEP1 is also required for the increased resistance of dsApo-II/I and dsCLIPA2 mosquitoes to systemic E. coli infections, we designed an assay that could test for a TEP1 role and at the same time provide some insight into the mechanism by which TEP1 mediates the clearance of E. coli in these mosquito genotypes. The assay is based on measuring the amount of
GFP present in the mosquito hemolymph at 3 h after injection of live GFP-expressing *E. coli*. Since these bacteria express cytoplasmic GFP, their lysis is expected to release GFP into the hemolymph which can then be detected by Western blot using an anti-GFP antibody. Hence, if TEP1 is indeed engaged in bacterial lysis similar to mammalian complement, then reducing or enhancing TEP1 activity should decrease or increase, respectively, the amount of GFP released from lysed bacteria. Our results revealed a clear GFP band in the hemolymph extract of ds*LacZ* mosquitoes and a very faint band in those silenced for TEP1 (fig. 3b), suggesting that TEP1 indeed contributes to early bacterial lysis in systemic infections. In dsApo-II/I and dsCLIPA2 mosquitoes the GFP signal was clearly enhanced relative to ds*LacZ* controls, indicating increased bacterial lysis; this increased lysis required TEP1 since the GFP signal in ds*TEP1*/ds*Apo-II/I* and ds*TEP1*/ds*CLIPA2* mosquitoes was again very faint and similar to that in the single TEP1 *kd* (fig. 3b). Cosilencing Apo-II/I and CLIPA2 did not enhance the GFP signal over that of the
single knockout of either gene. Altogether, our data indicate that both CLIPA2 and Apo-II/I negatively regulate the TEP1-mediated immune response and that TEP1 contributes significantly to bacterial lysis in systemic infections. However, this does not exclude that phagocytosis may also be enhanced in dsApo-II/I and dsCLIPA2 mosquitoes, despite not being addressed in our assay, since TEP1 is known to act as an opsonin [4, 29].

**Distinct Signaling Pathways Contribute to CLIPA2 and Apo-II/I Upregulation after B. bassiana Infections**

The fact that Apo-II/I and CLIPA2 exhibit similar RNAi phenotypes that are both TEP1 dependent prompted us to test whether these two genes are similarly regulated during systemic infections in a manner to impose a coordinated control over the complement response. To address this point, we monitored Apo-II, CLIPA2 and TEP1 protein levels in the hemolymph of *A. gambiae* mosquitoes at 48, 72 and 96 h after natural infection with *B. bassiana*. Our data revealed an increase in CLIPA2 protein levels in the hemolymph at 48, 72 and 96 h after natural infections with *B. bassiana*, with the strongest expression detected at the 96-hour time point (fig. 4a). Using real-time PCR, we showed that this strong increase at the latter time point correlates with an approximately 7-fold increase in CLIPA2 transcript levels (fig. 4b). Apo-II protein levels were also increased at 72 and 96 h after *B. bassiana* infection. At the transcript level, the Apo-II/I gene exhibited a more modest increase of approximately 2-fold at the 96-hour time point with respect to noninfected controls; despite showing no statistical significance by two-way ANOVA in this experiment, this increase was observed consistently. This is also supported by the clear and reproducible increase in Apo-II proteins in the hemolymph (fig. 4a). Surprisingly, *B. bassiana* did not alter the TEP1 protein (fig. 4a) or its transcript levels (fig. 4b).
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Fig. 4. Distinct signaling pathways control the upregulation of CLIPA2 and Apo-II/I following B. bassiana infections. a Representative Western blot showing Apo-II, CLIPA2 and TEP1 protein levels in hemolymph collected from wild-type mosquitoes at the indicated time points after B. bassiana infection. The protein concentration in samples was determined by Bradford protein assay and 0.9 μg of hemolymph proteins were loaded per lane. Additionally, the membrane was probed with αPPO6 to confirm equal loading. b Expression levels of Apo-II/I, CLIPA2 and TEP1 genes in whole mosquitoes treated with dsLacZ, dsRel1 or dsRel2 at 96 h after B. bassiana infection. The graph represents the mean expression levels in infected mosquitoes relative to noninfected controls (black bars) for which the mean was adjusted to a value of 1. Data are from three independent biological experiments. Error bars represent SEM. Statistical analysis was performed using two-way ANOVA and means were considered significantly different at p < 0.05. *** p < 0.001.

during the course of infection, suggesting the existence of a tight control over TEP1 gene expression during systemic infections. Silencing either of the NF-κB transcription factors Rel2 or Rel1 reduced significantly CLIPA2 transcript (fig. 4b) and protein levels (online suppl. fig. S2) after B. bassiana infection relative to dsLacZ control, indicating that CLIPA2 gene is regulated by both Toll/Rel1 and Imd/Rel2 immune signaling pathways. Neither Rel1 nor Rel2 kd altered significantly Apo-II/I expression following infection (fig. 4b; online suppl. fig. S2), suggesting that it is controlled by another signaling pathway.

Apo-II/I but Not CLIPA2 kd Upregulates TEP1 Expression during Systemic Infections

CLIPA2 kd does not alter TEP1 expression following systemic bacterial infections, but rather enhances TEP1 protein consumption during immune responses, as previously reported [13]. To address at what level Apo-II/I proteins regulate TEP1, we initially measured the transcript levels of the latter in dsLacZ-, dsApo-II/I- and dsCLIPA2-naive and E. coli-infected mosquitoes. Interestingly, TEP1 exhibited a significant 3-fold (p < 0.05) increase in dsApo-II/I relative to dsLacZ mosquitoes at 12 h after E. coli injection (fig. 5a), whereas this increase was not observed in naive conditions, suggesting that TEP1 upregulation in dsApo-II/I mosquitoes is infection dependent. In dsCLIPA2 mosquitoes, TEP1 expression was similar to that in dsLacZ controls whether in naive or infected conditions, which is in agreement with our previous data [13]. We tested in parallel the expression of four AMPs in these genotypes to determine whether other classes of effector molecules are also upregulated after silencing Apo-II/I. None of the AMPs tested showed a significant change in transcript levels either in dsCLIPA2 or in dsApo-II/I mosquitoes relative to dsLacZ controls, in naive or infected conditions (fig. 5a). CEC1 was strongly upregulated in response to E. coli infections; however, to similar levels in dsLacZ, dsCLIPA2 and dsApo-II/I mosquitoes. DEF1 exhibited a similar expression pattern as CEC1 but with a more modest upregulation. We then tested whether this enhanced upregulation of TEP1 in E. coli-infected dsApo-II/I mosquitoes is also observed fol-
Fig. 5. Apo-II/I kd upregulates TEP1 expression during systemic infections. **a** Expression levels of TEP1, Gambicin (GAM1), Defensin 1 (DEF1), Cecropins 1 (CEC1) and 3 (CEC3) in dsLacZ-, dsCLIPA2- and dsApo-II/I-naive (n) or E. coli-infected (i) mosquitoes. Shown are mean expression levels relative to the dsLacZ-naive control (plain white bars) for which the mean value was adjusted to a value of 1. Error bars represent the SEM of three independent biological experiments. Statistical analysis was performed using two-way ANOVA and the means were considered significantly different at p < 0.05. * p < 0.05, ** p < 0.01, *** p < 0.001.

**b** Expression levels of the same genes listed in **a** in dsLacZ and dsApo-II/I mosquitoes at 72 h after infection with B. bassiana. Mean expression levels in infected mosquitoes relative to noninfected controls for which the mean was adjusted to a value of 1, indicated by the dashed red line, are shown. Error bars represent the SEM of three independent biological experiments. Statistical analysis was performed as in **a**.

**c** Representative Western blot showing TEP1 protein levels in dsLacZ and dsApo-II/I mosquitoes at the indicated time points after E. coli and B. bassiana infections. Protein concentrations in hemolymph samples were determined by Bradford protein assay; 0.9 and 0.7 μg of hemolymph proteins were loaded per lane in the B. bassiana- and E. coli-infected samples, respectively. Additionally, membranes were probed with αPPO6 to confirm equal loading.
Following *B. bassiana* infections. Indeed, TEP1 transcripts exhibited a significant 3.2-fold increase (*p < 0.01*) in these mosquitoes at 72 h postinfection with *B. bassiana* relative to infected dsLacZ controls (fig. 5b). Again, none of the tested AMPs showed significant changes in gene expression in dsApo-II/I relative to dsLacZ after *B. bassiana* infections. TEP1 upregulation was also manifested at the protein level in hemolymph extracts from *B. bassiana*- and *E. coli*-infected dsApo-II/I mosquitoes (fig. 5c). These data suggest that Apo-II/I proteins negatively control TEP1 expression during systemic infections, but not in naive conditions. Hence, CLIPA2 and Apo-II/I are acting at different levels to control TEP1-mediated immunity, possibly to avoid a cost effect inflicted on the host by an exaggerated systemic immune response.

**TEP1 Upregulation in *B. bassiana*-Infected dsApo-II/I Mosquitoes Requires Signaling from the JNK Pathway**

The TEP1 gene seems to be subject to complex transcriptional regulation involving Rel1 [6], Rel2 [6, 7, 30] and JNK [8] signaling pathways. Among these, we hypothesized that JNK could be the main pathway responsible for TEP1 upregulation in dsApo-II/I mosquitoes following systemic infections for the following reasons: first, neither Rel1 nor Rel2 kd significantly altered TEP1 expression at a late time point (96 h) during *B. bassiana* infection (fig. 4b); second, the JNK pathway was shown to contribute substantially to TEP1 basal expression levels in hemocytes [8]. To address the role of the JNK pathway we questioned whether cosilencing Apo-II/I and Jun (dsJun/dsApo-II/I), the transcription factor activated downstream of the JNK signal transduction pathway, abolishes TEP1 upregulation in response to *B. bassiana* infections. Indeed, while TEP1 exhibited almost a 4-fold upregulation in dsApo-II/I-infected relative to naive mosquitoes, its expression returned to basal levels in dsJun/dsApo-II/I-infected mosquitoes (fig. 6a). No change in TEP1 expression was observed in dsLacZ- or dsJun-infected mosquitoes relative to naive controls, suggesting that fungal infection itself does not alter TEP1 transcript levels in wild-type mosquitoes, which seem to be maintained at basal levels independent of JNK signaling. On the other hand, fungal infection induced a significant 2.2-fold increase in Apo-II/I expression in dsLacZ mosquitoes relative to naive controls (fig. 6a). This infection-induced upregulation seems to depend on JNK signaling since Apo-II/I expression returned to basal levels in infected dsJun mosquitoes. To address whether JNK signaling is indeed required for the enhanced immunity of dsApo-II/I mosquitoes to systemic infections, dsApo-II/I, dsJun and

**Fig. 6.** TEP1 upregulation in dsApo-II/I mosquitoes is dependent on the JNK pathway. **a** Expression levels of TEP1 and Apo-II/I in dsLacZ (control), dsApo-II/I, dsJun and dsJun/dsApo-II/I mosquitoes at 72 h after infection with *B. bassiana*. Mean expression levels in infected (i) mosquitoes relative to noninfected controls (c) for which the mean was adjusted to a value of 1 are shown. Statistical analysis was performed using two-way ANOVA and the means were considered significantly different at *p < 0.05*. **b** Jun is required for the enhanced survival of dsApo-II/I mosquitoes to fungal infections. The indicated mosquito genotypes were sprayed with a suspension of 2 × 10⁸ spores/ml of *B. bassiana* 4 days post-dsRNA injection. Dead mosquitoes were counted daily over a period of 9 days after fungal challenge. Percent survival was calculated using the Kaplan-Meier method and statistical significance was calculated using the log-rank test. Survival curves were considered to be significantly different at *p < 0.05*. The survival curve is representative of three independent experiments.

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dsJun/dsApo-II/I mosquitoes were challenged with *B. bassiana* and their survival compared to that of infected dsLacZ controls. While dsApo-II/I mosquitoes exhibited significantly enhanced endurance to infection relative to dsLacZ, cosilencing jun abolished that phenotype (fig. 6b), clearly indicating that Jun is required for the enhanced immune response exhibited by Apo-II/I kd mosquitoes.

**Discussion**

Insect apolipophorins are complex assemblies of multifunctional molecules that are mainly involved in lipid transport. The lipophorin particle is composed of two obligate proteins, Apo-I and Apo-II, which are encoded by a single gene Apo-II/I, in addition to an exchangeable Apo-III protein that binds to and stabilizes the interaction of diacylglycerol with Apo-I and II forming the low-density lipophorins that serve as lipid transport vehicles [31]. In addition to their classical role in lipid metabolism, several reports pointed to a role for insect lipophorins, particularly Apo-III, in innate immunity. For instance, Apo-III has been involved in microbial recognition [32] and enhancement of humoral and cellular responses [33]. On the other hand, little information is available on the involvement of Apo-II/I proteins in insect immunity. Here, we show that silencing Apo-II/I renders *A. gambiae* mosquitoes more resistant to systemic infections with *E. coli* and *B. bassiana* by upregulating TEP1 expression in a JNK pathway-dependent manner, providing a mechanistic insight into the enhanced immune phenotype associated with this gene kd. Our data are in agreement with a previous report showing the key role of the JNK pathway in controlling TEP1 expression [8]. The RNAi phenotype of Apo-II/I suggests that Apo-II/I proteins likely play an immune regulatory role during systemic infections by attenuating or moderating the JNK pathway in mosquito hemocytes, the main, if not only producers of hemolymph TEP1. Interestingly, similar roles have also been ascribed to certain mammalian apolipoproteins [34].

It remains unclear why silencing Apo-II/I triggers this enhanced TEP1-mediated immune response. It is tempting to speculate that Apo-II/I kd may be triggering a stress response due to a defect in lipid metabolism. Since Apo-II/I proteins play an essential role in lipid transport, their absence may lead to excess diacylglycerol or triacylglycerol deposition in sites of lipid absorption (midgut) or storage (fat body) while depriving other tissues, such as hemocytes, ovaries and flight muscles, from these molecules. In both cases, this may lead to ER (endoplasmic reticulum) stress, which can increase JNK pathway activity. In fact, studies in mammals have revealed that JNK activity is increased in response to ER stress [35] and to excessive accumulation of lipids in tissues [36]. Alternatively, we cannot exclude that apolipophorins may moderate the JNK pathway through bioactive lipids they might carry. A recent study in *A. gambiae* revealed that a lipid carrier protein of the lipocalin family carries the lipid lipoxin that acts as a hemocyte differentiation factor in the mosquito *A. gambiae* [37], in a manner dependent on the Toll, STAT and JNK pathways [38]. Future biochemical analysis of the composition of these apolipophorin particles in infected mosquitoes may provide new insight into their functional interaction with the immune system.

Rono et al. [14] have previously shown that the reduction in the number of *P. berghei* oocysts in Apo-II/I kd mosquitoes is TEP1 dependent; however, TEP1 expression was not altered in these mosquitoes relative to dsLacZ controls, in contrast to the robust upregulation observed in our systemic infections of dsApo-II/I mosquitoes. A plausible explanation to this apparent discrepancy is that during systemic infections the microbes in the hemolymph may deliver stronger immune stimulatory signals to hemocytes, the main producers of TEP1, than oocysts, which are physically separated from hemocytes by the midgut epithelium. In other words, the robust TEP1 upregulation in dsApo-II/I mosquitoes during systemic infections may require, in addition to JNK, contribution from other signaling pathways that may not be stimulated in hemocytes during *Plasmodium* midgut invasion. The identity of these pathways and the molecular basis of moderation of the JNK pathway by these apolipophorins remain to be determined.

It was interesting to note that *E. coli* and *B. bassiana* infections in dsLacZ mosquitoes did not trigger any significant upregulation of TEP1 compared to dsLacZ-naive controls. Our results are in agreement with the study of Baton et al. [39], in which TEP1 expression was not altered in the hemocyte transcriptome of wild-type *A. gambiae* infected with *E. coli* and *Micrococcus luteus*, suggesting that a tight control over TEP1 expression is imposed during systemic infections possibly to avoid an exaggerated immune response that may be costly to the host. Such a cost was previously reported in dsCLIPA2 mosquitoes in the form of compromised female fecundity [13].

While dsApo-II/I mosquitoes exhibited enhanced resistance to *E. coli* infections, their resistance to *S. aureus* was similar to controls despite the robust TEP1 upregula-
tion, suggesting that mosquito complement is more effective in dealing with Gram-negative than Gram-positive bacteria. These results may seem intriguing because TEP1 was shown to be required for the phagocytosis of both *E. coli* and *S. aureus* in vivo [29]; however, they also suggest that mosquito complement elicits additional effector responses that are more effective in killing Gram-negative (such as lysis) than Gram-positive bacteria. We have shown that TEP1 contributes significantly to *E. coli* lysis; however, we were not able to address whether it has the same effect on *S. aureus* since we lack a GFP-expressing strain. Nevertheless, it would not be unexpected for *S. aureus* in particular to resist TEP1-mediated lysis, since the thick peptidoglycan layer surrounding these Gram-positive bacteria is one of the arsenals they use to escape lysis by mammalian complement [40]. Also, the fact that the gut microbiome of field caught *A. gambiae* mosquitoes is composed largely of Gram-negative bacteria [41] and that systemic bacterial infections in the field are likely to occur mainly through the oral route indicate that mosquito complement may have evolved to deal more efficiently with Gram-negative bacteria.

The identification of apolipophorins among the proteins that coimmunoprecipitated with CLIP2 was slightly surprising but may support early observations that the lipid particles nucleated by lipid carrier proteins in the hemolymph may in fact serve as platforms for recruiting immunity proteins [42]. We have shown using the bioparticle surface extraction assay that Apo-II binds to the surface of *E. coli* bioparticles in a TEP1-independent manner (online suppl. fig. S3). This could indicate that Apo-II/I proteins may either shuttle immune proteins to microbial surfaces, possibly by interacting with lipids exposed on these surfaces, or contribute to microbial clearance, or detoxify immune-stimulatory cell wall components, as has been ascribed to Apo-III. Identifying the molecular composition of lipophorin particles from mosquitoes coupled with genome-wide gene expression studies in *Apo-II/I*kd genotypes challenged with different microbes is expected to reveal novel insight into the mechanisms that link lipid metabolism to immune gene regulation.

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