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Plasmodium falciparum* suppresses the host immune response by inducing the synthesis of insulin-like peptides (ILPs) in the mosquito *Anopheles stephensi

Jose E. Pietri^a, Eduardo J. Pietri^a, Rashaun Potts^a, Michael Riehle^b, and Shirley Luckhart^{a,*}

Jose E. Pietri: jepietri@ucdavis.edu; Eduardo J. Pietri: ejpietri@ucdavis.edu; Rashaun Potts: rapotts@ucdavis.edu; Michael Riehle: mriehle@ag.arizona.edu

^aDepartment of Medical Microbiology and Immunology, 3437 Tupper Hall, One Shields Avenue, School of Medicine, University of California, Davis, CA 95616

^bDepartment of Entomology, 410 Forbes, College of Agriculture and Life Sciences, University of Arizona, Tucson, AZ 85721

Abstract

The insulin-like peptides (ILPs) and their respective signaling and regulatory pathways are highly conserved across phyla. In invertebrates, ILPs regulate diverse physiological processes, including metabolism, reproduction, behavior, and immunity. We previously reported that blood feeding alone induced minimal changes in *ILP* expression in *Anopheles stephensi*. However, ingestion of a blood meal containing human insulin or *Plasmodium falciparum*, which can mimic insulin signaling, leads to significant increases in *ILP* expression in the head and midgut, suggesting a potential role for *AsILPs* in the regulation of *P. falciparum* sporogonic development. Here, we show that soluble *P. falciparum* products, but not LPS or zymosan, directly induced *AsILP* expression in immortalized *A. stephensi* cells *in vitro*. Further, *AsILP* expression is dependent on signaling by the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) and phosphatidylinositol 3'-kinase (PI3K)/Akt branches of the insulin/insulin-like growth factor signaling (IIS) pathway. Inhibition of *P. falciparum*-induced ILPs *in vivo* decreased parasite development through kinetically distinct effects on mosquito innate immune responses. Specifically, knockdown of *AsILP4* induced early expression of immune effector genes (1–6 hours after infection), a pattern associated with significantly reduced parasite abundance prior to invasion of the midgut epithelium. In contrast, knockdown of *AsILP3* increased later expression of the same genes (24 hours after infection), a pattern that was associated with significantly reduced oocyst development. These data suggest that *P. falciparum* parasites alter the expression of mosquito *AsILPs* to dampen the immune response and facilitate their development in the mosquito vector.

*Corresponding author: sluckhart@ucdavis.edu, Tel: (530)-754-6963.

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Keywords

Mosquito; *Anopheles*; immunity; insulin; insulin-like peptide; malaria; *Plasmodium*; NF- κ B

1. Introduction

The human malaria parasite, *Plasmodium falciparum*, is transmitted by mosquitoes of the genus *Anopheles* and causes over 500,000 deaths annually (WHO, 2014). Transmission of *Plasmodium* parasites requires ingestion of an infectious blood meal by a mosquito. In the mosquito midgut, ingested *Plasmodium* gametocytes undergo a series of developmental changes in the context of robust immune responses that contribute to marked losses in parasite numbers (Clayton et al., 2014; Yassine et al., 2010). This process is regulated in part by insulin/insulin-like growth factor signaling (IIS) and mitogen-activated protein kinase (MAPK) pathways (Corby-Harris et al., 2010; Drexler et al., 2013; Drexler et al., 2014; Hauck et al., 2013; Horton et al., 2010; Luckhart et al., 2013; Pakpour et al., 2012; Surachetpong et al., 2009; Surachetpong et al., 2010).

The regulation and function of IIS are highly conserved among vertebrate and invertebrate species (Luckhart & Riehle, 2007; Wu & Brown, 2006). In mammals, insulin biosynthesis is regulated by autocrine positive feedback that controls both insulin gene transcription and peptide secretion through activation of IIS proteins (Khoo et al., 2003; Leibiger et al., 1998; Leibiger et al., 2000; Melloul et al., 2002). Following secretion, insulin and two insulin-like growth factors (IGFs) can signal through receptor tyrosine kinase homodimers to regulate a variety of physiological processes. Additionally, mammals utilize seven other relaxin family insulin-like peptides (ILPs) that signal through G-protein coupled receptors (Halls et al., 2007). Varied numbers of ILPs have been identified in the fruit fly *Drosophila melanogaster* (Brogiolo et al., 2001), the nematode *Caenorhabditis elegans* (Duret et al., 1998), the silkworm *Bombyx mori* (Mizoguchi & Okamoto, 2013), as well as the mosquitoes *Aedes aegypti* (Riehle et al., 2006), *Anopheles gambiae* (Krieger et al., 2004), and *Anopheles stephensi* (Marquez et al., 2011). Invertebrate ILPs regulate behavior (Nassel, 2012), metabolism (Broughton et al., 2008; Gulia-Nuss et al., 2011; Zhang et al., 2009), reproduction (Brown et al., 2008; Gulia-Nuss et al., 2011; Sim & Denlinger, 2009), aging (Evans et al., 2008; Broughton et al., 2010) and immunity (Evans et al., 2008; Garsin et al., 2003).

In *A. stephensi*, ILP expression does not change significantly with age or upon ingestion of a sugar or blood meal. However, ingestion of a *P. falciparum*-infected blood meal profoundly increased expression of *AsILP2*, 3, 4, and 5 in the head and midgut (Marquez et al., 2011). In support of a role for *AsILPs* in parasite infection, studies of field-collected *A. gambiae* from Mali identified a single nucleotide polymorphism (SNP) in *AgILP3* that was significantly associated with natural *P. falciparum* infection (Horton et al., 2010). We have since identified additional infection-associated SNPs in *AgILP3* and *AgILP4* that are predicted to alter protein function (unpublished data). These observations provide further evidence that mosquito ILPs – and likely *Anopheles* ILP3 and 4, in particular – contribute to the regulation of parasite development.

Strong mechanistic links between IIS and immunity have been well documented in other invertebrate species and these observations suggest that IIS and immune signaling are inversely correlated (Demas et al., 2011). That is, immune activation inhibits IIS, perhaps to allocate resources from storage and growth to the immune response, while up- and down-regulated IIS can inhibit or activate immunity to protect the host during infection and non-pathological physiological conditions, respectively. For example, activation of Toll signaling in *D. melanogaster* reduces endogenous IIS, leading to decreased nutrient storage during infection (DiAngelo et al., 2009). Similarly, *Mycobacterium marinum* infection-induced activation of Toll and IMD signaling in *D. melanogaster* resulted in a loss of metabolic stores (Dionne et al., 2006). In contrast, virulence factor-mediated increases in IIS during *Pseudomonas aeruginosa* infection in *C. elegans* suppressed anti-microbial peptide (AMP) gene expression, increasing bacterial colonization (Evans et al., 2008), while insulin receptor *daf-2* mutants were resistant to bacterial pathogens (Garsin et al., 2003). In more recent studies, pathogen resistance in *C. elegans* was observed to occur independently of the DAF-2 pathway, resulting instead from direct pathogen activation of the IIS downstream target DAF-16, a forkhead box O (FOXO) transcription factor (Zou et al., 2013). Our own studies affirmed that PI3K/Akt signaling is responsible for human insulin-induced repression of the innate immune responses of *A. stephensi* to *P. falciparum* infection (Pakpour et al., 2012), demonstrating that mosquito IIS regulates innate immune responses in a manner similar to that observed in *D. melanogaster* and *C. elegans*. However, little is known regarding the extent to which endogenous mosquito ILPs regulate host immunity.

Here, we build upon our previous studies demonstrating that *P. falciparum* infection can induce *AsILP* expression in the midgut to determine the mechanism and significance of this phenomenon (Marquez et al., 2011). Our results indicate that *AsILP* gene expression during *P. falciparum* infection results from parasite stimulation of IIS and not from immune activation by microbial signals that induce Toll/Immune deficiency (IMD) pathways. Induced *AsILP*3 and 4 cooperate temporally to facilitate parasite development in the mosquito by dampening NF- κ B-mediated immune responses. Parasite-induced activation of IIS, therefore, suggests that *P. falciparum* has co-opted *AsILP* function in part for immune suppression in the mosquito host. Further, *AsILP*-dependent suppression of anti-parasite defenses extends our understanding of the regulation of immunity by IIS to include the activity of functionally distinct neuropeptides.

2. Results

2.1 Soluble *P. falciparum* products (PfPs) induced the transcription of *AsILPs*

We previously showed that expression levels of *AsILP*2, 3, 4 and 5 are induced in the mosquito midgut and head following ingestion of a *P. falciparum*-infected blood meal (Marquez et al., 2011). Although *AsILP* expression was not induced in response to ingestion of blood alone, the *A. stephensi* midgut is populated with Gram-positive and Gram-negative bacteria (Djadid et al., 2011; Rani et al., 2009), including dominant Gram-negative *Asaia* sp. that can expand with blood feeding (Favia et al., 2007; Hughes et al., 2014), and with yeast symbionts (Ricci et al., 2011) that could contribute to induction of *AsILP* expression during parasite infection. To isolate and study these signals in a manner that is not possible *in vivo*,

we stimulated immortalized *A. stephensi* (ASE) cells *in vitro* with lipopolysaccharide (LPS; Horton et al., 2011; Pakpour et al., 2012; Pakpour et al., 2013) and zymosan (Han et al., 1999; Heard et al., 2005) as representative Gram-negative and fungal microbe-associated molecular patterns (MAMPs), with soluble *P. falciparum* products (Pakpour et al. 2012), or with human insulin. ASE cells express *AsILP1*, 3, and 5, genes that are induced by physiological concentrations of human insulin and *P. falciparum* infection in *A. stephensi* (Marquez et al., 2011). However, ASE cells express only very low levels of *AsILP2* and do not express *AsILP4* (data not shown). Despite this fact, ASE signaling responses were surprisingly analogous to those in the midgut (Marquez et al., 2011). In particular, human insulin induced ASE expression of *AsILP1*, 3, and 5 (Figs. 1A, B, C). While stimulation with a low concentration (3.6 parasite equivalents/cell) of soluble *P. falciparum* products (*PfPs*; Pakpour et al., 2012) had no effect on *AsILP* expression, stimulation with a 10-fold higher *PfPs* concentration (36 parasite equivalents/cell) significantly increased expression of *AsILP1*, 3, and 5 relative to untreated controls (Figs. 1A, B, C). In contrast, stimulation with LPS, an activator of IMD signaling, significantly decreased expression of *AsILP1* and *AsILP3* relative to control (Figs. 1A, B), while zymosan, an activator of Toll signaling, had no significant effect on *AsILP* expression, suggesting that bacterial and fungal MAMPs that induce Toll/IMD signaling do not contribute to induction of *AsILP* expression during *P. falciparum* infection in *A. stephensi*.

2.2 *AsILP* expression was dependent on both IIS-associated MEK/ERK and PI3K/Akt signaling

We previously observed that *P. falciparum* induction of *nitric oxide synthase (NOS)* expression in ASE cells (Lim et al., 2005) and in the *A. stephensi* midgut is dependent on activation of both MEK/ERK (Surachetpong et al., 2009) and PI3K/Akt (Corby-Harris et al., 2010; Luckhart et al., 2013) signaling. Here we have demonstrated that *PfPs* and human insulin induce *AsILP* transcription and that this signaling is distinct from pathways activated by LPS (Imd) and zymosan (Toll) that decrease or do not induce *AsILP* expression, respectively (Fig. 1). Thus, we reasoned that expression of *AsILPs* in *A. stephensi* could be regulated by MEK/ERK and PI3K/Akt signaling. Our analyses revealed that stimulation of ASE cells with *PfPs* led to increased phosphorylation of the MEK/ERK branch of IIS (Fig. 2A), with statistically significant increases in phosphorylated ERK levels relative to control at all timepoints after stimulation. In contrast, no changes in phosphorylation of Akt or the downstream transcription factor FOXO were observed in two independent replicates. These observations suggested that inducible MEK/ERK signaling regulates *AsILP* expression in response to *PfPs* stimulation, but did not preclude the involvement of steady-state PI3K/Akt signaling in the synthesis of *AsILP* transcripts for storage and later translation. To test these hypotheses, we utilized two approaches established in our previous studies for inhibition of MEK/ERK and PI3K/Akt signaling. We used the MEK inhibitors PD98059 and U0126 (Surachetpong et al., 2009) and the PI3K inhibitor wortmannin (Lim et al., 2005; Pakpour et al., 2012) as well as overexpression of *A. stephensi* phosphatase and tensin homolog (PTEN, Hauck et al., 2013), the endogenous inhibitor of PI3K/Akt signaling, to query regulation of inducible and baseline *AsILP* expression, respectively. Pre-treatment of ASE cells with PD98059 for 1 hour prior to stimulation, prevented *PfPs*-induced ERK phosphorylation (Fig. 2B) and significantly repressed *AsILP3* expression (Fig. 2C). MEK inhibition using

PD98059 also reduced *AsILP1* expression (Fig. 2C), though this finding was not statistically significant. While stimulation with *PfPs* had no effect on Akt phosphorylation (Fig. 2A), treatment with the PI3K inhibitor wortmannin significantly reduced the expression of *AsILP1* and *AsILP5* relative to untreated controls, suggesting that constitutive PI3K/Akt signaling contributes to basal *AsILP* expression (Fig. 2D). To determine whether analogous signaling was evident *in vivo*, we provided *A. stephensi* with a *P. falciparum*-infected bloodmeal supplemented with either UO126 or diluent as a control. As in ASE cells (Fig. 2C), chemical inhibition of MEK in the midgut (Fig. S1) significantly decreased *P. falciparum*-induced *AsILP3* expression, with non-significant downward trends for *AsILP1*, 2, and 5 (Fig. 2E). PTEN overexpression inhibited baseline *AsILP* expression in the midgut epithelium of non-fed *A. stephensi* relative to non-transgenic controls with significant repression of *AsILP2*, 3, and 5 and non-significant downward trends for *AsILP1* and 4 (Fig. 2F). Collectively, these data revealed that inducible *AsILP* expression requires MEK-ERK signaling while PI3K/Akt signaling controls steady-state *AsILP* expression.

2.3 Treatment with antisense morpholinos directed against *AsILP3* or *AsILP4* decreased *P. falciparum* development in *A. stephensi*

In ASE cells (Fig. 1B) and in the *A. stephensi* midgut and head (Marquez et al., 2011), *AsILP3* expression is induced by parasite signaling. *AsILP4* expression is further restricted, with induction only in the midgut and only by *P. falciparum* infection (Marquez et al., 2011). In field-collected *Anopheles gambiae*, we identified coding sequence SNPs in *AgILP3* (Horton et al., 2010) and *AgILP4* (unpublished data) that are significantly associated with natural *P. falciparum* infection, suggesting functional involvement of these ILPs in the host response. To determine whether *AsILP3* and 4 were functionally associated with *P. falciparum* infection in *A. stephensi*, we treated *A. stephensi* with translation blocking antisense morpholinos (Pietri et al., 2014) that repress *AsILP3* and 4 peptide levels *in vitro* and *in vivo* (Cator et al., 2015). Treatment of mosquitoes with each morpholino significantly reduced the prevalence (percentage of mosquitoes infected) and intensity (oocysts/infected midgut) of *P. falciparum* infection. Specifically, treatment with anti-*AsILP3* morpholino reduced infection prevalence from 68.1% to 51.5% (Fig. 3A) and reduced infection intensity by more than 30% (2.54 to 1.81 oocysts/infected midgut; Fig. 3B). Similarly, treatment with anti-*AsILP4* morpholino decreased infection prevalence from 60.9% to 48.2% (Fig. 3C) and infection intensity from 2.0 to 1.51 oocysts/midgut (Fig. 3D). Given that we have previously shown morpholinos efficiently target midgut proteins for knockdown (Pietri et al., 2014), these effects are likely due to a reduction in midgut ILPs, but we cannot fully discount contributions from knockdown in other tissues. Nevertheless, these data suggested that *AsILP3* and *AsILP4* function in a manner similar to that of ingested human insulin (Pakpour et al., 2012) to promote *P. falciparum* development in the midgut.

2.4 Treatment with antisense morpholinos directed against *AsILP3* and *AsILP4* increased immune effector gene expression and parasite killing in *A. stephensi*

To determine whether decreased oocyst numbers in morpholino-treated *A. stephensi* were associated with increased anti-parasite responses, we quantified transcript levels for *A. stephensi* orthologs of several gene targets known to regulate the *A. gambiae* anti-parasite

response at 1, 6, and 24 hours post-infection in antisense morpholino-treated mosquitoes relative to controls. We also quantified transcript levels for *Pfs16* (a sexual stage-specific expression marker for *P. falciparum* gametocyte to zygote stages; Berry et al., 2009; Luckhart et al., 2013) and *Pfs25* (a marker for gametocyte to oocyst stages; Berry et al., 2009; Luckhart et al., 2013) in the same samples as an indication of relative abundance of sexual stage parasites in the midgut lumen. Interestingly, treatment with antisense morpholinos directed against AsILP3 and AsILP4 produced distinctly different effects on expression of immune mediators and on relative parasite abundance (Fig. 4). In particular, treatment with anti-AsILP3 morpholino had no effect on immune gene expression at 1 or 6 hours post-infection relative to controls. However, by 24 hours post-infection, expression of *NOS*, *APL1*, *TEP1*, and *LRIM1* were significantly increased (Fig. 4A). In contrast, treatment with anti-AsILP4 morpholino was associated with rapid and significant induction of *NOS* expression at 1 hour and of *APL1*, *TEP1*, and *LRIM1* expression at 1 and 6 hours post-infection relative to controls, while only *NOS* expression was significantly induced at 24 hours post-infection (Fig. 4B). There were no significant differences in expression markers for relative parasite abundance between anti-AsILP3 morpholino-treated mosquitoes and controls at any of the timepoints examined (Fig. 4C). However, expression of *Pfs25* was significantly repressed at 24 hours post-infection in mosquitoes treated with anti-AsILP4 morpholino relative to controls (Fig. 4D), a pattern consistent with early induction of immune genes in these mosquitoes (Fig. 4B). Collectively, these observations suggested that AsILP3 and AsILP4 regulate mosquito immune gene expression and parasite killing through kinetically distinct mechanisms, with AsILP3 acting on parasites during or after invasion of the midgut epithelium and AsILP4 affecting parasites in the midgut lumen prior to invasion.

3. Discussion

Our data affirm previous observations that *P. falciparum* can induce the expression of *AsILPs* (Marquez et al., 2011), but extend these observations by demonstrating that IIS is functionally required for parasite-induced expression of *AsILPs*. The synthesis of *AsILPs*, in turn, represses NF- κ B-dependent host immune defenses through as yet unknown signaling pathways to facilitate parasite survival (Fig. 5). We summarize these observations below in the context of published studies.

Using ASE cells as a surrogate for the host response to parasite signaling *in vivo*, we affirmed that inducible *AsILP* expression is tuned to signaling by human insulin and *P. falciparum* and showed that *AsILP* expression is not activated by sensing of prototypical MAMPs that activate the Toll (zymosan) and IMD (LPS) pathways. These MAMPs did not activate and, in fact, repressed *AsILP* expression in ASE cells. In particular, zymosan had no significant effect on *AsILP* expression and stimulation with LPS decreased the expression of *AsILP1* and *AsILP3* (Figs. 1A, B). Given that activation of Toll and IMD signaling in *D. melanogaster* can decrease IIS and metabolic stores (DiAngelo et al., 2009, Dionne et al., 2006), LPS-mediated reductions in *AsILP* expression could contribute mechanistically to these phenomena. Conversely, stimulation with *PfPs* increased *AsILP* expression (Fig. 1), indicating that *PfPs* signaling is distinct from that of Toll/IMD activators. In particular, *PfPs* induced *AsILP* expression through IIS, akin to induction of *AsILP* expression by physiological concentrations of human insulin (Marquez et al. 2011). In support, stimulation

with *Pf*Ps activated the MEK/ERK branch of the IIS pathway (Fig. 2A). Chemical inhibition of MEK attenuated this signaling event (Fig. 2B) and reversed the effects of *Pf*Ps and *P. falciparum* infection on *AsILP* expression (Figs. 2C, E). While Akt phosphorylation was not induced by *Pf*Ps *in vitro* (Fig. 2A), chemical inhibition of PI3K using wortmannin reduced *AsILP* expression in unstimulated cells (Fig. 2D). Further, overexpression of the PI3K/Akt signaling inhibitor PTEN significantly reduced *AsILP* expression in the midgut of non-fed *A. stephensi* relative to non-transgenic controls (Fig. 2F), suggesting that MEK/ERK signaling regulates inducible *AsILP* expression for response to infection and stress, while PI3K/Akt signaling regulates steady-state *AsILP* expression for normal cellular growth and metabolism. Collectively, these data also suggest that feed-forward induction of ILPs by IIS activation is conserved from invertebrates to mammals (Khoo et al., 2003; Leibiger et al., 1998).

Although we have not identified the parasite ligand(s) responsible for inducing *AsILP* synthesis in the mosquito, parasite glycosylphosphatidylinositols (GPIs), which are insulin-mimetic and can activate IIS in the *A. stephensi* midgut within 30 minutes after ingestion (Lim et al., 2005), could function in this capacity. The induction of *AsILP* expression by *P. falciparum* infection (Marquez et al., 2011) and by *Pf*Ps (Fig. 1) also affirms that live pathogen signaling – similar to *P. aeruginosa*-induced immunosuppression via DAF2 signaling in *C. elegans* (Evans et al., 2008) – can induce *AsILPs*, but is not necessary for this response. Further, the levels of *AsILP* induction *in vitro* by *Pf*Ps (Fig. 1) and *in vivo* following infection (Marquez et al., 2011) – both roughly 2–3 fold relative to control – suggest that signals from parasite GPIs or other factors are predominant in regulating *AsILP* expression. Accordingly, we suggest that any other midgut-associated MAMPs that could activate *AsILP* expression likely contribute minimally if at all to *AsILP* expression *in vivo*. This speculation is supported by our observations that an uninfected blood meal, which can induce significant proliferation of *A. stephensi* microbiota (Hughes et al., 2014; Wang et al., 2011), has no effect on *AsILP* expression relative to non-fed controls (Marquez et al., 2011). Despite the concordance of these data, we cannot ultimately exclude minor contributions from other microbial factors, as these can activate a wide network of pathways, including multiple MAPKs (Lee & Kim, 2007).

The concept that parasite insulin mimicry activates mosquito IIS during infection is suggestive of immune evasion by the parasite, given the known inverse relationship between IIS and immunity (Demas et al., 2011; DiAngelo et al., 2009). To test this hypothesis of immunosuppression, we used anti-*AsILP* morpholino treatments to query the effects of *P. falciparum*-induced *AsILP3* and *AsILP4* (Marquez et al., 2011) on the expression of *A. stephensi* orthologs of known anti-parasite immune effectors in *A. gambiae*, and on parasite development *in vivo*. Treatment with morpholinos directed against both *AsILPs* significantly decreased the prevalence and intensity of *P. falciparum* infection (Fig. 3). Further analysis revealed that *AsILP4* appears to affect early expression (1–6 hours post-infection) of immune effectors (Fig. 4B), while the effects of *AsILP3* are delayed, only appearing at 24 hours post-infection (Fig. 4A). These patterns were temporally associated with parasite killing in the midgut lumen (Figs. 4C, D), suggesting that parasite-induced *AsILPs* are in fact immunosuppressive.

The upregulation of an array of anti-parasite immune effectors by AsILP3 and 4 knockdown suggests that AsILPs can broadly inhibit NF- κ B-dependent signaling. Among these anti-parasite effectors, inducible nitric oxide synthase (NOS) and the production of reactive oxygen and nitrogen species contribute to *P. falciparum* killing in the *A. stephensi* midgut lumen (Ali et al., 2010; Hurd & Carter, 2004; Luckhart et al., 1998; Peterson et al., 2007), while a complex of NF- κ B-regulated IMD pathway components including complement-like TEP1 (Blandin et al., 2004), the leucine-rich repeat protein LRIM1 (Fraiture et al., 2009) and APL1 (Mitri et al., 2009) have been demonstrated to act maximally against ookinetes that have completed passage through the *A. gambiae* midgut epithelium and have come to rest in the basal labyrinth under the basal lamina. Activity of mosquito defensin against oocyst development has been reported in some but not all mosquito-parasite combinations (Antonova et al., 2009; Blandin et al., 2002; Kokoza et al., 2010). In our studies, the upregulation of these anti-parasite effectors following AsILP knockdown (Fig. 4) was associated with reduced abundance of both early and invading ookinetes as well as early oocysts based on expression of the sexual stage-specific markers *Pfs16* and *Pfs25* (Berry et al., 2009; Luckhart et al., 2013) and direct parasite counts. Detailed studies of the interaction between *P. falciparum* ookinetes and the *A. stephensi* midgut using electron microscopy have shown that at 24 hours post-infection ookinetes are present at this time only in the midgut lumen, with invasion of the epithelium occurring between 32–36 hours post-infection (Meis & Ponnudurai, 1987). Therefore, we suggest that induction of *AsILP4* suppresses early anti-parasite gene expression during the first 24 hours of infection, a time when ookinetes have formed in the lumen but have not yet invaded the midgut epithelium and that *AsILP3* induction suppresses later anti-parasite gene expression to protect parasites during and after invasion.

In general, our data suggest that AsILPs negatively regulate anti-*P. falciparum* inducible NOS and IMD signaling, both of which are conserved among *Anopheles* species (Garver et al., 2009; Gupta et al., 2009; Herrera-Ortiz et al., 2004; Luckhart et al., 1998; Vijay et al., 2011), but with distinct kinetics as shown here with *A. stephensi*. These differences may be due to AsILP-specific differences in the timing of peptide secretion or to differential activation of distinct signaling pathways, biology that we are now exploring. In particular, *A. aegypti* ILP3 (*AaILP3*) has been shown to bind to the insulin receptor, while *AaILP4* binds to an uncharacterized mosquito membrane protein (Wen et al., 2010), suggesting that AsILPs could signal through multiple receptors to regulate anti-parasite effector genes. Alternatively, AsILP-specific activation of IIS pathway proteins, most notably FOXO, ERK and glycogen synthase kinase 3 (GSK3) could facilitate diverse direct effects on effector gene transcription as well as modulation of NF- κ B-dependent signaling (Becker et al., 2010; Pakpour et al., 2012; Zou et al., 2013; Surachetpong et al., 2009; Martin et al., 2005). Hence, further study of AsILPs and mechanisms whereby AsILPs signal host mosquito cells are likely to provide novel insights into parasite manipulation of mosquito defenses against infection.

Beyond host immunity, the functional diversity of mosquito ILPs is likely to impact vectorial capacity through effects on feeding behavior (Cator et al., 2015; Nassel, 2012), longevity (Evans et al., 2008; Broughton et al., 2010) and reproduction (Brown et al., 2008;

Gulia-Nuss et al., 2011; Sim & Denlinger, 2009). Notably, phylogenetic analysis revealed that *AsILPs* are more divergent as a group than are *D. melanogaster* ILPs (DILPs) (Marquez et al., 2011). *AaILPs* are similarly diverse, as ILP3 and ILP4 divergently regulate carbohydrate and lipid metabolism while having overlapping effects on gonadotropic activity and IIS activation (Wen et al., 2010). DILPs, in contrast, are highly redundant, with compensatory effects observed upon knockdown, and appear to function in networks. For example, RNAi-mediated knockdown of *DILP2* increased *DILP3* and *DILP5* expression (Broughton et al., 2010) and knockdown of multiple *DILPs* was necessary to observe a measurable effect on fly growth (Zhang et al., 2009). While it is likely that *AsILPs* cooperate to regulate mosquito physiology, our data also suggest that *AsILPs* regulate parasite development as well through distinct effects on host immune effector genes. Hence, it may be possible to precisely manipulate specific *AsILPs* to generate genetically modified mosquitoes that exhibit enhanced resistance coupled with enhanced survivorship and reproduction for sustainable malaria transmission control.

On a broader scale, our work has implications for understanding the evolutionary biology of both *P. falciparum* and IIS. In particular, *P. falciparum*-derived signals and insulin can activate *A. stephensi* IIS to induce immunosuppressive ILPs that facilitate parasite survival (Fig. 5). Given the highly conserved nature of IIS (Luckhart & Riehle, 2007), immunosuppressive ILPs could facilitate *P. falciparum* infection in other *Anopheles* species. A connection with biology in the human host is also likely. Human malaria is associated with hyperinsulinemia (Phillips et al., 1993; White et al., 1983), perhaps due to GPI signaling (Schofield and Hackett, 1993). Taken together, these observations highlight the existence of a co-evolved host interface that facilitates immune evasion by malaria parasites. Specifically, hyperinsulinemia of ingested blood can decrease mosquito anti-parasite defenses directly (Pakpour et al., 2012) and can also induce the synthesis of immunosuppressive mosquito *AsILPs* through a heterologous feedback loop (Marquez et al., 2011). Further, *P. falciparum* can directly activate *AsILP* expression to dampen immunity through mosquito IIS, as shown here, collectively suggesting that *P. falciparum* manipulates insulin and ILP synthesis in mammals and mosquitoes to facilitate their development and transmission at this critical interface that defines malaria (Fig. 5).

4. Materials and Methods

4.1 *AsILP* gene expression

Immortalized, embryo-derived *A. stephensi* (ASE) cells were maintained in modified minimal essential medium (MEM; Gibco, Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum at 28°C under 5% CO₂. Cells were plated at a density of 250,000 cells/mL and allowed to rest for 24 hours prior to stimulation with human insulin (1.7 µM; Sigma Aldrich, St. Louis, MO), *Escherichia coli* lipopolysaccharide (LPS, 10 µg/mL; Sigma Aldrich), *Saccharomyces cerevisiae* zymosan (10 µg/mL; Sigma Aldrich), or purified soluble *P. falciparum* products (*PfPs*, 3.6 or 36 parasite equivalents/cell; Pakpour et al., 2012) for 2 hours. Ingestion of 1–2 µl of blood by *A. stephensi* from a host with a 10% parasitemia, typical in peripheral blood samples from life-threatening *P. falciparum* infections (Bruce-Chwatt, 1971), would lead to midgut infections of 250 to 550 parasites per

midgut epithelial cell (Lim et al., 2005). Our *in vitro* assays of 3.6 or 36 parasite equivalents/cell, therefore, would be typical of mosquito feeding on hosts with lower (0.5 to 1%) parasitemias (Markell et al., 1999). Following stimulation, cells were transferred into TriZOL (Invitrogen) and total RNA was isolated according to the manufacturer's protocol. Quantitative real-time PCR was performed on an ABI 7300 Sequence Detection System (Applied Biosystems; Foster City, CA) using *AsILP1-5* primers as previously described (Marquez et al., 2011). Data were analyzed using the C_t method (Schmittgen et al., 2008) and *AsILP* expression in stimulated cells was normalized against *A. stephensi ribosomal protein S7 (RPS7)* for loading and then against expression levels in matched, untreated controls. These experiments were replicated 4–6 times using independent preparations of ASE cells.

For analyses of MEK/ERK and PI3K/Akt-dependent *AsILP* expression *in vitro*, cells were pre-treated with the MEK inhibitor PD98059 (10 μ M; Surachetpong et al., 2009; Selleckchem, Houston, TX) for 1 hour prior to stimulation with *PfPs* (36 parasite equivalents/cell) or treated for 3 hours with the PI3K inhibitor wortmannin without further stimulation (10 μ M; Lim et al., 2005, Selleckchem) and data were analyzed as described above. For analyses of MEK/ERK-dependent *AsILP* expression *in vivo*, midguts from 15–20 *A. stephensi* were dissected into TriZOL (Invitrogen) 24 hours after being fed either an unsupplemented, *P. falciparum* infected blood meal or a *P. falciparum*-infected blood meal supplemented with the MEK inhibitor UO126 (10 μ M; Surachetpong et al., 2009; Selleckchem). For analyses of PI3K/Akt-dependent *AsILP* expression *in vivo*, midguts from a total of 15–20 three day old, phosphatase and tensin homolog (PTEN) transgenic (Hauck et al., 2013) or non-transgenic sibling *A. stephensi* females were dissected for quantitative real-time PCR as described above. Data are reported as mean $- C_t$ values $\pm$ SEM on a Log2 scale (1 C_t value = 2 fold change). MEK/ERK and PI3K/Akt-dependent *AsILP* expression experiments *in vitro* and *in vivo* were replicated with 3–5 independent preparations of ASE cells and 3–5 separate cohorts of *A. stephensi*, respectively.

4.2 Western blotting of soluble *P. falciparum* product (PfPs)-stimulated *A. stephensi* cells

ASE cells were maintained as described above and stimulated with *PfPs* (36 parasite equivalents/cell) for 5, 15, or 30 minutes. Cells were harvested into cell extraction buffer (Invitrogen), incubated at 4°C for 1 hour and periodically vortexed. Cell lysates were then cleared at 16,000 g for 10 min and protein supernatants were mixed with sample loading buffer (125 mM Tris-HCl pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, 130 mM dithiothreitol) and boiled for 5 minutes. Proteins were separated on 10% SDS-PAGE polyacrylamide gels and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline/Tween-20 (TBS-T) for 1 hour at room temperature. For detection of phosphorylated ERK (pERK), membranes were incubated overnight at 4°C with a 1:10,000 dilution of anti-pERK antibody (Cell Signaling, Danvers, MA) in 5% non-fat dry milk. Membranes were then washed 3X with TBS-T for 5 minutes and incubated with a 1:10,000 dilution of secondary HRP conjugated rabbit anti-mouse antibody (Sigma Aldrich) in 5% non-fat dry milk overnight. Detection of phosphorylated FOXO (pFOXO; Cell Signaling, 1:1000 primary, 1:2000 goat anti-rabbit secondary), phosphorylated Akt (pAkt; Cell Signaling, 1:1000 primary, 1:3000

goat anti-rabbit secondary) and GAPDH (Cell Signaling, 1:10,000 primary, 1:20,000 goat anti-rabbit secondary) followed the same protocol. Proteins were visualized by incubating membranes with SuperSignal West Dura chemiluminescent reagent (Pierce, Rockford, IL) for 3 minutes and exposing on an Image Station 4000 Pro digital imager (Kodak, Rochester, NY) for 1–5 minutes. Densitometry of pERK western blots was performed using the ImageJ (<http://rsbweb.nih.gov/ij/>) gel analysis tool. Data were normalized to untreated control groups as well as a GAPDH loading control. These analyses were replicated 2–4 times with independent preparations of ASE cells.

4.3 Morpholino-mediated knockdown of AsILPs in vivo

Knockdown of AsILP3 and AsILP4 in adult female *A. stephensi* was performed by feeding translation blocking anti-*AsILP3* or anti-*AsILP4* morpholinos (Gene Tools, Philomath, OR) as previously described and validated *in vivo* (Pietri et al., 2014) and *in vitro* (Cator et al., 2015). In brief, anti-*AsILP3* (5'-TCGTGGACGACATCTTGACAGAGGT-3') or anti-*AsILP4* (5'-CGTGGAACTTTCATCTCAAGGACCT-3') morpholinos at a stock concentration of 500 μ M were diluted to 10 μ M in saline (15 mM NaCl, 10 mM NaHCO₃, pH 7.0) with ATP (Sigma Aldrich) added to a final concentration 1 mg/ml as a feeding stimulant. Immediately prior to feeding, morpholino solutions were warmed in a water bath at 37°C for 10 minutes. A morpholino standard control, targeting a human beta-globin intron (5'-CCTCTTACCTCAGTTACAATTTATA-3') was provided at 10 μ M in saline-ATP to control mosquitoes. Cohorts of 50–60 newly emerged female mosquitoes were provided with water only (no sucrose) for 24 hours and then provided control-morpholino, anti-*AsILP3* morpholino, or anti-*AsILP4*-morpholino in saline-ATP via a Hemotek artificial circulation system (Discovery Workshops, Accrington, UK). After 30 minutes, unfed mosquitoes were removed from experimental cohorts. Mosquitoes were maintained on cotton pads soaked in 10% sucrose and on day 3 post-morpholino feeding were provided a meal of human *P. falciparum*-infected red blood cells (RBCs).

4.4 *P. falciparum* culture and mosquito infection

The NF54 strain of *P. falciparum* was initiated at 1% parasitemia in 10% heat-inactivated human serum and 6% washed RBCs in RPMI 1640 with HEPES (Gibco/Invitrogen) and hypoxanthine. At days 15–17, stage V gametocytes were evident and exflagellation was evaluated at 200X magnification with phase-contrast or modified brightfield microscopy the day before and day of feeding by observation of blood smears and before addition of fresh media. Female *A. stephensi* were provided water only (no sucrose) for 24 hours and then fed a meal of *P. falciparum*-infected RBCs using a Hemotek artificial circulation system (Discovery Workshops). Mosquitoes were allowed to feed for 30 minutes and were then maintained on cotton pads soaked in 10% sucrose until day 10 post-infection. On day 10, midguts were dissected and stained with 0.5% mercurochrome for visualization of *P. falciparum* oocysts by microscopy. Analyses of morpholino effects on parasite infection were replicated with four independent infections consisting of 14–29 female *A. stephensi* per group.

4.5 qPCR detection of immune gene expression in the mosquito

Cohorts of 15–20 morpholino-fed female *A. stephensi* were dissected 1, 6, or 24 hours after provision of a *P. falciparum*-infected blood meal and midguts were collected into TriZOL (Invitrogen) for extraction of total RNA according to the manufacturer's protocol. cDNA was synthesized using the QuantiTec Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Quantitative real-time PCR was performed on an ABI 7300 Sequence Detection System (Applied Biosystems) using gene-specific primers for *A. stephensi* orthologs of *defensin* (ASTE011281), *NOS* (ASTE008593), *TEP1* (ASTE010227), *APL1* (ASTE0102571), *LRIM1* (ASTE000814), and *S7* with SYBR Green PCR Mastermix (Applied Biosystems) as previously described (Pakpour et al., 2013; Hauck et al., 2013). For relative quantitation of *P. falciparum* in the same samples, qPCR was performed using gene-specific primers for *Pfs16* (gametocyte-zygote; Berry et al., 2009; Luckhart et al., 2013) and *Pfs25* (gametocyte-oocyst; Berry et al., 2009; Luckhart et al., 2013) with SYBR Green PCR Mastermix (Applied Biosystems) as previously described (Luckhart et al., 2013). Since expression of *18S* rRNA is high in all parasite stages and varies with the parasitemia, this gene and the ribosomal protein *S7* gene (*A. stephensi*) were used to normalize parasite gene target data. Data were analyzed using the $\Delta\Delta C_t$ method (Schmittgen et al., 2008). These assays were completed with three separate cohorts of *P. falciparum*-infected *A. stephensi*.

4.6 Statistical analyses

All statistical analyses were conducted using Graphpad Prism 5.02 (Graphpad Software, La Jolla, CA). For *in vitro* analysis of *AsILP* expression in response to PAMPS (Fig. 1), data were normalized and compared to untreated controls by t-test. *AsILP* expression levels following chemical inhibition of MEK or PI3K (Fig. 2) were analyzed relative to matched controls (*PfPs* only or untreated) by t-test. *AsILP* expression levels in PTEN transgenic mosquitoes were analyzed relative to matched non-transgenic siblings by t-test following rank transformation. ERK phosphorylation in ASE cells in response to stimulation with *PfPs* (Fig. 2A) was analyzed by ANOVA with Dunnett's multiple comparison test following rank transformation. Oocyst data from multiple replicates were pooled based on the determination by ANOVA that oocyst intensities did not differ among controls (individual replicate data provided in Table S1). Infection prevalence and intensity data were then analyzed using goodness of fit (chi-squared) and unpaired t-test with Welch's correction, respectively. Analyses of immune gene expression and sexual stage parasite marker expression in the midgut (Fig. 4) were analyzed by ANOVA followed by Fisher's exact test to analyze differences between control mosquitoes and mosquitoes treated with antisense morpholinos targeting *AsILPs* at matched timepoints. For all analyses, differences were considered significant when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ILP expression is induced by *P. falciparum* activation of mosquito insulin signaling
- ILPs, like human insulin, promote *P. falciparum* development in *A. stephensi*
- ILP3 and ILP4 produce kinetically distinct effects on the mosquito immune response
- *P. falciparum* may have co-opted mosquito insulin signaling to increase its survival

Figure 1. Soluble *P. falciparum* products and human insulin, but not LPS or zymosan, induced *AsILP* gene expression

ASE cells were stimulated for 2 hours and homogenized in TriZOL for RNA extraction.

AsILP1, 3, and 5 transcript levels were quantified by qRT-PCR using the $-\Delta\Delta Ct$ method and were analyzed relative to matched controls by t-test. Gene expression data are represented as mean $-\Delta\Delta Ct$ values $\pm$ SEM from 4–6 biological replicates on a Log2 scale (1 $\Delta\Delta Ct$ value = 2 fold change, untreated controls set at 0). *p < 0.05

Figure 2. Soluble *P. falciparum* products signal through IIS to induce *AsILP* expression
(A) Representative western blots from ASE cells stimulated with *PjPs* for 5, 15, or 30 min and analyzed for pERK, pAkt, and pFOXO levels relative to GAPDH as a loading control.
(B) ASE cells were pre-treated with the MEK inhibitor PD98059 for 1 hour, stimulated for 15 min with *PjPs* and then harvested for western blot analysis or **(C)** pre-treated with PD98059 for 1 hour, stimulated for 2 hours and harvested for qRT-PCR analysis of *AsILP* gene expression relative to matched untreated and *PjPs* only treated controls. **(D)** ASE cells were treated with the PI3K inhibitor wortmannin for 3 hours and harvested for qRT-PCR analysis of *AsILP* gene expression. **(E)** Midguts from mosquitoes provided a meal of *P. falciparum* infected RBCs +/- the MEK inhibitor UO126 were dissected 24 hours after feeding for qRT-PCR analysis of *AsILP* gene expression. **(F)** Midguts from 3 day old, PTEN transgenic and sibling non-transgenic control *A. stephensi* were dissected for qRT-PCR analysis of *AsILP* gene expression. Gene expression data are represented as mean – Ct values $\pm$ SEM from 3–5 biological replicates on a Log2 scale (1 Ct value = 2 fold change, untreated controls set at 0). Data from **(A, F)** were normalized by rank transformation. Western blot densitometry data **(A)** and gene expression data **(C–F)** were analyzed relative to matched controls by ANOVA or t-test, respectively. Western blots **(A)** were independently replicated twice for pAkt and pFOXO or 4 times for pERK. *p 0.05

Figure 3. Treatment with anti-AsILP3 and anti-AsILP4 morpholinos reduced the prevalence and intensity of *P. falciparum* infection in *A. stephensi*

Mosquitoes were fed either anti-AsILP3 (A, B) or anti-AsILP4 (C, D) morpholinos in saline-ATP and then provided a meal of human RBCs infected with *P. falciparum* three days later. Ten days later, midguts were dissected and stained with mercurochrome for visualization of oocysts to record both infection prevalence (A, C, percentage of mosquitoes infected) and infection intensity (B, D, number of oocysts/infected midgut). Data are reported as means for 4 replicates with separate cohorts of *A. stephensi*. Infection prevalence and intensity data were analyzed using goodness of fit (chi-square) and unpaired t-test with Welch's correction, respectively. *p 0.05

Figure 4. Treatment with anti-*AsILP3* and anti-*AsILP4* morpholinos increased immune effector gene expression and parasite killing in the mosquito midgut following *P. falciparum* infection Mosquitoes were fed either anti-*AsILP3* (A, C) or anti-*AsILP4* (B, D) morpholinos in saline-ATP and then provided a meal of human RBCs infected with *P. falciparum* three days later. Midguts were dissected at 1, 6, and 24 hours post-feeding for RNA extraction and qRT-PCR analysis of: (A, B) expression of immune effector genes or (C, D) markers of sexual state parasites (*Pfs16*, gametocyte-zygote; *Pfs25*, gametocyte-oocyst). Gene expression data from 3–7 replicates with separate cohorts of *A. stephensi* are represented as mean $\pm$ SEM on a Log2 scale (1 Ct value = 2 fold change, infected controls set at 0). Data were analyzed by ANOVA followed by Fisher's test. *p < 0.05

Figure 5. A model for IIS regulation of AsILP-dependent control of *P. falciparum* development
P. falciparum-derived signals activate MEK/ERK in the context of basal PI3K/Akt signaling to induce the expression of AsILPs (Panel 1). In the midgut, AsILP signaling decreases the expression of immune effector genes through distinct mechanisms (Panel 2), creating a more favorable environment for infection and increasing parasite survival (Panel 3). Inhibition of PI3K/Akt represses steady-state AsILP synthesis, while inhibition of MEK/ERK signaling abrogates *P. falciparum*-induced AsILP expression (Panel 4). AsILP knockdown results in increased defense responses to *P. falciparum* (Panel 5), killing parasites and decreasing infection levels in the mosquito (Panel 6).