Novel factors of Anopheles gambiae haemocyte immune response to Plasmodium berghei infection

Fabrizio Lombardo\textsuperscript{1,2,*} and George K. Christophides\textsuperscript{1}

Abstract

Background: Insect haemocytes mediate cellular immune responses (e.g., phagocytosis) and contribute to the synthesis of humoral immune factors. In previous work, a genome-wide molecular characterization of Anopheles gambiae circulating haemocytes was followed by functional gene characterization using cell-based RNAi screens. Assays were carried out to investigate the role of selected haemocyte-specific or enriched genes in phagocytosis of bacterial bio-particles, expression of the antimicrobial peptide cecropin1, and basal and induced expression of the mosquito complement factor LRIM1 (leucine-rich repeat immune gene I).

Findings: Here we studied the impact of a subset of genes (37 candidates) from the haemocyte-specific dsRNA collection on the development of Plasmodium in the mosquito by in vivo gene silencing. Our screening identifies 10 novel factors with a role in the mosquito response to Plasmodium. Analysis of in vivo screening phenotypes reveals a significant anti-correlation between the prevalence of oocysts and melanised ookinetes.

Conclusions: Among novel immune genes are putative pattern recognition proteins (leucine-rich repeat, fibrinogen-domain and R-type lectins), immune modulation and signalling proteins (LPS-induced tumor necrosis factor alpha factor, LITAF and CLIP proteases), and components of extracellular matrix such as laminin and collagen. Additional identified proteins such as the storage protein hexamerin and vesicular-type ATPase (V-ATPase) are associated for the first time with the mosquito response against Plasmodium.

Keywords: Anopheles gambiae, Innate immunity, Plasmodium, RNAi, Melanisation

Findings

Background

Plasmodium parasites must overcome several barriers before they can successfully establish infection in their anopheline mosquito vector. They include the microbiological barrier of the mosquito midgut microbiota, two physical barriers involving the peritrophic matrix and the midgut epithelium, and the immunological barrier of the mosquito innate immune system. The latter plays a critical role immediately after a Plasmodium ookinete crosses the midgut epithelium and before it develops into an oocyst. Circulating haemocytes are important contributors to the haemolymph immune response \cite{1, 2}. They take part in defense against invading microorganisms, both through cellular processes like phagocytosis and through the production and secretion of soluble humoral factors, such as antimicrobial peptides, complement-like proteins and components of proteolytic enzymes that control melanisation \cite{3, 4}.

To identify novel factors of the mosquito immune response and derive further insights into the function of haemocytes, we have recently developed in vitro, cell-based, double-stranded RNA (dsRNA) screens of about 100 Anopheles gambiae genes specifically or predominantly expressed in haemocytes \cite{5}. Using these screens, we have identified several novel modulators of phagocytosis, antimicrobial peptide expression, and expression of the complement factor, LRIM1. Here, we use a subset of this dsRNA collection to identify genes affecting An. gambiae infection with the rodent parasite Plasmodium berghei in vivo. This study extends our earlier published work and concludes the in vivo screening \cite{2}. The data
obtained from our screen are integrated with in vitro results obtained previously, highlighting a role of several genes in haemocyte innate immune responses to *Plasmodium* infection.

**In vivo RNAi screen to identify Plasmodium modulators**

We selected a subset of 39 dsRNAs corresponding to 37 putative immune modulators from an *An. gambiae* haemocyte-specific dsRNA library we had previously generated (Additional file 1: Table S1). These genes exhibit enriched expression in haemocytes, are differentially regulated by immune challenges, and/or have immune-related InterPro domains, signal peptides or transmembrane domains [2, 5]. Experimental procedures, such as gene knockdown (KD), mosquito infections with *P. berghei* and parasites assessment in the midguts, were performed according to standard protocols, detailed in Additional file 2 (primers used are listed in Additional file 3: Table S2). KD efficiency was assessed for a representative group of candidates and results are summarized in Additional file 4: Table S3.

Four successive screening rounds were implemented of 39, 29, 20 and 3 dsRNAs, respectively. DsRNAs were included in the next round of testing if they showed significant effects or at least a constant trend on either parasite intensity or prevalence of live oocysts or melanised ookinetes. Quality control and replicate pooling criteria were applied before performing statistical analyses (described in Additional file 2). Results are summarized in Table 1 and records of parasite counts of each gene KD are reported in Additional file 5: Table S4.

Silencing AGAP007540, AGAP009201 and SNAP_A-NOPHELES00000017730 (long version of VectorBase predicted AGAP010658, henceforth AGAP010658*) resulted in a significant increase of oocyst intensities (and also melanised ookinetes, as for AGAP009201). AGAP007540 and AGAP010658* silencing also led to a significant decrease in melanised ookinete prevalence (and intensity as for AGAP010658*). Silencing AGAP003960, AGAP004017, AGAP004928 and AGAP004993 caused a decrease in oocyst intensity. AGAP004993 silencing also significantly decreased the oocyst prevalence. AGAP011223 silencing decreased oocyst and melanised ookinete prevalence, while AGAP003879 silencing resulted in a decrease of oocyst intensity and prevalence and an increase of melanised ookinete intensity and prevalence. Lastly, silencing AGAP012034 significantly reduced the intensity and prevalence of melanised ookinetes and increased the number of developing oocysts.

### Novel modulators of the mosquito immune response to *Plasmodium*

The RNAi screen of 37 genes specifically or predominantly expressed in *An. gambiae* haemocytes identified ten novel modulators of mosquito infection with *P. berghei*. Below is a brief summary of the main characteristics of these genes, such as sequence similarities with known immune factors or domains and comparisons with phenotypes of orthologs in other insects (see Additional file 1).

AGAP007540 encodes a putative von Willebrand factor-type A domain (vWF) protein. The vWF domain can serve various biological functions in insects including haemolymph coagulation and haemostasis, wound healing and other innate immunity functions [6].

AGAP009201 is highly expressed in circulating haemocytes [2] and encodes for a collagen type IV protein, thought to be involved in the extracellular matrix, such as the basal lamina. Laminin and collagen are components of the basal lamina and interact with invading parasites [7]. Previous in vivo and cell-based RNAi assays have shown that laminin silencing leads to reduced oocyst intensity and increased phagocytosis capacity [5]. A role of laminin was proposed in regulating the expression of the complement factor LRIM1 during an immune challenge [5]. Here we reveal that additional putative components of the basal lamina are involved in these reactions, as recently described in the greater wax moth, *Galleria mellonella* [8], and the flour beetle *Tribolium castaneum* [9].

AGAP010658* encodes a homolog of the hexamerin 2 beta of *An. darlingi* Root and *Aedes aegypti* (Linneaus) and the larval serum protein 1 (LSP1.1) of *Culex quinquefasciatus* Say, which serve as major storage proteins [10]. The strong activation after blood meal suggests that hexamersins are a source of amino acids for the synthesis of vitellogenin in the fat body. A function of storage proteins and vitellogenin (Vg) in various facets of arthropod innate immunity has been described [11]. It has also been shown that depletion of the lipid carrier protein lipophorin (Lp) reduces the number of developing *Plasmodium* oocysts in the mosquito midgut, while both *An. gambiae* Lp and Vg are required for the function of the complement factor TEP1 (thioester-containing protein 1) against *Plasmodium* ookinetes [12].

AGAP003960 encodes a putative transmembrane protein encompassing peptidase and trypsin-like domains, possibly involved in immune regulation through proteolytic processing. AGAP003960 transcripts are enriched in haemocytes and up-regulated upon bacterial challenge in mosquito cell cultures [13].

The Leucine-Rich Repeat (LRR) domain protein-encoding gene, AGAP004017, is specifically expressed in circulating haemocytes [2]. It carries a predicted signal peptide and a transmembrane domain, and does not belong to the LRIM family of proteins [14]. AGAP004017 clusters with AGAP004016, another LRR-containing protein that is also highly expressed in haemocytes and acts as a *Plasmodium* agonist [2].

AGAP004928 (LL6) encodes a LITAF (LPS-induced tumor necrosis factor alpha factor) domain, a membrane-associated
motif possibly involved in immune signalling pathways [15]. We previously showed that this gene plays a role in bacterial phagocytosis [5]. Recently, additional members of this family were associated with the defence against Plasmodium [16]. Indeed, An. gambiae LITAF-like 3 (AGAP009053) expression is up-regulated in response to midgut invasion by both rodent and human malaria parasites, and its KD analysis reveals a role in anti-Plasmodium defence [17]. Four members of the LITAF family i.e. LL1, LL2, LL3 and LL4 are closely related, while LL5 and LL6 are more divergent. LL6 (AGAP004928) clusters with Drosophila melanogaster CG13559, a member of the fruit fly LITAF family expressed in the haemocytes and modulated by immune challenge [18].

AGAP004993 encodes an An. gambiae homolog of D. melanogaster laminin (LanA), an extracellular matrix protein with several functions. Six laminin paralogs are found in the An. gambiae genome: netrin 1 (AGAP000228), netrin 1 homolog (AGAP000225), laminin gamma 1 (AGAP007629), multiple epidermal growth factor-like domains 10 (AGAP007256), laminin alpha 1/2 (AGAP007849) and laminin beta 1 (AGAP001381). We previously showed that the latter regulates both phagocytosis and basal and induced expression of LRIM1 [5], while laminin gamma 1 and LanB2

Table 1 In vivo RNAi screen results. Gene KDs affecting the number of developing oocysts and the prevalence of developing oocysts and/or melanised oocinetes are listed. Descriptive statistics (arithmetic mean ± standard error) and P values as results of statistical tests to compare parasite intensity and prevalence of each group with corresponding LacZ control are reported here. Upper part of the Table (above the double line): genes affecting oocyst intensity; lower part of the Table (below the double line): genes affecting prevalence of infection and/or oocyst / melanised oocinete intensities. Significant P values (P < 0.05) are reported. ns: P > 0.05

| Gene KD         | Rep. | N.  | Developing oocysts | Melanised oocinetes |
|----------------|------|-----|--------------------|---------------------|
|                |      |     | Intensity          | Prevalence          |
|                |      |     | Mean ± SE          | Fd  | P§ | Mean ± SE | Fd  | P§ |
| AGAP010658°    | 3    | 51  | 64.4 ± 11.8        | 3.1 | 0.0004 | 0.2 ± 0.2 | 0.1 | 0.035 |
| LacZ           | 63   | 80  | 20.8 ± 4.4         | 8   | 2.4 ± 1.0 | 18 |
| AGAP007540     | 3    | 45  | 77.3 ± 12.1        | 2.1 | 0.002  | 1.4 ± 0.6 | 0.6 | 13 |
| LacZ           | 64   | 83  | 37.3 ± 6.2         | 83  | 2.2 ± 1.1 | 29 |
| AGAP009201     | 3    | 58  | 56.6 ± 9.4         | 1.9 | 0.032  | 9.0 ± 5.4 | 6.5 | 0.040 |
| LacZ           | 84   | 87  | 30.5 ± 5.0         |     | 1.4 ± 0.6 | 17 |
| AGAP004017     | 3    | 62  | 23.7 ± 5.0         | 0.7 | 0.036  | 5.1 ± 1.7 | 0.6 | 39 |
| LacZ           | 59   | 76  | 34.8 ± 5.5         |     | 8.0 ± 5.2 | 27 |
| AGAP004928     | 3    | 64  | 32.5 ± 4.9         | 0.7 | 0.042  | 3.5 ± 1.7 | 3.9 | 24 |
| LacZ           | 68   | 95  | 45.9 ± 5.8         |     | 0.9 ± 0.3 | 19 |
| AGAP004993     | 4    | 60  | 32.7 ± 7.9         | 0.6 | 0.002  | 11.3 ± 4.4 | 1.5 | 33 |
| LacZ           | 58   | 85  | 54.0 ± 7.5         |     | 7.3 ± 2.7 | 39 |
| AGAP003960     | 3    | 52  | 18.7 ± 3.8         | 0.5 | 0.044  | 4.0 ± 0.8 | 0.2 | 48 |
| LacZ           | 47   | 78  | 38.5 ± 7.6         |     | 16.5 ± 7.1 | 39 |
| AGAP011223     | 3    | 51  | 60.7 ± 12.9        | 1.4 | ns     | 9.6 ± 5.3 | 3.2 | 22 |
| LacZ           | 47   | 96  | 44.8 ± 11.5        |     | 3.0 ± 1.1 | 36 |
| AGAP003879     | 3    | 76  | 40.2 ± 8.3         | 0.9 | 0.011  | 8.6 ± 2.6 | 1.5 | 40 |
| LacZ           | 71   | 87  | 44.3 ± 5.6         |     | 5.9 ± 4.3 | 19 |
| AGAP012034     | 3    | 37  | 70.7 ± 25.9        | 2.0 | ns     | 0.7 ± 0.7 | 0.3 | 4 |
| LacZ           | 47   | 81  | 35.9 ± 9.5         |     | 2.6 ± 0.9 | 29 |

|                |      |     | Intensity          | %    | Fd  | %    | Fd  |
|                |      |     | Mean ± SE          |      |     |      |     |
| AGAP001223     | 3    | 51  | 60.7 ± 12.9        | 1.4 | ns     | 9.6 ± 5.3 | 3.2 |
| LacZ           | 47   | 96  | 44.8 ± 11.5        |     | 3.0 ± 1.1 | 36 |
| AGAP003879     | 3    | 76  | 40.2 ± 8.3         | 0.9 | 0.011  | 8.6 ± 2.6 | 1.5 |
| LacZ           | 71   | 87  | 44.3 ± 5.6         |     | 5.9 ± 4.3 | 19 |
| AGAP012034     | 3    | 37  | 70.7 ± 25.9        | 2.0 | ns     | 0.7 ± 0.7 | 0.3 |
| LacZ           | 47   | 81  | 35.9 ± 9.5         |     | 2.6 ± 0.9 | 29 |

|                |      |     | Prevalence          | %    | Fd  | %    | Fd  |
|                |      |     | Mean ± SE          |      |     |      |     |
| AGAP001223     | 3    | 51  | 60.7 ± 12.9        | 1.4 | ns     | 9.6 ± 5.3 | 3.2 |
| LacZ           | 47   | 96  | 44.8 ± 11.5        |     | 3.0 ± 1.1 | 36 |
| AGAP003879     | 3    | 76  | 40.2 ± 8.3         | 0.9 | 0.011  | 8.6 ± 2.6 | 1.5 |
| LacZ           | 71   | 87  | 44.3 ± 5.6         |     | 5.9 ± 4.3 | 19 |
| AGAP012034     | 3    | 37  | 70.7 ± 25.9        | 2.0 | ns     | 0.7 ± 0.7 | 0.3 |
| LacZ           | 47   | 81  | 35.9 ± 9.5         |     | 2.6 ± 0.9 | 29 |

Gene KD: ID of silenced genes (VectorBase Gene IDs)
Rep.: number of independent replicates
N: sample size (total number of mosquitoes across replicates)
Mean ± SE: arithmetic mean ± standard error (SE) of parasite intensities per midgut in each group after pooling replicate data
Fd: fold difference, ratio between mean oocyst (or melanised oocinete) value of a specific gene KD and the LacZ KD
P§: statistical significance according to Mann–Whitney U-Test on oocyst or melanised oocinete intensity of pools (gene-specific KD vs LacZ KD)
%: prevalence (infected mosquitoes/total mosquitoes) of developing oocysts or melanised oocinetes calculated as geometric means of the prevalence of each replicate
P¶: statistical significance according to Fisher’s Exact Test on oocyst or melanised oocinete prevalence (gene-specific KD vs LacZ KD)
° SNAP_ANOPHELES00000017730
(AGAP007629, Q9U3U7) were shown to promote oocyst development in the mosquito midgut, possibly by inhibiting their melanotic encapsulation [19].

AGAP011223 encodes the fibrinogen-related FBN8 (also known as FREPS7) that is shown to play a role in anti-Plasmodium defence [20]. We previously demonstrated that FBN8 promotes phagocytosis of bacterial bio-particles [5], highlighting the complex networks regulating mosquito innate immunity.

AGAP003879 encodes a vesicular-type ATPase, a transmembrane protein involved in several cellular processes [21]. V-ATPase utilizes ATP to actively transport H⁺, regulating osmotic changes in mosquito cells and osmoregulatory tissues, including the stomach, malpighian tubules, gut and rectum. A role of a V-ATPase in Plasmodium infection in Aedes and Anopheles was previously suggested, since the typical distribution of oocysts in the posterior half of the midgut overlaps with the spatial distribution of V-ATPase-overexpressing epithelial cells [22]. We previously showed that KD of V-ATPase reduces phagocytosis of E. coli bio-particles [5]. Here we reveal for the first time that KD of V-ATPase decreases the oocyst intensity and prevalence. It remains to be elucidated whether this effect of V-ATPase is caused by altered cellular equilibrium of water and ions (as for phagosome acidification and maturation) or by more conventional immune mechanisms.

Finally, AGAP012034 encodes a potential new member of the subfamily B of CLIP-domain serine proteases. The role of CLIPBs and their putatively inactive homologs, CLIPAs, as activators or suppressors of the An. gambiae melanisation response against P. berghei is well known [23]. AGAP012034 maps to a genomic cluster of four highly conserved CLIPBs, including CLIPB20 that is regulated after Serratia marcescens infection [24].

In conclusion, our results identify 10 novel regulators of the haemocyte immune response to Plasmodium. A complex role in different immune responses is discovered for some proteins (for instance, V-ATPase and LL6), as it arises by comparing results of this work with KD phenotypes from previous screens [5]. Finally, additional proteins such as the storage protein hexamerin and the V-ATPase are associated for the first time with the mosquito response against the malaria parasite.

Parasite melanisation is linked to parasite killing
We assessed the overall correlation of the prevalence of oocysts and melanised ookinetes across the dataset. By sorting one of the two phenotypes from highest to lowest, we observed a clear trend of anti-correlation between the

![Fig. 1 Correlation between prevalence of live oocysts and melanised ookinetes. A. Oocyst prevalence data from the first round of screening of 39 dsRNA targeting 37 genes sorted from highest (100 %) to lowest (37.50 %). Linear regression and correlation between prevalence of live oocysts and melanised ookinetes assessed in: B, 22 groups of mosquitoes injected with dsLacZ, which were used as controls in the four rounds of screenings (correlation coefficients: Pearson r: −0.1686; Spearman r: −0.2376, P = 0.2869); C, 10 groups of mosquitoes injected with dsRNAs that showed effects on parasite development (correlation coefficients: Pearson r: −0.6351; Spearman r: −0.6991, P = 0.027) and D, 29 groups of mosquitoes injected with dsRNAs that did not show effects on parasite development (correlation coefficients: Pearson r: −0.1912; Spearman r: −0.2727, P = 0.1523). Insets in graphs B, C and D report Pearson R² values and the relative statistical significance (P).](image-url)
two variables (Fig. 1a) that led us to partition the data according to whether effects on parasite development were observed or not. Data obtained in the first round of screening for 39 dsRNA targeting 37 genes were considered (Fig. 1a, c and d) as well as data from 22 dsLacZ injections in the four successive rounds of screenings to be used as a reference (Fig. 1b). A statistically significant anti-correlation between the two phenotypes was detected when genes that produced KD phenotypes were analysed (Fig. 1c) while no correlation was observed when genes that did not cause KD phenotypes and dsLacZ controls were evaluated (Fig. 1b and d). The dual role of melanisation in both parasite killing and clearance has been well established [25–27]. Our data corroborate the function of melanisation as a clearance mechanism that follows parasite killing by the mosquito immune system. Recently, novel insights into the balance between immune tolerance and resistance, as well as into the ability of the innate immune system to recognize and combat different pathogens using different strategies allowed the definition of innate immunity as a combination of both microbe clearance (melanisation) and damage control (pathogen survival) [28].

Ethics statement

Animals were cared for in accordance with the guidelines reported in the revised Animals (Scientific Procedures) Act 1986 (UK).

Additional files

Additional file 1: Table S1. dsRNA haemocyte-specific library. The main features of target genes and dsRNAs used in this study are presented. DsRNA IDs, KD phenotypes in cell-based RNAi screens [5], VectorBase gene IDs, Affymetrix probe codes and previous ENSEMBL IDs are listed in columns A, B, C, D and E, respectively. Circulating haemocyte microarray information from [2] are summarized in columns F (cluster), G (normalized haemocyte value), H (normalized carcass value) and I (normalized head value). Comments (name and/or homology of An. gambiae genes) and InterPro domain data are reported in columns J and K, respectively. Drosophila melanogaster orthologs are shown in column L, and corresponding FlyBase IDs in column M. D. melanogaster KD phenotypes, according to the GeneMetaRNAi database are reported in column N. This table is modified from Lombardo et al. 2013 [5]. (XLS 81 kb)

Additional file 2: Detailed description of the experimental procedures and statistical analyses utilized. Evaluation of gene knock down efficiency. (PDF 62 kb)

Additional file 3: Table S2. List of primers used in this study. (PDF 86 kb)

Additional file 4: Table S3. Measurement of knock down efficiency. (PDF 53 kb)

Additional file 5: Table S4. Oocysts and melanised ookinetes dataset. (XLSX 195 kb)

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

FL and GKC conceived and designed the study and the experiments; FL performed the experiments; FL and GKC analysed the data; FL and GKC drafted the manuscript. Both authors have read and approved the final manuscript.

Acknowledgements

We thank Tibebu Habtewold and Katarzyna Sala for their help with mosquito rearing. This work was supported by Wellcome Trust grants (G077229 and WT093587MA) and a NIH/NIAID grant (2P01AI044220-06A1).

Received: 9 October 2015 Accepted: 3 February 2016

Published online: 09 February 2016

References

1. Lavine MD, Strand MR. Insect hemocytes and their role in immunity. Insect Biochem Mol Biol. 2002;32(10):1295–309.

2. Pinto SB, Lombardo F, Koutouso AC, Waterhouse RM, McKay K, An C, et al. Discovery of Plasmodium modulators by genome-wide analysis of circulating hemocytes in Anopheles gambiae. Proc Natl Acad Sci U S A. 2009;106(50):21270–5.

3. Lemaître B, Hoffmann J. The host defense of Drosophila melanogaster. Annu Rev Immunol. 2007;25:697–743.

4. Christophides GK, Vlachou D, Kafatos FC. Comparative and functional genomics of the innate immune system in the malaria vector Anopheles gambiae. Immunol Rev. 2004;198:127–48.

5. Lombardo F, Ghani Y, Kafatos FC, Christophides GK. Comprehensive genetic dissection of the hemocyte immune response in the malaria mosquito Anopheles gambiae. PLoS Pathog. 2013;9(8):e1003145.

6. Lesch C, Goto A, Lindgren M, Bida G, Dushay MS, Theophilou P. A role for Hemolectin in coagulation and immunity in Drosophila melanogaster. Dev Comp Immunol. 2007;31(12):1255–63.

7. Gare DC, Pietney SB, Billingsley PF. Anopheles gambiae collagen IV genes: cloning, phylogeny and midgut expression associated with blood feeding and Plasmodium infection. Int J Parasitol. 2003;33(7):681–90.

8. Altincicek B, Vilcinskas A. Metamorphosis and collagen IV fragments stimulate innate immune response in the greater wax moth (Galleria mellonella) Dev Comp Immunol. 2006;30(12):1108–18.

9. Knor E, Schmidbauer H, Vilcinskas A, Altincicek B. MMPs regulate both development and immunity in the tribolium model insect. PLoS One. 2009;4(10):e7451.

10. Martins LA, Fogaca AC, Bijvokt AT, Carballar-Lejarazu R, Marinotti O, Cardoso AF. Culex quinquefasciatus storage proteins. PLoS One. 2013;8(10):e77664.

11. Lourenço AP, Martins JR, Bitondi MM, Simoes ZL. Trade-off between immune stimulation and expression of storage protein genes. Arch Insect Biochem Physiol. 2008;71(2):80–87.

12. Rono MK, Whitten MM, Oulad-Abdelghani M, Levashina EA, Marois E. The major yolk protein vitelligenin interferes with the anti-plasmodium response in the malaria mosquito Anopheles gambiae. PLoS Biol. 2010;8(7):e1000434.

13. Dimpopulos G, Christophides GK, Meister S, Schultz J, White KP, Barillas-Mury C, et al. Genome expression analysis of Anopheles gambiae responses to injury, bacterial challenge, and malaria infection. Proc Natl Acad Sci U S A. 2002;99(13):8814–9.

14. Waterhouse RM, Powellones M, Christophides GK. Sequence-structure-function relations of the mosquito leucine-rich repeat immune proteins. BMC Genomics. 2010;11:531.

15. Igai T, Kanda H, Yamamoto-Goto Y, Kanuka H, Kuranaga H, Aigaki T, et al. Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. Embo J. 2002;21(11):3009–18.

16. Smith RC, Eappen AG, Radcliffe AJ, Jacobs-Lorena M. Regulation of anti-Plasmodium immunity by a LITAF-like transcription factor in the malaria vector Anopheles gambiae. PLoS Pathog. 2012;8(10):e1002965.

17. Smith RC, Barillas-Mury C, Jacobs-Lorena M. Hemocyte differentiation mediates the mosquito late-phase immune response against Plasmodium in Anopheles gambiae. Proc Natl Acad Sci U S A. 2015;112(26):E3412–3420.

18. Irving F, Ubeda JM, Doucet D, Troxler L, Laguez M, Zachary D, et al. New insights into Drosophila larval haemocyte functions through genome-wide analysis. Cell Microbiol. 2005;7(3):335–50.

19. Warburg A, Stent A, Cohen N, Daham N, Lammin and a Plasmodium oocyst surface protein inhibit melanotic encapsulation of Sephadex beads in the hemocoel of mosquitoes. Microbes Infect. 2007;9(2):192–9.
20. Dong Y, Dimopoulos G. Anopheles fibrinogen-related proteins provide expanded pattern recognition capacity against bacteria and malaria parasites. J Biol Chem. 2009;284(15):9835–44.

21. Rund SS, Gentile JE, Duffield GE. Extensive circadian and light regulation of the transcriptome in the malaria mosquito Anopheles gambiae. BMC Genomics. 2013;14:218.

22. Cociancich SO, Park SS, Fidock DA, Shahabuddin M. Vesicular ATPase-overexpressing cells determine the distribution of malaria parasite oocysts on the midguts of mosquitoes. J Biol Chem. 1999;274(18):12650–5.

23. Barillas-Mury C. CLIP proteases and Plasmodium melanization in Anopheles gambiae. Trends Parasitol. 2007;23(7):297–9.

24. Stathopoulos S, Neafsey DE, Lawniczak MK, Muskavitch MA, Christophides GK. Genetic dissection of Anopheles gambiae gut epithelial responses to Serratia marcescens. PLoS Pathog. 2014;10(3):e1003897.

25. Povelones M, Bhagavatula L, Yassine H, Tan LA, Upton LM, Osta MA, et al. The CLIP-domain serine protease homolog SPCLIPI regulates complement recruitment to microbial surfaces in the malaria mosquito Anopheles gambiae. PLoS Pathog. 2013;9(9):e1003623.

26. Collins FH, Sakai RK, Vennix KD, Pakewitz S, Seeley DC, Miller LH, et al. Genetic selection of a Plasmodium-refractory strain of the malaria vector Anopheles gambiae. Science. 1986;234(4776):607–10.

27. Shiao SH, Whitten MM, Zachary D, Hoffmann JA, Levashina EA. Fz2 and cdc42 mediate melanization and actin polymerization but are dispensable for Plasmodium killing in the mosquito midgut. PLoS Pathog. 2006;2(12):e133.

28. Chambers MC, Schneider DS. Pioneering immunology: insect style. Curr Opin Immunol. 2012;24(1):10–4.