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Dennis Klug, Stephanie Blandin

► **To cite this version:**

Dennis Klug, Stephanie Blandin. Activation of complement-like antiparasitic responses in *Anopheles* mosquitoes. *Current Opinion in Microbiology*, 2023, 72, pp.102280. 10.1016/j.mib.2023.102280 . hal-04017092

HAL Id: hal-04017092

<https://hal.science/hal-04017092v1>

Submitted on 6 Mar 2023

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1 **Activation of complement-like antiparasitic responses in *Anopheles* mosquitoes**

2
3 Dennis Klug and Stephanie A. Blandin

4
5
6 Address

7
8 Université de Strasbourg, CNRS, Inserm, UPR9022/U1257, Mosquito Immune Responses
9 (MIR), F-67000, Strasbourg, France

10
11 Corresponding author: Stephanie A Blandin (sblandin@unistra.fr)

12 13 14 **Abstract**

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17 During their development in mosquitoes, malaria parasites undergo massive losses that are
18 in part due to a potent antiparasitic response mounted by the vector. The most efficient and
19 best characterized response relies on a complement-like system particularly effective against
20 parasites as they cross the mosquito midgut epithelium. While our vision of the molecular
21 and cellular events that lead to parasite elimination is still partial, our understanding of the
22 steps triggering complement activation at the surface of invading parasites has considerably
23 progressed, not only through the identification of novel contributing genes, but also with the
24 recent in-depth characterization of the different mosquito blood cell types, and the ability to
25 track them in live mosquitoes. Here, we propose a simple model based on the time of
26 invasion to explain how parasites may escape complement-like responses during midgut
27 infection.

28 29 30 **Glossary**

31
32
33 **Hemocytes:** Immune cells in insects. They circulate in the body cavity, bathed in the
34 hemolymph. Some of them are sessile, i.e. stay attached to tissues. In mosquitoes three
35 main types can be distinguished: prohemocytes, oenocytoids and granulocytes. The last type
36 is composed of several subtypes presenting specific transcriptomic signatures, including
37 larger cells called megacytes. They fulfill several essential functions such as phagocytosis
38 and melanisation.

39
40 **Hemolymph:** extracellular fluid in insects, analogous to vertebrate blood. Insect have an
41 open circulatory system with hemolymph bathing all tissues and kept in movement by a
42 heart. Beside hemocytes, hemolymph carries important soluble immune factors such as
43 antiparasitic TEP1, LRIM1 and APL1C, and antimicrobial peptides.

44
45 **Melanisation:** deposition of a dark pigment called melanin. Physiological process playing
46 important roles in insects, including wound healing, clot formation, encapsulation of invading
47 pathogens. It results from the activation of a proteolytic cascade involving prophenoloxidas
48 (produced by oenocytoids) that synthesize melanin.
49

Detection of parasite invasion in the mosquito gut

Plasmodium parasites causing human malaria are transmitted by mosquitoes of the genus *Anopheles*. *P. falciparum* is responsible for the most severe symptoms of the disease, and for most deaths. While feeding on a parasite carrier, mosquitoes ingest infected red blood cells, of which only gametocytes, the sexual forms of the parasite, are able to further develop in the vector. Gametocytes become activated in the blood bolus, and release gametes that proceed to mate, forming a zygote that becomes motile and is then called ookinete. Within 18 to 32h, the ookinete traverses the midgut epithelium (Figure 1) and undergoes meiosis. It then resides between the midgut epithelium and the basal lamina, forming a cyst where it goes through multiple rounds of replication for 8-14 days before cytokinesis occurs, giving rise to sporozoites. These egress in batches upon rupture of the oocyst membrane, resulting in the release of thousands of sporozoites in the mosquito open circulatory system. Part of these invade the salivary glands and can then be transmitted to a naive host upon a subsequent blood meal. Still, mosquitoes are not mere needles that transmit parasites, they mount potent immune responses that limit their development, especially during the first days after infection, when most parasite losses occur [1]. After a blood meal, midgut cells secrete a peritrophic matrix, an acellular semi-permeable layer of chitin polymers, to protect cells from direct contact with the blood bolus and proliferating bacteria during digestion. This protection is reinforced by the Duox/IMPer module (Dual oxidase / IMMune heme Peroxidase Hpx15) that catalyzes crosslinking of mucins in the ectoperitrophic space located between the peritrophic matrix and the midgut epithelium, further protecting midgut cells from contact with immune elicitors [2]. Hence, after forming inside the mosquito midgut, ookinetes first traverse the peritrophic matrix before crossing the epithelium (Figure 1). This breaching of mosquito intestinal barriers allows both parasites and bacterial elicitors to come in contact with the midgut cells, triggering a series of cellular and humoral reactions that ultimately lead to complement activation at the surface of ookinetes.

Nitric oxide synthase (NOS), whose expression is rapidly induced in cells that are traversed by ookinetes [3], produces nitric oxide (NO) that can diffuse in nearby tissues and possibly act as a signaling molecule. Locally, NO and derivatives (such as nitrites) can also react with H₂O₂ produced by the NADPH oxidase NOX5 and heme peroxidase Hpx2 whose expression is upregulated in infected midguts, leading to protein nitration at the site of parasite invasion [4] (Figure 2). This modification has been evidenced in some midgut cells that were traversed by parasites [5] and was proposed to affect the parasite surface, making it visible to the complement-like system [4]. However, whether the parasite surface itself is a target of nitration remains to be demonstrated experimentally. In addition, parasites traverse the midgut epithelium within minutes [6], often invading several cells in a row before exiting and resting below the basal lamina, which is not compatible with the time required for their nitration. Indeed, the upregulation of NOS, NOX5 and Hpx2 activity in invaded cells likely takes several hours, which is consistent with the observation that nitro-tyrosines are usually detected only in the first invaded cell of a series at 24h post infection [5] and suggests protein nitration occurs long after the parasite has traversed the midgut cells (Figure 1). Alternatively, nitration of midgut cells could be detected as a danger signal by patrolling **hemocytes** that would activate the complement system, or even represent a mark of extensive cellular damage with no specific role in the antiparasitic response. In the latter case, the fact that NOX5 and Hpx2 are essential for an efficient antiparasitic response [4] would indicate that they may contribute to it in a different manner. For instance, parasite infection or bacteria ingestion also trigger secretion of prostaglandins (PGs) by midgut cells, and silencing of Hpx7 or Hpx8 decreases PG release, suggesting these enzymes are involved in PG synthesis [7] (Figures 1, 2). It would be interesting to investigate the contribution of NOX5, Hpx2 and other induced Hpxs in PG synthesis. Among PGs, prostaglandin E2 seems to have the strongest effect on parasite infection: (1) it contributes to recruit **hemocytes** to the infected midgut, (2) it signals to fat body cells to produce a **hemocyte** differentiating factor (HDF) that is composed of a lipid, lipoxin A4, bound to Evokin, a proteic lipid carrier [6–8].

105 **Hemocytes, key actors of the TEP1-mediated response**

106
107 **Hemocytes** are immune cells of insects that circulate in the **hemolymph**, the equivalent of
108 vertebrate blood. Single cell transcriptome profiling has identified two main **hemocyte**
109 lineages in *Anopheles gambiae*, the oenocytoids expressing prophenoloxidasases that are
110 involved in the **melanisation** cascade, and several granulocyte subtypes including large
111 megacytes and expressing distinct sets of effector genes [9,10]. Both lineages are likely
112 derived from prohemocyte precursors [10].

113
114 Upon infection, midgut cells increase their secretion of PGE2, a strong and rapid attractant to
115 hemocytes that also enhances their mobility. Impressive *in vivo* images of granulocytes
116 patrolling around the site of infection have been obtained [6]. Importantly, some granulocytes
117 attracted to the parasite- or the bacteria-infected midgut, undergo apoptosis and release
118 **hemocyte**-derived microvesicles (HdMv) that are strongly associated with parasite-infected
119 midgut cells [6,11] (Figure 1). Uptake of synthetic beads by **hemocytes** reduces the number
120 of midgut-associated HdMvs, but also the labeling of parasites by the key antiparasitic
121 effector ThioEster-containing Protein 1 (TEP1) (see below) [11], the later phenotype being
122 also observed upon hemocyte depletion by clodronate liposomes [12]. As **hemocytes** do not
123 produce large quantities of TEP1 [13], it is tempting to speculate HdMvs may instead deposit
124 factors that are essential for TEP1 activation on the microbial surface. It would be interesting
125 to determine the exact content of HdMvs by mass spectrometry.

126
127 At later time points, the PGE2-induced and fat-body derived HDF triggers major changes in
128 circulating **hemocytes**, notably their proliferation and an increase in the proportion of
129 circulating oenocytoids and granulocytes [8] (Figure 1). These changes likely occur too late
130 to have an effect on parasite development during a primary infection, but they are long-
131 lasting and provide extended protection to mosquitoes during subsequent infections or upon
132 **hemolymph** transfer from previously infected mosquitoes, a phenomenon termed immune
133 priming [8].

134 135 **Parasite labeling and elimination mediated by complement-like protein TEP1**

136
137
138 The mosquito complement system is composed of soluble proteins that are mainly produced
139 by the fat body [13] and circulate in the **hemolymph**. Its central component is TEP1, a
140 structural and functional homologue of vertebrate complement factor C3 [14]. Like C3, TEP1
141 opsonizes the surface of invading microorganisms (bacteria, parasites, fungi), marking them
142 for lysis and/or melanotic encapsulation [1]. TEP1 circulates constitutively in the **hemolymph**
143 as a full-length and an activated, cleaved form. The protease(s) responsible for TEP1
144 cleavage are not known, however stability of the circulating cleaved form requires a complex
145 comprising two leucine-rich repeat proteins (LRRs), LRIM1 and APL1C. Deposition of TEP1
146 on foreign surfaces is mediated by a covalent thioester bond and must be tightly regulated,
147 as experimental depletion of LRIM1 or APL1 triggers uncontrolled deposition on self-tissues
148 [15]. According to current models [1], the accumulation of TEP1 on the ookinete surface is
149 initiated by precocious, rapid binding of small amounts of circulating cleaved TEP1. In a
150 second and slower step, TEP1 deposition is amplified by mobilizing and processing the pool
151 of circulating full length TEP1. This depends on the recruitment at the target surface of
152 SPCLIP1 (CLIPA30), a serine protease homolog that is not catalytically active. Therefore,
153 additional protease(s) are likely necessary to cleave further TEP1 molecules and expose
154 their thioester site, that can then react with nearby surfaces. The protein complex necessary
155 for this cascade of events, comprising some TEP1cut associated to (or freed from) LRRs;
156 SPCLIP1; and unknown proteases(s), is called TEP1 convertase complex. An attractive
157 hypothesis is that HdMvs deliver factors that potentiate, or take part in, this convertase.
158 Notably, genetic polymorphism in the *TEP1* locus contributes to determine parasite killing
159 efficiency, with the *TEP1**R allele being more efficient to kill *P. berghei* parasites [16].

160 Oocysts are relatively protected from TEP1 attack. Indeed, once formed, they undergo
161 limited losses despite their long development time: over 10 days, only few oocysts are
162 labeled with TEP1 and their elimination is TEP1-independent [17,18]. It is possible that
163 oocyst proteins such as Cap380 [19], but also the circumsporozoite protein CSP [20], act as
164 a shield, or as complement inhibitors, on the oocyst surface. In addition, the mosquito basal
165 lamina under which oocysts reside could also provide some protection from soluble immune
166 factors. Indeed, in the case of *P. berghei* but not *P. falciparum*, a subsequent blood meal 4
167 days after infection stretches the midgut basal lamina and makes it more permeable to
168 **hemolymph** factors, leading to increased TEP1 labeling and elimination of oocysts [21].
169 These results suggest that *P. falciparum* oocysts are further protected from TEP1 activation.
170 Finally, despite their release in the **hemolymph** with no apparent protection, sporozoites are
171 not labeled with TEP1, possibly because they are likewise shielded by CSP, a protein that
172 also protects them from melanisation [22].
173
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175 **Parasite success in crossing the midgut epithelium: a question of time?**

177 *Plasmodium* and *Anopheles* are engaged in a co-evolution arm race to circumvent each
178 other's defense mechanisms. We propose a simple model, derived from the "time bomb
179 theory" [3], where invasion speed rather than inhibition of mosquito immunity during midgut
180 traversal, may be key to *Plasmodium* success. This model predicts that the mosquito
181 immune system is activated by an infectious blood meal, however with a certain delay,
182 offering ookinetes a short window of time to invade and transform into protected oocysts.
183 Published data can be explained under this light. (i) P47, an ookinete surface protein, exists
184 as different alleles in *P. falciparum* that determine escape of the mosquito complement
185 system depending on the presence of a matching Pfs47 receptor, P47rec, on mosquito
186 midgut cells, this receptor being itself variable among *Anopheles* species [23–25]. Pfs47 is
187 thought to suppress activation of the JNK defense pathway, resulting in increased nitration
188 and parasite recognition by TEP1. However, artificially boosting JNK signaling by silencing a
189 negative regulator has no effect on *P. falciparum* [26]. An attractive alternative explanation is
190 that the Pfs47 / P47rec physical interaction may simply promote entry and traversal of the
191 parasite, speeding up the process. In agreement with this, P47rec aggregates at the site of
192 parasite entry [25], and Pfs47 belongs to the family of 6-cystein domain proteins comprising
193 notably the surface protein P36 expressed by sporozoites and mediating interactions with
194 CD81 and SR-BI, two liver cell receptors used by sporozoites to penetrate hepatocyte [27].
195 We propose that if Pfs47 does not match its receptor, or if one of the two is absent or
196 expressed at low levels, traversal would be slower using alternative receptors, rendering
197 ookinetes more vulnerable to the TEP1-dependent response, especially to the very efficient
198 *TEP1*^R* allele. Of note, the increased JNK activation and nitration observed upon infection
199 with *Pfs47-KO* parasites [26] may be a consequence rather than a cause of the increased
200 parasite killing, or a consequence of prolonged midgut invasion. (ii) An alternative receptor
201 could be the PIMMS43 ookinete surface protein whose depletion also sensitizes parasites to
202 elimination by the mosquito complement system [28]. (iii) The different temperatures at which
203 infections with *P. falciparum* and *P. berghei* are taking place (26 vs. 21°C, respectively), and
204 possibly less efficient Pfs47 and P47Rec combinations (not co-evolved), could also lead to
205 slower midgut traversal by *P. berghei* and explain why *P. falciparum* shows little sensitivity to
206 the complement pathway in contrast to *P. berghei*. Consistently with this, decreasing
207 temperature from 27 to 19°C for *P. falciparum* infection elevates ookinetes susceptibility to
208 **melanisation** in *CTL4*-knockout mosquitoes [29]. In addition, temperature differences likely
209 also affect midgut microbiota and mosquito immune reactions. (iv) Transgenic expression in
210 mosquito midgut cells of the Magainin peptide, decreasing mitochondrial activity and
211 therefore ATP production on which ookinete movement crucially depends, decreases oocyst
212 formation [30]. The two latter observations are consistent with increased vulnerability of
213 parasites to mosquito defense mechanisms when their speed is decreased, either by a drop
214 in temperature or by synthetic depletion of energy. In addition, delays in ookinete to oocyst

215 transformation are detrimental for oocyst development after both Magainin expression and
216 PIMMS43 knockout, suggesting that slow ookinete traversal could also result in long-lasting
217 parasite growth defects.

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220

221 **Conclusion**

222

223 Here we sought to propose an alternative, simple model to explain how parasites may
224 escape mosquito immune responses upon midgut invasion, which does not necessarily
225 exclude other models positing that parasites actively dampen the antiparasitic response. In
226 our view, as in an earlier model called the time bomb theory, the speed of parasite invasion
227 is crucial for parasites to escape immune attack by mosquito soluble factors. However, rather
228 than escaping nitration during midgut cell traversal, speed would allow parasites to timely
229 reach the basal side and transform into oocysts, before hemocytes are activated and
230 stimulate parasite labeling with TEP1 which promotes their subsequent elimination.
231 Temperature during the invasion process may act as a first important determinant of parasite
232 successful escape, with faster and more successful invasion at higher temperature.
233 Receptors on the parasite surface, such as Pfs47 and PIMMS43, if efficiently interacting with
234 their mosquito partner (a process dependent on genetic diversity in both organisms) would
235 also speed up the invasion process and promote maximal ookinete survival. Live imaging of
236 midgut invasion with *P. falciparum* at different temperatures to visualize and count ookinetes
237 that have crossed the epithelium as a function of time, ideally *in vivo* and concomitant with
238 the tracking of fluorescence-expressing hemocytes, would shed more light on these complex
239 parasite-vector interactions. For this, genetic modifications that decrease cuticle
240 pigmentation, combined with the expression of fluorescent reporters in specific cell lineages
241 or tissues, will be key to optimize visualization of mosquito/parasite interactions at the cellular
242 level [31]. Should nitration of invaded midgut cells have no specific role in the antiparasitic
243 response, other functions of NOX5 and Hpx2, e.g. in prostaglandin signaling and hemocyte
244 activation, should be investigated. Finally, delaying midgut invasion by ookinetes may be
245 considered as a promising transmission blocking strategy, for instance by interfering with
246 P47 [32,33] and PIMMS43 interactions with their respective receptors, ideally both at the
247 same time, through human vaccination against these receptors, or in genetically modified
248 mosquitoes producing single chain antibodies targeting them in the midgut lumen.

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251 **Conflict of interest statement**

252 We declare no conflict of interest.

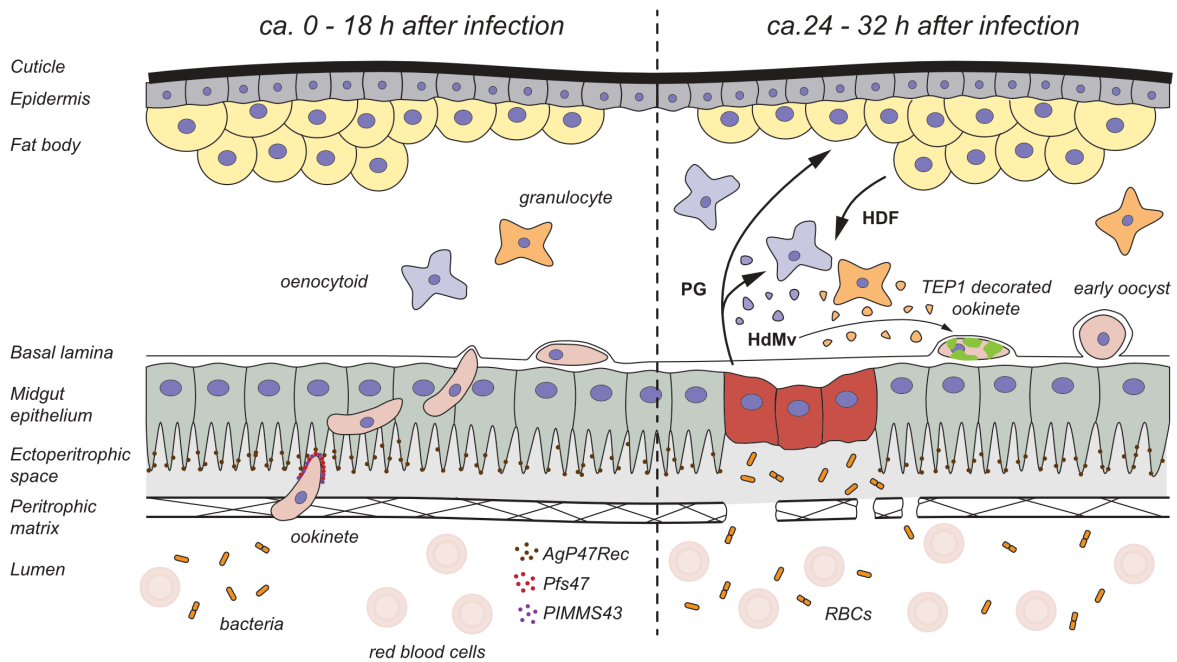
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255 **Acknowledgements**

256 The authors would like to thank Eric Marois for critical reading and editing of the manuscript,
257 and the Mosquito Immune Responses lab for fruitful discussions. They received funding from
258 the Laboratoire d'Excellence ParaFrap (ANR-11-LABX-0024 to SAB), the ERC Starting
259 Grant Malares (N°260918 to SAB), and from CNRS, Inserm, and the University of
260 Strasbourg. Additional funding was awarded to DK by the DFG as a postdoctoral fellowship
261 (KL 3251/1-1).

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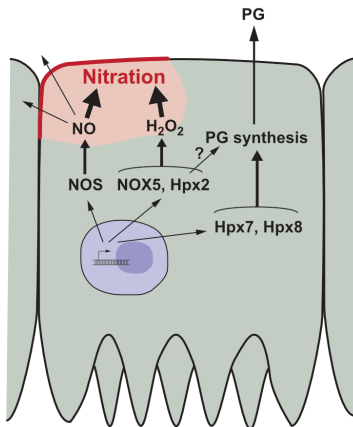
Figure 1



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Figure 1. How *Plasmodium* invasion speed could determine evasion from the TEP1-dependent antiparasitic response in *A. gambiae*. Within the first 18h post infection, ookinetes breach through the peritrophic matrix and come in contact with the midgut epithelium. Efficient entry into midgut cells is promoted by interactions of parasites receptors (e.g. P47, PIMMS43) with midgut cells apical surface proteins (e.g. AgP47Rec). By 24-32 hpi, bacteria and parasite elicitors contact midgut cells and trigger a massive immune response. The release of prostaglandins (PG) by invaded midgut cells recruits hemocytes to the site of infection and induces the secretion of a hemocyte differentiation factor (HDF) by fat body cells. At the site of infection, granulocytes release hemocyte derived microvesicles (HdMVs), that could promote TEP1 binding (green dots on parasite surface) to late ookinetes that have not yet reached the less sensitive oocyst stage. Some cells, damaged by parasite traversal, undergo massive nitration (indicated in red) and will be extruded towards the midgut lumen. At later time points, HDF is responsible for an increase in the number of circulating hemocytes, notably granulocytes and oenocytoids, which will be protective upon a subsequent infection.

Figure 2



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Figure 2. Processes induced in midgut cells damaged by parasite invasion. Ookinete invasion of a midgut cell induces the expression of several genes encoding: (1) the nitric oxide synthase (NOS) producing NO that can diffuse to nearby cells; (2) the NADPH oxidase NOX5 and heme peroxidase Hpx2, producing H₂O₂. Reaction of NO with H₂O₂ can lead to massive nitration. (3) Heme peroxidases Hpx7, Hpx8 are induced as well, contributing the synthesis of prostaglandins (PG). Whether Hpx2 is also involved in PG production remains to be tested.

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