Fz2 and Cdc42 Mediate Melanization and Actin Polymerization but Are Dispensable for Plasmodium Killing in the Mosquito Midgut

Shin-Hong Shiao, Miranda M. A. Whitten, Daniel Zachary, Jules A. Hoffmann, Elena A. Levashina

Institut de Biologie Moléculaire et Cellulaire, UPR 9022 du CNRS, Equipe “Avenir” INSERM, Strasbourg, France

The midgut epithelium of the mosquito malaria vector Anopheles is a hostile environment for Plasmodium, with most parasites succumbing to host defenses. This study addresses morphological and ultrastructural features associated with Plasmodium berghei ookinete invasion in Anopheles gambiae midguts to define the sites and possible mechanisms of parasite killing. We show by transmission electron microscopy and immunofluorescence that the majority of ookinetes are killed in the extracellular space. Dead or dying ookinetes are surrounded by a polymerized actin zone formed within the basal cytoplasm of adjacent host epithelial cells. In refractory strain mosquitoes, we found that formation of this zone is strongly linked to prophenoloxidase activation leading to melanization. Furthermore, we identify two factors controlling both phenomena: the transmembrane receptor frizzled-2 and the guanosine triphosphate–binding protein cell division cycle 42. However, the disruption of actin polymerization and melanization by double-stranded RNA inhibition did not affect ookinete survival. Our results separate the mechanisms of parasite killing from subsequent reactions manifested by actin polymerization and prophenoloxidase activation in the A. gambiae–P. berghei model. These latter processes are reminiscent of wound healing in other organisms, and we propose that they represent a form of wound-healing response directed towards a moribund ookinete, which is perceived as damaged tissue.

Introduction

Few infectious diseases carry heavier economic and social burdens than malaria. Within Anopheles mosquitoes, the only natural vectors of human malaria, immune responses to the Plasmodium parasite are highly species and strain specific. However, two universal bottlenecks stand out: parasites suffer very heavy losses as they traverse the epithelia of the midgut and as they journey from the midgut to the salivary glands. The attrition of ookinetes in the mosquito midgut can therefore serve as an excellent model for the study of host–parasite interactions.

The Plasmodium sporogonic cycle is initiated when gametocytes are ingested by the mosquito during a blood meal. Gametocytes swiftly give rise to gametes, which fertilize in the midgut lumen. The zygotes thus formed develop into motile, banana-shaped ookinetes, which invade and traverse the midgut epithelium approximately 24 h after blood ingestion (depending on the host–parasite species combination). During this trip the majority of ookinetes are destroyed by host responses even in susceptible mosquito strains, but a few surviving ookinetes reach the basal side of the midgut and transform into oocysts, which mature over the next 10–12 d to release thousands of sporozoites into the mosquito hemocoel. Sporozoites then journey through the hemolymph (blood) and invade the salivary glands. Successful salivary gland sporozoites are finally transmitted to a new vertebrate host via an infective bite.

In general terms, mosquitoes are able to mount efficient cell-mediated and humoral immune responses, and are equipped with an array of antimicrobial molecules, coagulation factors, opsonins, and recognition factors (for reviews, see [1,2]). Although the mechanisms of anti-Plasmodium defense are still poorly understood, the characterization of the Anopheles gambiae genome [3] has provided powerful new tools for functional analysis. Recent research has highlighted a role for a complement-related thioester-containing protein 1 (TEP1) in ookinete lysis and subsequent melanization [4]. However, the exact mode of TEP1-mediated killing is yet to be determined and probably involves further downstream factors. An additional molecule shown to be induced by Plasmodium infection is leucine-rich repeat immune protein 1 [5], whose silencing leads to a phenotype similar to that of TEP1. Depletion of the hemolymph-derived C-type lectin 4

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Abbreviations: AZ, organelle-free actin zone; Cdc42, cell division cycle 42; dpi, days post-infection; dsRNA, double-stranded RNA; Fz2, frizzled-2; GFP, green fluorescent protein; hpi, hours post-infection; PPO, prophenoloxidase; SRPN, serine protease inhibitor; RNAi, RNA interference; TEM, transmission electron microscopy; TEP1, thioester-containing protein 1

* To whom correspondence should be addressed. E-mail: e.levashina@ibmc.u-strasbg.fr

† These authors contributed equally to this work.

‡ Current address: Department of Entomology, University of California Riverside, Riverside, California, United States of America
Synopsis

A dangerous journey awaits malaria \textit{Plasmodium} parasites ingested by a mosquito. Most parasites are destroyed by host responses in the midgut, and in parasite-resistant refractory strains of mosquito the mortality can reach 100%. This midgut “bottleneck” represents an appealing target for reducing malaria transmission by the genetic control of wild mosquitoes. However, the killing mechanisms are still unclear. In this study, electron microscopical analyses followed the entire midgut invasion process in mosquitoes to identify the major site(s) and ultrastructural features of \textit{Plasmodium} killing. The authors found that invasion can be divided into two steps: a swift passage through a midgut cell, followed by establishment of the parasite in the basal extracellular space, where it becomes an important target for destruction by soluble immunity factors. In refractory mosquito parasites, dead parasites are associated with the formation of organelle-free zones of actin in adjacent midgut cells, and melanin deposition on the parasite surface. The authors identify two genes, called \textit{frizzled-2} and \textit{cell division cycle 42}, that control these phenomena. Actin zone formation and melanization are generally thought to be killing mechanisms; however, the authors show by gene silencing that neither is lethal to \textit{Plasmodium}. Instead, these mechanisms may represent a form of mosquito wound-healing response that is triggered by the presence of a moribund parasite.

Early studies have reported organelle-free cellular zones formed by midgut epithelial cells at their point of contact with invading ookinetes. This event was first described by Vernick et al. [14] in \textit{P. gallinaceum} infections of a refractory \textit{A. gambiae} strain, and has very occasionally been seen in a fully susceptible strain, Fam5. Vernick et al. observed a finely fibrillar zone protruding from the epithelial cell basal labyrinth that encircled dead ookinetes. These organelle-free, actin-rich cytoplasmic formations, for which we propose the term “organelle-free actin zone” (AZ), have since been noted by other authors investigating different host–parasite species combinations (e.g., the “purse-string” [15,16]; the “lamellipodial hood” or “collar” [17]; and the “actin cone” or “ring” [18]). AZ-like structures are generally assumed to be involved in parasite killing or a consequence of an intracellular parasite exiting a damaged midgut cell, but a functional role in parasite killing has not been formally demonstrated.

We are interested in determining the predominant mode of ookinete killing in the \textit{A. gambiae–P. berghei} infection model, and in redressing a paucity of electron microscope studies in this host–parasite species combination. Here, we aim to identify the precise location of dead ookinetes and to estimate the relative numbers of ookinetes that die in each compartment. In so doing, we hope to establish whether parasites are killed via exposure to soluble hemocyte-derived immunity factors in the extracellular milieu, or by midgut cell-mediated killing responses, or a combination of both. There is also a very clear need to resolve the functional significance of the AZ in terms of ookinete killing and its relationship with melanization. To address these points, we have used transmission electron microscopy (TEM) and confocal immunofluorescence microscopy in combination with RNA interference (RNAi)–dependent gene silencing, and have investigated AZ/ookinete co-localization, the composition of the AZ, its control by TEP1 or proteins involved in cytoskeletal rearrangement, and its potential role in parasite killing.

We show that the majority of ookinetes are killed by lysis in the extracellular space, rather than succumbing to intracellular killing. Moreover, in the refractory strain, we noted that the majority of dead and dying ookinetes were surrounded by melanin and a zone of polymerized actin within the adjacent midgut cells (the AZ). We show that both features are linked and dependent on TEP1 activity. We identified two genes controlling actin polymerization and melanization, \textit{frizzled-2} and \textit{cell division cycle 42} (Cdc42). Silencing of both genes by RNAi did not affect parasite survival, demonstrating that AZ formation and melanization do not constitute the ookinete killing mechanism. Actin polymerization and PPO activation are both processes reminiscent of wound healing in other organisms (e.g., [19,20]). We propose that \textit{Plasmodium} infection induces a wound-healing response in \textit{A. gambiae}, which includes actin polymerization and PPO activation. However, these responses do not account for major parasite losses during invasion of the midgut.

Results

Ookinete Killing Is Predominantly Extracellular

Two major mechanisms have been described to account for ookinete killing in the mosquito midgut epithelium: the first involves destruction of intracellular parasites in apoptotic

and of serine protease inhibitor (SRPN) 2 provokes spontaneous melanization of ookinetes [5] in non-melanizing mosquito strains. This suggests that these molecules control the early stages of prophenoloxidase (PPO) activation, which leads to ookinete melanization [6,7].

Genetic variability within a single mosquito species leads to substantial differences in individuals’ permissiveness towards malaria parasites, and this has allowed laboratory selection and study of highly refractory mosquito strains (discussed by [8,9]). From \textit{A. gambiae}, the most important malaria vector of sub-Saharan Africa, the L3–5 refractory strain has been selected. In this strain, ookinete development is completely terminated and accompanied by subsequent melanization of the parasite [10]. The parental G3 strain, from which L3–5 was derived, is considered “susceptible” since approximately 20% of invading ookinetes survive to the oocyst stage in the absence of melanization. Knockdown of \textit{TEP1} or \textit{leucine-rich repeat immune protein 1} by double-stranded RNA (dsRNA) completely abolishes melanotic refractoriness in the refractory L3–5 strain [4,5] and leads to hyper-parasitization in the susceptible G3 strain. All of the above immune factors are soluble and secreted by the hemocytes (blood cells) in the hemocoel, and as there is no direct contact between the hemocytes and the ookinetes, these factors have to pass through the midgut basal lamina to interact with the parasites located in the basal labyrinth [4] (reviewed by [11]).

Anti-\textit{Plasmodium} responses may also be mediated by midgut factors. SRPN6 is produced by midgut epithelial cells in response to malaria infection [12]. The dsRNA knockdown of \textit{SRPN6} results in a complex phenotype: it temporarily slows down ookinete killing in L3–5 mosquitoes, whilst simultaneously enhancing melanization [12]. Furthermore, the transcriptional responses of the \textit{A. gambiae} midgut epithelium to \textit{Plasmodium berghei} infection involve extensive regulation of genes controlling actin and microtubule cytoskeleton remodeling, of which some appear to modestly impair ookinete survival [13].

Mosquito Midgut Responses to \textit{Plasmodium}
cells of the infected midgut (the “time bomb” model [15,16,21–25]); in the second, ookinetes are proposed to be lysed in intercellular or extracellular spaces by soluble factors secreted from hemocytes [4,5]. To gain a better understanding of the killing mechanism in the *A. gambiae–P. berghei* model, we first identified the compartments occupied by parasites during traversal of the epithelium, and most importantly the location(s) in which ookinetes are killed. We examined ultra-thin midgut sections by TEM from both susceptible (G3) and refractory (L3–5) mosquito strains between 18 and 48 hours post-infection (hpi). An important overall observation was that the great majority (at least 80%)
of ookinetes, particularly dead ones, were located outside the midgut epithelial cells in the intercellular or extracellular spaces of the epithelium at the level of the basal labyrinth (Figures 1 and 2).

The major features of successful ookinete invasions are represented in Figure 1, and were very similar between susceptible and refractory insects. Ookinete invasion of the midgut epithelium occurred between 18 and 33 hpi and involved a complex route, beginning at the apical (microvillous) surface and ending at the membranous labyrinth beneath the basal lamina (hemocoel side) where the oocyst developed. Ookinetes were found in intra-, inter-, and extracellular locations within the midgut epithelium (not necessarily representing consecutive steps), but predominantly in the extracellular compartment. We observed that in this infection system, the invasion of the midgut was often complete before full polymerization of the peritrophic matrix. Of note, peritrophic matrix development is significantly retarded at the relatively low temperature (20.5 °C) necessary for P. berghei development [26].

Only 11% of the ookinetes in susceptible mosquitoes (15 out of 137) and none (out of 93) in refractory mosquitoes were judged to be intracellular (Figure 1F), indicating either that this phase is extremely transient or that only a minority of ookinetes invade the cytoplasm of midgut cells. Intracellular parasites are characterized by the absence of the midgut epithelial membrane covering the double parasite pellicle membrane (Figure 1A), signifying that ookinetes are in direct contact with the cytoplasm of the epithelial cells. Approximately 80% of the intracellular ookinetes observed were located near the apical side of the invaded midgut cell, usually just beneath the microvilli and close to the junctional complex.

**Figure 2. A Filamentous AZ Surrounds Extracellular Ookinetes**

(A and B) A thin AZ (asterisks) forms from the cytoplasm of midgut cells to partially cover an ookinete in a susceptible (G3) mosquito. The ookinete is apparently healthy. Boxes: detail of the ookinete/midgut cell border showing the triple membrane indicative of an extracellular parasite. Note the proximity of the parasite to the basal lamina.

(C) A lysed ookinete in a susceptible mosquito surrounded by a thin AZ (asterisk). The parasite is extracellular (inset), and the membranes remain intact even though the organelles of the ookinete appear lysed.

(D) In refractory (L3–5) mosquitoes, a thicker AZ forms around dead or dying ookinetes, but not live parasites. The AZ is associated with melanin deposition. Melanization occurs in the extracellular space between the parasite and the midgut cell membrane (black arrow in detail box) and is thicker on the basal side closest to the hemocoel. Note that the midgut cells on either side of the ookinete are alive.

(E) A second melanized, dead ookinete associated with a thick AZ. The putative intercellular path of invasion is indicated by a trail of melanin that reacted with shed ookinete proteins (white arrowhead).

(F) High magnification of AZs from refractory and susceptible mosquitoes showing identical ultrastructural features. The zone is characterized by finely granular or filamentous material and is devoid of organelles (scale bar = 200 nm).

(G) The percentage of dead ookinetes associated with an AZ is higher in L3–5 mosquitoes than in the G3 strain.

(H) In both strains, the vast majority of dead parasites visible by TEM were extracellular and none appeared to be intracellular.

bl, basal lamina; la, basal labyrinth; mel, melanin; ook, ookinete; *, AZ.

Scale bars = 1 μm in main pictures and 0.1 μm in detail boxes, unless stated otherwise.

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Intracellular parasites represent ookinetes at the earliest stages of penetration of the midgut epithelium. The time-points of midgut dissections were sufficiently early to detect intracellular phases of invasion, because ookinetes were still observed in the midgut lumen approaching the microvilli (Figure 1F).

The vast majority of ookinetes in both mosquito strains were observed to be outside the midgut cells, but in extremely intimate contact with the midgut cell membranes (Figure 1C–1E), based on the observation of several sections. A triple membranous layer surrounding the ookinete is indicative of an inter- or extracellular location—that is, the outer membrane is contributed by the host midgut cell, and the inner two compose the pellicle membrane complex of the parasite. Importantly, *P. berghei* ookinetes were extracellular (55.5% in G3 and 71% in L3–5) or intercellular (23% in G3

Figure 3. Biochemical Composition and Characteristics of the AZ

Confocal sections and 3-D reconstructions of *P. berghei*-infected midgut tissues.

(A–D) Ookinetes in susceptible (G3) mosquitoes, fixed at 28 hpi and showing ookinetes without associated AZs (A and B) and with AZs (C). (A) Non-polymerized (globular) α-actin is detected on both live (GFP-expressing) and dead ookinetes (stained red for the killing factor TEP1). (B) Red phalloidin dye labels polymerized actin and heavily stains midgut muscle tissue. Live (GFP) parasites are stained blue with monoclonal antibody against β-tubulin. (C) A rare example of an AZ associated with a live (GFP) ookinete in a susceptible mosquito. The AZ is labeled red with phalloidin and does not co-localize with the blue β-tubulin signal, nor does its distribution pattern follow that of α-actin (A). The shape of the ookinete is indicated by a white outline. Note the parasite shape constriction at the AZ. (D) Cartoon of a typical ookinete indicating the arrangement of major cytoskeletal elements and organelles.

(E–I) Dead ookinetes in refractory (L3–5) mosquitoes, fixed at 28 hpi. DIC, differential interface contrast. (E) A red polymerized actin “halo” forms around a melanized ookinete that is stained in blue for the TEP1 killing factor. The ookinete shape is outlined in white. Note that the intensity of melanization is not uniform across the parasite but is concentrated at the same (basal) end as the halo. This concentration effect is seen more clearly by TEM ([F], yellow arrows), suggesting that the AZ can block melanin dispersal, which remains extracellular (see circle, bottom left). (G) The blue β-tubulin signal on a melanized ookinete does not co-localize with the red phalloidin-stained polymerized actin of the AZ (muscles are also phalloidin stained). (H) AZs containing polymerized actin form a range of shapes but do not uniformly cover the entire parasite, which may also display a range of shape constrictions. A long phalloidin-stained trail is seen behind this ookinete. Patterns of phalloidin staining are also affected by the viewing angle. (I) Dead ookinetes that are non-melanized are not covered by an AZ. Surrounding muscle is stained red with phalloidin, but the parasites (identified by dense clusters of hemozoin) remain unstained.

Scale bars = 5 μm unless otherwise stated.

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and 25% in L3–5) during the time of peak parasite death (24–33 hpi) [4].

Apoptotic and necrotic cells were occasionally observed in both uninfected (not shown) and infected (Figure 1G) midguts, and healthy adjacent midgut cells extended filamentous cytoplasmic regions towards the dying cells. These extensions were organelle free and resembled the crawling lamellipodia or actin aggregates (described by [17,18]) that have been implicated in repair of the midgut epithelium. These zones often exhibited extensive membrane ruffling. Intercellular and extracellular ookinetes were sometimes found near extruding apoptotic midgut cells (e.g., Figure 1G), which did not appear to contain intracellular ookinetes. These observations suggest that the time bomb mechanism is not the predominant mode of P. berghei killing in A. gambiae.

In contrast to the projections associated with apoptotic midgut cells, we frequently observed additional filamentous zones surrounding lysed and dying ookinetes. Therefore, subsequent analyses were focused on the features of these zones.

A Filamentous AZ Surrounds Extracellular Ookinetes

A striking feature of ookinete presence in the basal extracellular midgut space is the appearance of a finely granular or filamentous, membrane-surrounded zone that forms as an extension of midgut cells immediately adjacent to dead or dying ookinetes (Figure 2A–2F).

This zone, which we call the AZ, is an organelle-free region of the midgut epithelial cell cytoplasm and is contained within its plasma membrane. We observed the AZ at the basal side of both susceptible (G3) and refractory (L3–5) A. gambiae midguts infected with P. berghei, and noted that the zones originated from healthy looking cells. The fine structure of the AZ was identical in L3–5 and G3 mosquitoes (Figure 2F). However, there were important differences between the strains in terms of zone thickness, frequency, and association with dead ookinetes. Few ookinetes in G3 mosquitoes displayed an AZ, and when they did, the AZ was usually thin (<200 nm) and surrounded both live (Figure 2A and 2B) and dead, lysed (Figure 2C) ookinetes. However, melanization did not occur. In contrast, AZs were more frequently observed in L3–5 mosquito midguts, and formed exclusively around dead or dying ookinetes (Figure 2D and 2E). The zones were normally much thicker (>500 nm) and always associated with melanization in this strain. Approximately 63% of all dead ookinetes within L3–5 midguts were surrounded by an AZ, compared with 53% of the dead parasites in G3 midguts, as judged by TEM (Figure 2G). Additionally, all of the dead ookinetes visible in our TEM study occurred in the extracellular or intercellular spaces (Figure 2H). Where a parasite contacted several cells, they all contributed to the AZ (e.g., Figure 2E).

The AZ and intracellular ookinetes did not co-localize in our study. We found that the AZ occurs only in the most basal region of the midgut, where the parasite is almost invariably extracellular. The triple membrane arrangement around the parasite (including both the apical and basal regions of the ookinete) indicated that the AZ did not form as a parasite exited a damaged midgut cell, but rather surrounded an extracellular parasite. The AZ did not usually cover the entire ookinete surface, and its extent and location on the ookinete surface varied (see Figures 2 and 3).

The AZ Is Composed of Polymerized Actin

To investigate the composition of the AZ, which is formed by healthy midgut cells, we used confocal and fluorescence microscopy with antibodies and specific dyes that discriminate between various cytoskeletal proteins. The AZ was not stained by an antibody raised against non-polymerized or globular forms of α-actin, nor by anti-β-tubulin. Both antibodies nevertheless recognized ookinete proteins at sites corresponding to microtubule and cytoskeleton assembly (Figure 3). However, polymerized or filamentous actin, as detected by the dye phalloidin, specifically stained the AZ (and midgut muscle fibers) in L3–5 and G3 mosquitoes, and did not stain the parasites. In G3 mosquitoes, the phalloidin staining of the thin AZ was often much fainter than in the L3–5 strain.

Phalloidin-stained collar- or halo-shaped zones on melanized (dead) ookinetes in the L3–5 mosquitoes are shown in Figure 3E, 3G, and 3H. These images also demonstrate that the AZ is often associated with shape constrictions of the parasite and can limit the dispersal of melanin pigment so that it appears concentrated at one end of the parasite (Figure 3E and 3F). Ookinetes are known to be melanized first at their apical tips closest to the basal lamina, reflecting the...
hemocoelic origin of the PPO cascade precursors [6]. Typically, melanin was deposited in the confined spaces between the AZ membrane and the ookinete in the extracellular space (Figure 2D and 3F). In some rare cases, however, a triple layer was observed, consisting of first an AZ, then a melanin, and finally a second AZ layer (not shown). The association of the AZ with melanization and dead parasites led us to suspect that AZ formation could be a refractory killing mechanism, a hypothesis also suggested by [14,17]. We therefore focused on the prevalence of AZ formation associated with dead parasites in the refractory L3–5 strain, using the more quantitative method of phalloidin staining with fluorescence microscopy.

AZ Formation and Melanization Are Linked in Refractory Mosquitoes

Figure 4 details the relative proportions of live, dead, melanized, and AZ-associated ookinetes in susceptible and refractory mosquito midguts, as determined by fluorescence and confocal microscopy. Not surprisingly, the majority (84.9%) of ookinetes in refractory L3–5 mosquitoes were already dead by 28 hpi (Figure 4B), and by 48 hpi, killing was almost total (99.5%), compared with just 13.4% at 24 hpi and 26.5% at 48 hpi in susceptible G3 mosquitoes (Figure 4A). In the G3 strain, only a small minority of parasites were surrounded by a phalloidin-stained AZ (1.8% at 24 hpi and 5.8% at 48 hpi overall). In contrast, in the L3–5 strain a phalloidin-positive AZ surrounded 41.2% and 22.3% of ookinetes at 24 hpi and 48 hpi, respectively. The relative weakness of the phalloidin staining in G3 mosquitoes meant that fewer dead parasites could be detected in association with the AZ than were observed by TEM (see Figure 2G). Melanization was absent in G3 mosquito midguts, but it was the predominant feature associated with ookinetes at 24 hpi and 48 hpi, respectively. The relative lack of melanization means that fewer dead parasites could be detected in association with the AZ than were observed by TEM (see Figure 2G). Melanization was absent in G3 mosquito midguts, but it was the predominant feature associated with ookinetes at 24 hpi and 48 hpi, respectively. The relative weakness of the phalloidin staining in G3 mosquitoes meant that fewer dead parasites could be detected in association with the AZ than were observed by TEM (see Figure 2G). Melanization was absent in G3 mosquito midguts, but it was the predominant feature associated with ookinetes at 24 hpi and 48 hpi, respectively. The relative weakness of the phalloidin staining in G3 mosquitoes meant that fewer dead parasites could be detected in association with the AZ than were observed by TEM (see Figure 2G). Melanization was absent in G3 mosquito midguts, but it was the predominant feature associated with ookinetes at 24 hpi and 48 hpi, respectively. The relative weakness of the phalloidin staining in G3 mosquitoes meant that fewer dead parasites could be detected in association with the AZ than were observed by TEM (see Figure 2G). Melanization was absent in G3 mosquito midguts, but it was the predominant feature associated with ookinetes at 24 hpi and 48 hpi, respectively. The relative weakness of the phalloidin staining in G3 mosquitoes meant that fewer dead parasites could be detected in association with the AZ than were observed by TEM (see Figure 2G). Melanization was absent in G3 mosquito midguts, but it was the predominant feature associated with ookinetes at 24 hpi and 48 hpi, respectively. The relative weakness of the phalloidin staining in G3 mosquitoes meant that fewer dead parasites could be detected in association with the AZ than were observed by TEM (see Figure 2G).
Mosquito Midgut Responses to *Plasmodium*

**A 28 hpi**

|               | dsRNA knockdown, (n) |
|---------------|----------------------|
| lacZ         | (1016)               |
| TEP1         | (348)                |
| Fz2          | (1048)               |
| Cdc42        | (1096)               |

**B 48 hpi**

|               | dsRNA knockdown, (n) |
|---------------|----------------------|
| lacZ         | (1967)               |
| TEP1         | (1691)               |
| Fz2          | (2836)               |
| Cdc42        | (1496)               |

**oocyst survival**

|               | live oocysts / midgut |
|---------------|-----------------------|
| lacZ         | (32)                  |
| TEP1         | (28)                  |
| Fz2          | (30)                  |
| Cdc42        | (33)                  |

**ookinete melanization**

|               | melanized ookinetes / midgut |
|---------------|-------------------------------|
| lacZ         | (32)                          |
| TEP1         | (28)                          |
| Fz2          | (30)                          |
| Cdc42        | (33)                          |

**G knockdown efficiency**

| Gene | fold induction |
|------|---------------|
| TEP1 |               |
| Fz2  |               |
| Cdc42|               |
Figure 6. Knockdown of Fz2 and Cdc42 Disrupts AZ Formation and Melanization, but Does Not Prevent Ookinete Killing

Refractory mosquitoes were injected with dsFz2 and dsCdc42 and infected with *Plasmodium berghei*. The percentage of melanized ookinetes surrounded by the AZ at (A) 28 hpi and (B) 48 hpi was reduced by injection of dsFz2 and dsCdc42, compared with the dslacZ-injected control insects. Killing was also delayed compared with the lacZ controls, in which no live parasites remained by 48 hpi (B). Consequently, the number of dead parasites that exhibit neither melanization nor a surrounding AZ was increased by Fz2 and Cdc42 knockdown, while the total amount of melanized ookinetes was reduced. Parentheses indicate number of ookinetes counted. Mean values from three independent experiments. Bars = standard error of the mean.

Ookinite survival at 7 dpi was gauged by counting GFP oocysts. Experiments. Bars the total amount of melanized ookinetes was reduced. Parentheses indicate number of ookinetes counted. Mean values from three independent experiments. Bars = standard error of the mean. Parentheses indicate number of ookinetes counted. Mean values from three independent experiments. Bars = standard error of the mean.

To disrupt actin polymerization, we selected to knock down the genes *Fz2* and *Cdc42*, which both control cytoskeletal rearrangements. The aim was to impair AZ formation whilst keeping the activity of *TEP1* intact, and thereby examine the functional role of the AZ in ookinete killing. In a parallel microarray study by our group, we identified *Fz2* as a gene up-regulated with *TEP1* in *P. berghei*-infected *A. gambiae* midguts (S. Wyder, P. Irving, S. H. Shiao, L. Troxler et al., unpublished data). Whole-body *Fz2* expression is also induced by a non-infectious blood meal in *A. gambiae* (*A. gambiae* Gene Expression Profile, http://www.angaged.bio.uci.edu). *Fz2* encodes a seven-pass transmembrane receptor protein with described roles in *Drosophila* development, namely in establishing epithelial cell polarity during embryogenesis (reviewed by [27]). We also chose to study the Rho family guanosine triphosphate–binding cytoplasmic protein Cdc42, primarily for its published roles in actin polymerization and filopodium formation in other animals (e.g., [28]). Cdc42 was not regulated in our microarray analysis and shows only a minor up-regulation after a non-infectious blood meal (http://www.angaged.bio.uci.edu). The roles of these genes in adult insects are, however, not well defined.

Our studies focused on refractory L3–5 strain mosquitoes in which we could observe prominent phalloidin staining for polymerized filamentous actin under fluorescence microscopy. The effects of AZ disruption were first investigated at early time-points (28 and 48 hpi) to correlate with the peak times of ookinete killing and appearance of melanin and the AZ. We performed dsRNA knockdown of the *Fz2* and *Cdc42* genes 4 d prior to *P. berghei* infection and made differential counts in L3–5 mosquito midguts of the following: (i) ookinetes positive for green fluorescent protein (GFP) (live), (ii) ookinetes surrounded by a phalloidin signal (polymerized actin), (iii) ookinetes surrounded by melanin (dead), and (iv) unstained ookinetes (dead but not melanized). The efficiency of *TEP1*, *Fz2*, and *Cdc42* knockdown by dsRNA was confirmed by quantitative real-time PCR analysis at 18 and 24 hours post-injection. Corresponding gene induction was reduced to 0.58, 0.30, and 0.40 times the pre-injection level, respectively, while the control, dslacZ, had little effect (see Figure 6).

*Fz2* or *Cdc42* knockdown did not have significant effects on the normal mosquito midgut morphology, as examined by TEM (an example is given in Figure 7F). This shows that the cell-polarizing developmental phenotype of *Fz2* is not disrupted by RNAi in adult midgut cells. There was also no unusual die-off of infected dsFz or dsCdc2 knockdown mosquitoes compared with the dslacZ controls (unpublished data).

Knockdown of *Fz2* or *Cdc42* resulted in both a significant decrease in the percentage of parasites surrounded by phalloidin-positive material, and a corresponding increase in the percentage of surviving ookinetes at 28 hpi and 48 hpi compared with the dslacZ control mosquitoes (Figure 6A and...
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Discussion

In this study we examined the ultrastructure of P. berghei ookinete invasion of the A. gambiae midgut, and possible mechanisms of P. berghei killing by refractory mosquitoes. Our TEM microscope studies consistently demonstrated that the ookinetes are predominantly located in the extracellular space, where they are exposed to hemocyte-derived soluble phalloidin staining) than the controls. In addition, we observed that melanization of ookinetes was often aberrant or incomplete in the Fz2 and Cdc42 knockdown mosquitoes, resulting in semi-transparent capsules or thin crescents of melanin limited to a small area of the parasite surface (not shown).

These results indicated that Fz2 and Cdc42 are important for the assembly of the AZ and (by an unknown relationship) for melanization. We therefore next investigated whether perturbation of these events by the knockdown of Fz2 and Cdc42 would result in increased numbers of surviving oocysts.

Interfering with AZ Formation and Melanization Does Not Affect Ookinete Survival

We gauged ookinete survival in dsCdc42 and dsFz2 knockdown L3–5 mosquitoes at 7 days post-infection (dpi) by counting the total numbers of GFP-expressing oocysts per midgut. Melanized (dead) ookinetes were also quantified at this time-point.

The important result of this experiment is that the knockdown of either Fz2 or Cdc42 did not ultimately lead to a significant increase in ookinete survival in refractory mosquitoes, compared with the control dslacZ-injected mosquitoes (Figure 6C and 6D).

In the L3–5 strain, where ookinete killing is virtually 100%, significantly fewer ookinetes were melanized in the Fz2 and Cdc42 knockdown mosquito midguts compared with the lacZ controls (melanization was reduced by 47% and 66%, respectively), indicating that although Fz2 and Cdc42 are important for the mosquito melanization response to parasites, killing can still occur even when melanization is suppressed.

The individual knockdown phenotypes for Fz2 and Cdc42 were remarkably similar, suggesting that these molecules could be part of the same functional process. We next examined whether these phenotypes would be exacerbated in double knockdowns (Figure 6E and 6F). The double knockdown of Fz2/Cdc42 did not result in a significant increase in live oocysts, nor in a reduction in melanized ookinetes, compared with the single knockdowns. This could be interpreted as Fz2 and Cdc42 belonging to the same pathway, but it is also possible that the knockdowns were not sufficiently complete to allow us to detect a difference.

Finally, we tested the effects of Fz2 and Cdc42 dsRNA knockdown on ookinete survival at 7 dpi in susceptible mosquitoes. Once again, ookinete survival was not significantly increased by knockdown of either Fz2 or Cdc42 compared with that of the control dslacZ-injected mosquitoes (Figure 7).

The essential result is that the processes of AZ formation and melanization are both partially disrupted by the knockdown of Cdc42 and Fz2. However, this disruption does not translate into improved ookinete survival.

Figure 7. RNAi of Fz2 and Cdc42 Does Not Prevent Killing of Ookinetes in Susceptible (G3) Mosquitoes

Mosquitoes were injected with dsRNA and infected with P. berghei, and parasite survival at 7 dpi was gauged by counting live GFP-expressing oocysts (A). TEP1 knockdown significantly increased parasite survival compared with dslacZ-injected controls (A–C). DsFz2 and dsCdc42 knockdowns did not significantly affect parasite survival (A, D, and E). Included are photos of representative infected midguts for each phenotype (B–E), showing GFP-expressing oocysts ([B], inset). Combined results for four independent experiments are shown. Bar in graphs indicates mean value; sample sizes shown in parentheses. (F) Midguts from dsFz2 (shown) and dsCdc42 (not shown) knockdown G3 mosquitoes appear to have normal morphology, as shown by TEM. Scale bars = (B–E), 200 μm (10 μm in detail box); (F), 1 μm. doi:10.1371/journal.ppat.0020133.g007

6B). The Fz2 knockdown mosquitoes also contained a lower percentage of melanized parasites overall, and at 48 hpi both Fz2 and Cdc42 knockdown mosquitoes had significantly fewer melanized parasites (of which even fewer were associated with
immunity factors, rather than in intracellular locations. We hypothesize that intracellular invasion is minimized to a very transient phase, and is localized to the most apical part of a single midgut cell at the microvillus (luminal) side of the epithelium and close to the cell junction. Thereafter, the ookinete rapidly exits the cell from the basal side, or moves laterally towards the basal lamina through the narrow intercellular space. Ookinetes finally reside in an extracellular space of the membranous labyrinth, next to the basal lamina. Significantly, dead ookinetes are found consistently in this compartment, and we conclude that the major site of ookinete killing is the extracellular space, where killing is mediated by hemocyte-derived soluble factors. The dead ookinetes are surrounded by a zone of polymerized actin (the AZ), which is formed in adjacent host epithelial cells. We used a combination of confocal fluorescent microscopy and TEM with RNAi technology to demonstrate that formation of the AZ structure is triggered specifically by the presence of a P. berghei ookinete (a dead one in refractory mosquitoes) at the basal side of the midgut, and in the absence of apparent epithelial damage, as judged by TEM.

We further demonstrated that formation of the AZ is dependent on TEP1, Fz2, and Cdc42. We were interested in the possibility that formation of the AZ could be an ookinete killing mechanism in refractory mosquitoes. However, RNAi knockdown experiments, where we partially abolished the AZ and melanization, clearly established that in contrast to TEP1, Fz2 and Cdc42 do not induce killing of parasites. Our results separate the mechanisms of parasite killing from subsequent reactions manifested by AZ formation and melanization.

We propose that, in response to Plasmodium infection, the mosquito mounts a form of wound-healing response that is directed towards a dead or dying ookinete. Classical midgut tissue repairhealing processes and AZ formation probably respond to a common basic stimulus (i.e., abnormal or damaged tissue), but in the case of AZ formation, it is a moribund parasite that provides this signal. The AZ reaction is characterized by actin polymerization and PPO activation, which is characterized by melanization in refractory mosquitoes.

We have now identified a component of the AZ as polymerized actin and shown that the presence of the AZ and melanin is a predominant feature associated with dead ookinetes in refractory mosquitoes. In our A. gambiae–P. berghei infection model, the AZ is morphologically distinct from other zones of polymerized actin that occur throughout the midgut, which assist the expulsion of dead epithelial cells and repair the remaining tissue [15–18]. In contrast to this tissue repair process, the AZ is specifically provoked in the presence of a parasite, is only formed by the most basal part of the midgut cell, does not appear to facilitate cell expulsion, and is linked in an unknown way to melanization in refractory mosquitoes. All the midgut cells in contact with the ookinete contribute to the AZ, and all the cells appear to be healthy. Apoptosis and the extrusion of infected cells was also minimal in our study, suggesting that intracellular modes of ookinete killing are less prevalent in A. gambiae infected with P. berghei than in A. stephensi infected with P. berghei [15,16,21–25]. However, it would be inappropriate to make direct comparisons between analyses involving different host–parasite species combinations. In our study, ookinetes did not undergo phagocytosis in the midgut. Midgut cells are non-phagocytic; furthermore, our TEM observations of ookinete phagocytosis by cultured mosquito cells in vitro were very distinct structurally from anything seen by us in the midgut (S. Shiao, unpublished data).

In our study, dsRNA knockdown of Cdc42 and Fz2 decreased AZ formation in refractory mosquitoes. Unexpectedly, melanization was also impaired, in terms of both intensity and the total number of melanized parasites. This strongly suggests a link between the AZ and melanization. The exact relationship between the AZ and melanization is complex and may also involve other factors, because a thinner AZ can form around a small percentage of non-melanized ookinetes in susceptible mosquitoes. In some arthropods the upstream phenoloxidase component of the melanization cascade can aid wound healing by cross-linking clot fibers, possibly via transglutaminase activity [20,29,30]. Should this reaction occur in G3 mosquitoes, it would not result in easily detectable melanin, which is in contrast to the L3–5 strain, where production of melanin is overtly exaggerated and demonstrable with the tools used in our study. It would be interesting in this respect to determine whether, in susceptible G3 strain mosquitoes, the strength of AZ formation could be increased by the knockdowns of C-type lectin 4, SRPN2, or SRPN6, which lead to massive spontaneous melanization reactions [5,12,31]. Furthermore, these knockdowns and double knockdowns of Fz2/SRPN6 and Cdc42/SRPN6 in L3–5 mosquitoes would provide more insight into the importance of melanization in AZ formation.

The role of Cdc42 in AZ actin polymerization in L3–5 mosquitoes was not unexpected, because in other animals this cytoplasmic Rho family guanosine triphosphate–binding protein controls a wide array of cellular processes, including filopodium formation (via activation of Wiskott–Aldrich syndrome protein, profilin, the Arp2/3 complex, and finally, actin nucleation; reviewed by [28,32,33]). To our knowledge, however, this is the first study to implicate Fz2 in the control of actin polymerization in adult insects, and Fz2 and Cdc42 in the melanization response. Frizzled proteins are seven-pass transmembrane cell surface receptors that mediate the Wnt protein signaling necessary for development and establishing cell polarity in several embryonic tissues in Drosophila, including the midgut epithelium and the developing primordium of the adult wing (e.g., [27,34–36]). Many of these processes require coordination or remodeling of the cytoskeleton, especially at the apical side of polarized cells.

The double knockdown of Fz2/Cdc42 did not significantly alter the single knockdown phenotype, implying that both molecules could be part of the same biochemical process, possibly with Fz2 acting as a transmembrane receptor upstream of the cytoplasmic Cdc42. Alternatively, the knockdowns were not sufficiently complete to allow us to detect a difference in the phenotype. The current data do not allow us to distinguish between these two possibilities. Clearly, the parasites did not utilize host actin polymerization or Cdc42 activities to achieve intracellular invasion, a mode of invasion recruited by certain bacteria (e.g., Shigella and Listeria [37–39]). The AZ was never associated with intracellular ookinetes, and we did not see unusually greater numbers of intracellular parasites in Cdc42 or Fz2 knockdown midguts (unpublished data). We do not exclude at this stage an alternative role of Fz2 and Cdc42 in controlling hemocyte cytoskeletal rearrange-
Materials and Methods

TEM. TEM ultrastructural studies were conducted to analyze (i) P. berghei invasion and compartmentalization, (ii) formation of the AZ and melanization in control and dsRNA knockedown mosquitoes, and (iii) the morphology of F2 and Cde-42 knockdown midguts.

Midguts were dissected in 0.1 M phosphate buffer at 18, 21, 22, 23, 24, 29, 29* (2, 3, 4, 5, and 6 dpi), and fixed with 4% glutaraldehyde for 6 h. The midguts were post-fixed in 1% osmium tetroxide, rinsed, dehydrated through a graded ethanol series, and embedded in araldite resin. A series of semi-thin sections were screened by toluidine blue staining, and several ultra-thin sections per sample were transferred to copper grids and stained with uranyl acetate and lead citrate. Sections were observed at 60 kV on a Hitachi (http://www.hitachi.com) 7500 transmission electron microscope.

In total, 137 individual oocytes in G3 mosquitoes and 93 in L3–5 mosquitoes were screened by TEM.

dsRNA injection and dsRNA infections. An F2 Xh1- EcoRI 450-bp-long fragment and a Cde-42 Xhol- Smal 500-bp-long fragment were cloned from the Gateway system library (Invitrogen, http://www.invitrogen.com) clones 33 DG01 (ENSANGT00000023104) and 20A06 (ENSANGT00000022573), respectively, into the pH.L10 vector. Plasmids pH.L10, pH.L17 (dsTEP1), and pH.L100 (dsLacZ), and the synthesis of dsRNAs, were constructed as previously described [4, 40].

A. gambiae–susceptible G3 and refractory L3–5 strains were maintained at 28 °C, 75–80% humidity, and a 12/12 h light/dark cycle. Female 2-d-emerged adult mosquitoes from the same cohort were injected with 0.2 μg of dsRNA using a Nanoinject II injector (Drummond, http://www.drummonddsci.com). Co-injection experiments were performed by injecting a double volume of 1:1 mixtures of 3-pg/ml solutions of dsRNAs.

Mosquitoes were dissected 4 d after dsRNA injection. In each infection experiment, mosquitoes were fed on an anesthetized ICR mouse that had been infected with P. berghei GFPCON 259/c12 clone 16 [41]. The parasitemia in mice was assayed using Diff-Quik I (Dade Behring, http://www.dadebehring.com)–stained blood smears for the proportion of infected red blood cells and differentiated gamocytes, and gametocytemia was established by FACs analysis of 10,000 infected red blood cells.

RNAi efficiency was assessed by quantitative real-time PCR. Total RNA from ten mosquitoes was extracted with Trizol reagent (Invitrogen) from head–emerged adult mosquitoes from the same cohort, and reverse transcribed using M-MLV enzyme with random primers (Invitrogen). Specific primers were designed using PrimerSelect (DNAStar, http://transcriptome.com). RNAi efficiency was assessed by quantitative real-time PCR. Total RNA from ten mosquitoes was extracted with Trizol reagent (Invitrogen) from head–emerged adult mosquitoes from the same cohort, and reverse transcribed using M-MLV enzyme with random primers (Invitrogen). Specific primers were designed using PrimerSelect (DNAStar, http://transcriptome.com).

Candidate gene selection by microarray analysis. Briefly, midguts taken at 24 and 48 hpi from P. berghei-infected transgenic TEP1 gain-of-function (7a), wild-type (G3), and TEP1 loss-of-function (7b) A. gambiae mosquitoes were subjected to Affymetrix chip microarray analysis (http://www.affymetrix.com). Arrays were screened for TEP1–coregulated candidate genes (7a > G3 > 7b) by a factor of at least 2-fold at 24 hpi using the dCHIP filter analysis (Genespring/Agilent Technologies, http://www.affymetrix.com).

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Author contributions. SHS and EAL conceived and designed the experiments. SHS, MMAW, and DZ analyzed the data. DZ and EAL contributed reagents/materials/analysis tools. MMAW, and DZ analyzed the data. SHS, MMAW, and DZ analyzed the data. DJH and EAL wrote the paper.

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