Targeting molecular interactions essential for \textit{Plasmodium} sexual reproduction

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Summary

Malaria remains one of the most devastating infectious diseases, killing up to a million people every year. Whereas much progress has been made in understanding the life cycle of the parasite in the human host and in the mosquito vector, significant gaps of knowledge remain. Fertilization of malaria parasites, a process that takes place in the lumen of the mosquito midgut, is poorly understood and the molecular interactions (receptor–ligand) required for \textit{Plasmodium} fertilization remain elusive. By use of a phage display library, we identified FG1 (Female Gamete peptide 1), a peptide that binds specifically to the surface of female \textit{Plasmodium berghei} gametes. Importantly, FG1 but not a scrambled version of the peptide, strongly reduces \textit{P. berghei} oocyst formation by interfering with fertilization. In addition, FG1 also inhibits \textit{P. falciparum} oocyst formation suggesting that the peptide binds to a molecule on the surface of the female gamete whose structure is conserved. Identification of the molecular interactions disrupted by the FG1 peptide may lead to the development of novel malaria transmission-blocking strategies.

Introduction

Malaria, caused by parasites of the genus \textit{Plasmodium}, is among the most devastating parasitic diseases in the world with an estimated loss of up to 1 million lives every year (Murray et al., 2012; WHO, 2013). During its life cycle, the parasite alternates between the human host and the anopheline mosquito vector. \textit{Plasmodium} sexual reproduction is an essential step of the parasite’s life cycle and takes place in the lumen of the mosquito midgut (Smith et al., 2014). \textit{Plasmodium} gametocytes are ingested by the mosquito when it feeds on blood of an infected individual. Soon thereafter, the gametocytes round-up (in the case of \textit{P. falciparum}), egress from the red blood cell and convert into gametes. Male gametes actively search for female gametes to attach, fuse and complete fertilization generating a zygote.

\textit{Plasmodium} fertilization is a promising target for the development of transmission-blocking vaccines (TBVs). Three members of the 6-cysteine protein family: P47, P48/45 and P230, appear to be important for gamete attachment and the latter two are currently being tested as TBV candidates (van Dijk et al., 2001; 2010; Eksi et al., 2006; Tachibana et al., 2011). Antibodies against these proteins inhibit zygote formation, thereby reducing parasite transmission (Vermeulen et al., 1985; Naotunne et al., 1990; Targett et al., 1990; Williamson et al., 1995; Roefen et al., 1996; Healer et al., 1997; Ouchtourov et al., 2008). Disruption of the corresponding genes resulted in a substantial reduction of gamete fertilization; however, 100% inhibition was never reached (van Dijk et al., 2001; 2010; Eksi et al., 2006). Moreover, complete block of fertilization was not achieved even after the simultaneous disruption of P47 (important for female gamete fertility) and P48/45 (important for male gamete fertility; van Dijk et al., 2010). These findings suggest that gamete adhesion is a multi-genic process. This is not surprising since a similar phenomenon has been reported for gamete attachment in several organisms (Nixon et al., 2007; Gadella, 2008; Hirohashi et al., 2008). Another protein, known as hapless 2 (HAP2)/generative cell specific 1 (GCS1), was shown to be essential for gamete fusion (Liu et al., 2008; Mori et al., 2010). Male gametes of a HAP2 knockout \textit{Plasmodium berghei} line are able to attach to females but fail to fuse and complete fertilization.
(Liu et al., 2008). HAP2 is also considered a TBV candidate; however, the molecular mechanism that results in gamete fusion is still unknown. The rationale for TBVs is that transmission-blocking antibodies will interfere with molecular interactions essential for parasite development in the mosquito. However, to date, no molecular interacting partners (receptor–ligand) have been identified for any of the above mentioned TBV candidates or any other Plasmodium gamete surface protein required for fertilization. This much-needed information would further our understanding of the biology of Plasmodium fertilization as well as improve the current efforts of developing a successful TBV.

Here we report on the identification of a peptide, named FG1 for Female Gamete peptide 1, which binds specifically to P. berghei female gametes. FG1 inhibits gamete fertilization and interferes with further development of the parasite in the mosquito. We provide evidence that the transmission-blocking activity of FG1 is sequence specific. We hypothesize that FG1 binds to a female gamete receptor thus blocking its interaction with a male ligand required for fertilization.

Results

Screening a phage display library for peptides that bind to female P. berghei gametes

A phage display library (Bonnycastle et al., 1996) was screened for peptides that bind to the surface of female P. berghei gametes (Fig. 1A). The library is composed of almost random 12 amino acid peptides fused to the major coat protein of the filamentous f88.4 phage (Bonnycastle et al., 1996). Every peptide in this library carries a cysteine in positions 2 and 11, while random amino acids occur at the remaining positions (Fig. 1B). The two cysteines interact via an S-S bond forming an 8 amino acid loop that provides structure to the peptides. The estimated complexity of the library is $10^9$ different peptides.

To select for peptides with affinity to the surface of female gametes, blood from mice infected with the P. berghei 820m1c1 line [female gametocytes/gametes express red fluorescent protein (RFP) and male gametocytes/gametes express green fluorescent protein (GFP)] (Ponzi et al., 2009) was incubated in P. berghei ookinete culture medium to induce differentiation of gametocytes into gametes (Fig. 1A). Aphidicolin, a DNA polymerase inhibitor, was added to the culture medium to block male gamete exflagellation and consequently avoid fertilization of female gametes. Red fluorescent protein-positive female gametes were isolated in pure form by cell sorting (Supporting Information Fig. S1) and then incubated with the phage library. After extensive washing to remove loosely attached phages, bound phages were recovered and amplified in Escherichia coli for use in the next round of selection (Fig. 1A). After three rounds of selection, close to half of the recovered phages displayed the same NCEDYLPGWFTC peptide, named FG1 for Female Gamete peptide 1 (Fig. 1C and Supporting Information Table S1). Sequencing of 18 random phages from the original library showed that there was no bias as each phage displayed a different peptide sequence (Supporting Information Table S2).
The FG1 peptide binds specifically to *P. berghei* female gametes

To test the binding affinity of the FG1 peptide to *P. berghei* gametes, sorted female (RFP-positive) and aphidicolin-treated male (GFP-positive) gametes of the *P. berghei* 820m1cl1 line were incubated with biotinylated FG1. Incubation with a biotinylated scrambled version of the FG1 peptide (FG1scr) was included as a negative control. The FG1scr peptide retained the same amino acid composition as FG1 but the amino acid sequence was randomized while maintaining the cysteines in positions 2 and 11 (Fig. 1D). Bound peptide was detected with streptavidin labelled with the red fluorescent dye Texas Red. The FG1 peptide bound strongly to female gametes (Fig. 2A and B). However, FG1 bound only
weakly to activated male gametocytes or to microgametes (Fig. 2A and B) and did not bind to uninfected red blood cells (Fig. 2C). Importantly, FG1scr bound weakly to female gametes, activated male gametocytes and microgametes, and to a comparable extent as binding of FG1 to activated male gametocytes (Fig. 2A and B). These results suggest that the FG1 peptide specifically binds to P. berghei female gametes and that this binding is sequence dependent.

The FG1 peptide inhibits Plasmodium sexual development in the mosquito

FG1 could bind to one of two classes of molecules: (i) to a molecule (receptor) that interacts with the male gamete during fertilization or (ii) to a molecule unrelated to the process of fertilization. In the first instance, binding of the peptide to a putative receptor is expected to inhibit fertilization as receptor occupancy by the peptide may prevent interaction with the male gamete. In the second instance, binding of the peptide should not affect fertilization. To distinguish the two possibilities, we performed passive administration feeding assays (PAFA) in the presence or absence of the FG1 phage or the FG1 peptide. For PAFA (Supporting Information Fig. S2), a group of Anopheles gambiae female mosquitoes (control) was fed on a P. berghei-infected mouse. Next, the mouse was injected intravenously with the phage or the peptide. After 15 min of recovery to allow peptide distribution in the mouse bloodstream, a second group of mosquitoes (experimental) was fed on the same mouse. Therefore, on average, control and experimental mosquitoes ingest the same number of parasites. Mosquito midguts were dissected 12 days post-infection to determine the number of oocysts per midgut. Administration of the FG1 phage or the FG1 peptide inhibited P. berghei oocyst formation by 79% and 60% respectively (Fig. 3A, C and Supporting Information Table S3). This reduction in oocyst numbers was not observed when mosquitoes were fed on mice injected with the wild-type phage or with the FG1scr peptide (Fig. 3B, D and Table S3).

To determine the effect of FG1 on P. falciparum oocyst formation, we performed standard membrane feeding assays (SMFA) with increasing concentrations of the peptides. The FG1 peptide inhibited development of P. falciparum oocysts in the mosquito from 57% at the lowest concentration (0.25 mg ml⁻¹; Fig. 3E and Supporting Information Table S4). The FG1scr peptide had no significant effect on P. falciparum oocyst development at all the concentrations tested (Fig. 3E and Supporting Information Table S4). These results show that the FG1 peptide hinders P. berghei and P. falciparum development in the mosquito, presumably by interfering with a conserved molecular interaction required for parasite fertilization.

FG1 inhibits fertilization

Oocyst formation is a process far ‘downstream’ from fertilization. To more precisely determine if the FG1 peptide affects Plasmodium gamete fertilization, we performed in vitro fertilization assays in the presence of FG1 or FG1scr. As a negative control, the P. berghei Δp48/45-Δp47 double knockout (low fertilization rate; van Dijk et al., 2010) was used. Efficiency of fertilization was determined using flow cytometry by measuring the DNA content of female gametes (1N) and zygotes (2N and 4N), all of which express the Pbs21 protein on their surface. Plasmodium berghei-infected blood was incubated in complete ookinete culture medium (containing or not the FG1 or FG1scr peptides) to induce gametogenesis, fertilization and zygote formation. At 4 h post-activation, female gametes and zygotes were labelled with a monoclonal anti-Pbs21 antibody (Winger et al., 1988) and parasite DNA was stained with DRAQ5 (Talman et al., 2010). Fertilization efficiency was determined by measuring the percentage of gametes (Pbs21 +, 1N DNA) that convert into zygotes (Pbs21 +, 2N/4N DNA). In the no-peptide control, 87.4% of the P. berghei ANKA2.34-activated females were fertilized while in the P. berghei Δp48/45-Δp47 double knockout negative control, only 10% of the females were fertilized (Fig. 4A, B and E). When P. berghei gametes were cultured in the presence of the FG1 peptide, the percentage of fertilized female gametes decreased with increasing concentrations of the peptide (Fig. 4C and E). Incubation of gametes with FG1scr resulted in a fertilization efficiency comparable with the blank control (Fig. 4D and E). These results suggest that the inhibitory effect of the FG1 peptide on Plasmodium development in the mosquito is due to inhibition of fertilization by specific peptide binding to a female gamete surface component.

FG1 does not affect gametocyte activation

After mosquitoes ingest an infected blood meal and before fertilization, Plasmodium gametocytes must undergo activation into gametes (gametogenesis). Plasmodium gametocyte activation is characterized by the egress of the parasite from the red blood cell (RBC) and for the male, by the exflagellation event. To determine if the FG1 peptide affects female gametocyte activation, we measured the egress of female gametes from the RBC in the presence of the FG1 and the FG1scr peptides. To measure female egress from the RBC, female gametocytes of the P. berghei 820cl1m1 line (females express RFP) were activated in ookinete culture medium...
Fig. 3. The FG1 peptide inhibits parasite sexual development in the mosquito. The effect of the FG1 peptide on sexual development of the parasite was determined by passive administration feeding assays (PAFAs) for *P. berghei* (A–D) or by standard membrane feeding assays (SMFAs) for *P. falciparum* (E).

A–D. For PAFAs (Supporting Information Fig. S2), a group of *A. gambiae* female mosquitoes (control group) were allowed to feed on a mouse infected with *P. berghei*. The mouse was then injected with either $10^{11}$ FG1 (A) or wild-type (B) phage or 600 μg of the FG1 (C) or FG1scr peptides (D). After injection, a second group of mosquitoes (experimental) was allowed to feed on the same mouse. Oocyst numbers for the two groups of mosquitoes were determined 12 days post-infection. The FG1 phage (A) or the FG1 peptide (C) significantly reduced the *P. berghei* oocyst numbers in mosquitoes when compared with the wild-type phage or the FG1scr peptide respectively. Percent inhibition = [(control median oocyst number − experimental median oocyst number)/control median oocyst number] × 100. *N*, number of mosquitoes analysed; Pre, before phage or peptide administration; Post, after phage or peptide administration. Horizontal bars represent the median number of oocyst from independent experiments shown in Supporting Information Table S3. Statistical significance was determined using Mann–Whitney U test.

E. For SMFAs, increasing concentrations of the FG1 or FG1scr peptides were added to *P. falciparum* gametocyte cultures and fed to *A. gambiae* female mosquitoes. A control group of mosquitoes (blank) was fed with cultures to which only buffer and no peptide were added. The FG1 peptide significantly reduced the *P. falciparum* oocyst numbers in the mosquito when compared with the no-peptide control or the FG1scr peptide. Bars represent the median number of oocyst formation from independent experiments shown in Supporting Information Table S4. Statistical significance was determined using one-way ANOVA with Bonferroni multiple comparison test. Percent inhibition calculation was done as for *P. berghei* PAFAs. NS, not significant.
Fig. 4. The FG1 peptide inhibits fertilization of *P. berghei* gametes. The effect of the FG1 peptide on gamete fertilization was determined using flow cytometry by measuring the DNA content of Pbs21-positive cells. Unfertilized female gametes have 1N DNA content and zygotes have a 2N or 4N DNA content after 4 h of *in vitro* culture in ookinete culture medium. DNA was stained with the far-red DNA dye DRAQ5 and Pbs21 was detected with the monoclonal antibody mAb 13.1. Female gametes and zygotes but not male gametes, express Pbs21 on their surface.

A and B. The fertilization assay was optimized by comparing the DNA content of Pbs21-positive female gametes in the ANKA 2.34 line (normal fertilization) and the Δp48/45-Δp47 double knockout (low fertilization; van Dijk *et al.*, 2010). Note that most of the Pbs21-positive gametes in the Δp48/45-Δp47 double knockout show a 1N DNA peak while for the ANKA 2.34 wild-type additional peaks are detected (2N and 4N).

C and D. Fertilization assays were performed in the presence of the FG1 and the FG1scr peptides (1 mg ml⁻¹). Note a reduction in fertilization efficiency with the FG1 peptide shown by the reduced DRAQ5 signal in the 2N/4N peak area.

E. Quantification of fertilization efficiency for *P. berghei* ANKA 2.34 with different concentrations of the FG1 or the FG1scr peptide. The Δp48/45-Δp47 double knockout was used as a negative control. Differences between control and treatment were compared by chi square test with a Bonferroni adjusted *P* value of 0.008 needed for 5% significance.
for 15 min. Immunofluorescence assays were performed with activated parasites using the RBC-specific anti-Ter-119 antibody to determine the presence or absence of the RBC surrounding the RFP-positive cells (Fig. 5A). Egress of female gametes from the RBC was not affected by incubation with either the FG1 or the FG1scr peptide (Fig. 5B).

The effect of the FG1 peptide on male gametocyte activation was determined by measuring the male gamete exflagellation centres in the presence or absence of the peptides. Plasmodium berghei gametocytes were incubated in complete ookinete culture medium to induce activation and the number of exflagellation centres per microliter of blood was determined by light microscopy using a haemocytometer. Neither the FG1 nor the FG1scr peptide significantly changed the number of male exflagellations when compared with the no-peptide control (Fig. 5C). These results indicate that the reduction in Plasmodium gamete fertilization induced by the FG1 peptide did not result from a reduction of gamete activation.

Discussion

Transmission of malaria by mosquitoes was discovered more than one century ago. However, our knowledge of the molecular mechanisms operating during malaria transmission, including sexual reproduction, remains scant. It has been known for decades that natural immunity acquired against Plasmodium sexual stages blocks parasite sexual reproduction in the mosquito, thereby reducing transmission to a human host (Baird et al., 1991; Mulder et al., 1994; Lensen et al., 1998; Arevalo-Herrera et al., 2005; Bousema and Drakeley, 2011). Furthermore, TBV candidates targeting Plasmodium sexual reproduction are currently being tested (van Dijk et al., 2001; 2010; Eksi et al., 2006; Tachibana et al., 2011). However, in most cases, the function of the target antigens or their interaction with other proteins is unknown.

By using a phage display library, we identified a peptide – FG1 – that binds specifically to Plasmodium female gametes. Scrambling the amino acid sequence of this peptide greatly reduces binding, indicating specificity of interaction. These results support the hypothesis that FG1 binds to a specific protein on the surface of the female gamete and makes unlikely the possibility of non-specific interaction. Importantly, the FG1 peptide inhibits P. berghei oocyst formation and interferes with fertilization, presumably by disrupting molecular interactions between the gametes. The relatively small size of the peptide renders unlikely the possibility of steric hindrance. The FG1 peptide also inhibits P. falciparum oocyst formation suggesting that the putative interacting molecule is conserved among Plasmodium species. We propose that the FG1 peptide binds to a receptor on the surface of the female gamete that interacts with a surface ligand from the male gamete during fertilization (Fig. 6A). We also hypothesize that the FG1 peptide mimics the structure of the male surface ligand and competes with it for binding to the female receptor, thereby inhibiting fertilization (Fig. 6B).

Sperm-oocyte recognition in any organism has remained intractable to molecular analysis. The recent discovery that oocyte surface Juno interacts with sperm
surface Izumo in mammals is the first receptor–ligand interaction required for gamete fertilization to be characterized in any organism (Bianchi et al., 2014). This finding promises to impact the development of new contraceptive and fertility treatments in humans. Likewise, the identification of molecular interactions between Plasmodium gametes would expand our understanding of sexual reproduction of malaria parasites and potentially shed light on the mechanisms of fertilization of other protozoan parasites such as Toxoplasma gondii and Cryptosporidium parvum.

The use of phage display screening provides a novel approach to characterize molecular interactions between male and female Plasmodium gametes during fertilization. One advantage of this approach is that it does not require any prior knowledge about the interacting molecules. This strategy was previously used to identify receptor–ligand interactions between malaria ookinete and sporozoites with the mosquito organs (midgut and salivary glands) that they invade (Ghosh et al., 2001; 2009; 2011; Vega-Rodriguez et al., 2014). Following our previously adopted strategy, we next plan to use the FG1 peptide to capture and identify (i) the protein to which the peptide binds on the surface of the female gamete (putative receptor) and (ii) the male gamete surface protein that structurally mimics the FG1 peptide (putative ligand). The identification of such molecules may lead to additional targets for malaria transmission-blocking interventions. Should the targets be conserved, it could lead to vaccine candidates that are effective for both major malaria pathogens, P. falciparum and P. vivax.

**Experimental procedures**

**Biological materials**

Plasmodium berghei (ANKA clone 2.34) was propagated in female CD1 mice (Jackson Laboratories). Plasmodium falciparum NF54 infectious gametocyte cultures were provided by the Johns Hopkins Malaria Research Institute Parasite Core Facility, and were diluted to 0.03% gametocytemia before feeding to the mosquitoes using an artificial membrane feeder. Anopheles gambiae (Keele) (Hurd et al., 2005) female mosquitoes were provided by the Johns Hopkins Malaria Research Institute Mosquito Core Facility.

**Mice**

Random-bred Swiss albino CD-1 female mice, weighting 20 to 25 g at the time of primary infection were used throughout the study. Mice were kept in a room with a temperature of 22°C and a 12 h light/12 h dark cycle. All studies involving laboratory animals were performed in accordance with the regulations of the Institutional Animal Care and Use Committee (IACUC).

**Isolation of P. berghei female gametes**

Mice were injected intraperitoneally with 200μl of 10 mg l⁻¹ phenylhydrazine (Sigma) in 1 x PBS solution 3 days before the infection with the parasite to induce anaemia and thus stimulate the production of reticulocytes. Mice were infected with the transgenic P. berghei 820m1c1 line (Ponzi et al., 2009) whose female gametocytes/gametes express RFP and male gametocytes/gametes express GFP. Two days after infection with the parasite, mice were injected intraperitoneally with 200μl of a 1 mg ml⁻¹ solution of pyrimethamine to eliminate asexual blood stages.
Gametocyte-containing blood was collected by cardiac puncture 24 h after the pyrimethamine treatment and incubated for 15 min at room temperature in complete ookinete culture medium (Vega-Rodriguez et al., 2014) to induce gamete activation. Aphidicolin (0.5 mM) was added to the culture medium to block exflagellation of male gametes and the subsequent fertilization by microgametes. The culture was cooled on ice and passed through a LS magnetic column (Miltenyi Biotec) kept at 4°C to enrich for sexual stages and remove non-infected RBCs and white blood cells. Red fluorescent protein-positive female gametes were subsequently sorted using a MoFlo Cell Sorter. The estimated purity of the sorted sample (99.9% female gametes) was verified by analysing the RFP-positive female gametes by fluorescence microscopy.

Selection of phages that bind to female P. berghei gametes

Sorted female gametes (≈5 × 10^6) were incubated with phages (1 × 10^11 colony-forming units; about 100 times the complexity of the library) in complete P. berghei ookinete culture medium (Vega-Rodriguez et al., 2014) at room temperature for 30 min. The gametes were washed six times with ice-cold RPMI. Excess E. coli K91 bacteria (Coli Genetic Stock Center; http://cgsc.biology.yale.edu) were added to the gametes to recover and amplify the gamete-bound phages. The amplified phages (1 × 10^11 colony-forming units) were used for the next round of selection. The process was repeated two more times to afford a total of three rounds of selection. After the third round, colony PCR was performed on individual phage-infected E. coli colonies using the forward primer: 5′-CTTGGTCTAGTCTGAAGCAGGAAGA-3′ and the reverse primer: 5′-AGTGCAGGAAGCCTGGTTTG-3′. Polymerase chain reaction products were purified using the DNA Clean & Concentrator^™^-5 (Zymo Research) and sequenced to determine the predicted amino acid sequence of the displayed peptide.

Detection of peptide binding

Sorted female gametes and aphidicolin-treated activated male P. berghei gametocytes were fixed on slides for 1 h with 4% paraformaldehyde at room temperature. Slides were washed three times with 1× PBS and blocked with 4% BSA in 1× PBS for 1 h at room temperature. Gametes were then incubated with 10 μg ml^-1 of biotynilated-FG1 or FG1-scr peptide diluted in blocking buffer. Samples were washed three times with 1× PBS for 5 min and then incubated with streptavidin-conjugated to Texas Red diluted in blocking buffer. Gametes were washed three times with 1× PBS for 5 min and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen Inc.) and visualized on a Leica DMLB microscope. Binding of the peptides to the gametes was quantified by measuring relative fluorescence using Image J software.

Sorted non-activated female gametocytes were fixed onto glass slides and permeabilized with methanol for 15 min at −20°C. Slides were washed with 1× PBS and peptide binding and detection was performed as described above.

For microgametes, P. berghei gametocytes were activated in complete ookinete culture medium (Vega-Rodriguez et al., 2014) for 15 min at room temperature. Microgametes were immediately fixed on slides for 1 h with 4% paraformaldehyde at room temperature. Microgametes were blocked, incubated with FG1 or FG1-scr peptides and then with streptavidin-conjugated to Texas Red diluted in blocking buffer as described above. Microgametes were then permeabilized with 0.1% Triton-X diluted in blocking buffer for 15 min at room temperature. Microgametes were washed three times with 1× PBS for 5 min and then blocked with 4% BSA in 1× PBS for 30 min. Samples were incubated with a monoclonal mouse anti-α-tubulin antibody (Sigma, T9026) and washed with 1× PBS for 5 min. Microgametes were then incubated with Alexa Fluor® 488 goat anti-mouse IgG antibody (Invitrogen) for 1 h at room temperature and then washed with 1× PBS for 5 min. Slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen Inc.) and visualized on a Leica DMLB microscope.

P. berghei PAFA

A group of A. gambiae female mosquitoes (control) were fed for 15 min on a mouse infected with P. berghei parasites with a male gametocyte exflagellation rate between 1 and 2 exflagellations per 10 fields at 400× magnification (Vega-Rodriguez et al., 2014). Then, the same mouse was injected intravenously with either 10^11 phages (wild-type or FG1 phage) or 600 μg synthetic peptide (FG1 or FG1-scr). Mice were allowed to recover for 15 min to allow for dissemination of the injected components in the bloodstream. Next, a second group of A. gambiae female mosquitoes (experimental) were fed for 15 min on the injected mouse. Mosquitoes were kept at 19°C in a humidified chamber. Midguts were dissected 12 days after the blood meal and oocyst numbers per mosquitoes midgut were determined after staining with 0.2% mercuric chloride in 1× PBS. Percent inhibition was defined as [(control median oocyst number – experimental median oocyst number)/control median oocyst number] × 100.

P. falciparum SMFA

Plasmodium falciparum gametocyte cultures were diluted to 0.03% gametocytemia. FG1 or FG1-scr peptides (experimental) were added to the gametocytes at a final concentration of 0.25, 0.5, 1.0 or 1.5 mg ml^-1 and then fed to A. gambiae female mosquitoes. For controls, gametocytes were mixed with 1× PBS/5% DMSO. Mosquitoes were kept at 24°C in a humidified chamber. Mosquito midguts were dissected 8 d after feeding and oocyst number per midgut was determined after staining with 0.2% mercuric chloride. Percent inhibition was defined as: [(control median oocyst number – experimental median oocyst number)/control median oocyst number] × 100.

Analysis of fertilization by flow cytometry

To determine the effect of FG1 on fertilization efficiency, we measured the number of unfertilized female gametes and the number of zygotes resulting from peptide treatments by flow cytometry based on staining with anti-Pbs21 antibody to identify female gametes/zygotes and the dye DRAQ5 to determine DNA content (1N to 4N). Mice were injected intraperitoneally with 200 μl of 10 mg l^-1 phenylhydrazine (Sigma) in 1× PBS 3 days before parasite infection to induce anaemia and consequently the
production of reticulocytes. Mice were infected by intraperitoneal injection of 200 μl of P. berghei ANKA 2.34-infected blood at a parasitemia of 10–20%. Two days after infection, mice were injected intraperitoneally with 200 μl of a 1 mg ml⁻¹ solution of pyrimethamine to eliminate the asexual blood stages. Gametocyte-containing blood was collected by cardiac puncture 24 h after pyrimethamine treatment and cultured in ookinete culture medium (containing or not the FG1 or the FG1scr peptides) for 4 h at 19°C with gentle agitation to induce gametocyte activation into gametocytes, fertilization and zygote formation. At 4 h post-activation, cultures were incubated with an anti-Pbs21 antibody (Winger et al., 1988) for 30 min at room temperature (Pbs21 is only expressed in female gametes and in zygotes). Samples were then incubated with anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen) for 30 min at room temperature. DNA was stained with 20 μM DRAQ5 (Cell Signaling Technology; Talman et al., 2010) for 30 min at 37°C and samples were analysed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson). Fertilization efficiency was determined by measuring the percentage of unfertilized gametes (Pbs21-positive and 1N DNA) that transform into zygotes (Pbs21-positive and 2N or 4N DNA).

Female gamete activation assay

Gametocytes of the P. berghei 820m1c1 line were incubated in ookinete culture medium for 15 min at room temperature in the presence or absence of 1 mg ml⁻¹ of FG1 or FG1scr peptide. As a negative control, gametocytes were incubated in 1× PBS on ice for 15 min. RBC membranes were stained with an anti-mouse Ter-119 antibody conjugated to FITC (Miltenyi Biotec) and analysed with a fluorescence microscope. Non-activated female gametocytes were defined as RFP and Ter-119-positive cells and activated female gametocytes were defined as RFP-positive and Ter-119-negative cells.

Male gamete exflagellation assay

Gametocytes of the P. berghei 820m1c1 line were incubated in ookinete culture medium for 15 min at room temperature in the presence or absence of 1 mg ml⁻¹ of FG1 or FG1scr peptide. Reactions were loaded on an improved Neubauer haemocytometer and analysed by light microscopy to determine the number of exflagellating centres per microliters of blood. The results were normalized to the no-peptide control.

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