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Host complement C3 promotes malaria transmission by killing symbiotic bacteria in the mosquito midgut

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Host-derived factors ingested during mosquito blood feeding are poorly understood modulators of malaria transmission. Here, we demonstrated that host complement C3, acquired by mosquitoes during *Plasmodium* infection, significantly enhanced rodent malaria infection in laboratory-reared mosquitoes. This effect was recapitulated in field-caught *Anopheles sinensis* mosquitoes, confirming its relevance to malaria transmission in a more natural setting. Moreover, host-derived C3 significantly reduced the efficacy of anti-Pfs25 antibodies in blocking malaria transmission. Mechanistically, host-derived C3 lyses the mosquito midgut symbiont *Elizabethkingia anophelis* (*E. anophelis*)—a bacterium that intrinsically suppresses parasite development by blocking the zygote-to-ookinete transition. Strikingly, host-derived C3 in mosquitoes appears to be activated by the alternative pathway, and inhibiting Factor B with Iptacopan (LNP023) reduced *Plasmodium falciparum* (*P. falciparum*) infection, while increased the efficacy of anti-Pfs25 antibodies to blocking *P. falciparum* transmission in the standard membrane-feeding assay. Therefore, this study describes a strategy of the malaria parasite to utilize host complement C3 to promote its transmission and provides us with an avenue to block malaria transmission and improve the blocking efficacy of anti-Pfs25 antibodies by the inhibition of C3 activation.

complement C3 | malaria parasite | microbiota | mosquitoes | transmission

Significance

Malaria remains one of the most devastating diseases worldwide. Blocking malaria transmission is considered a promising strategy for controlling and potentially eliminating the disease. In this study, we report that host complement C3 significantly enhances malaria transmission by modulating the abundance of the antimalarial bacterium *Elizabethkingia anophelis* in the mosquito midgut. In the mosquito midgut, host-derived C3 is activated via the alternative pathway, which directly destroys *E. anophelis*. Inhibition of this activation using the Factor B inhibitor LNP023 not only reduces the transmission of both rodent and human malaria but also enhances the blocking efficacy of anti-Pfs25 antibodies. These findings reveal a malaria parasite transmission strategy and propose microbiota modulation/complement inhibition to block spread.

Malaria is one of the most devastating diseases worldwide, responsible for approximately 263 million cases and 597 thousand deaths in 2023 (1). Malaria is caused by protozoans of the genus *Plasmodium* and transmitted by the bite of an infected female mosquito (genus *Anopheles*). Following ingestion by the definitive host (mosquito), sexual gametocytes, namely, microgametocytes (male) and macrogametocytes (female), fertilize to produce zygotes in the mosquito midgut. Subsequently, zygotes transform into mobile ookinetes, which penetrate epithelial cells of the midgut and develop into oocysts. Upon maturation, thousands of sporozoites are released from an oocyst into the hemolymph, ultimately invading the salivary glands. These sporozoites then become injected into the skin of the host upon mosquito feeding, initiating infection of healthy persons. Hence, blocking *Plasmodium* replication and development in mosquitoes is a promising strategy to control and eradicate malaria globally.

The malaria parasite is most vulnerable to the mosquito immune response in the midgut during the preoocyst stages, including gametocyte, zygote, and ookinete (2). Considerable progress has been made toward elucidating these immune responses (3–6) and defining the important roles of symbiotic bacteria in regulating *Plasmodium* development. Symbiotic bacteria, such as *Serratia ureilytica* Su_YN1 and *Enterobacter*, directly kill the parasite in the midgut during the sexual stages of development by secreting lipase or generating reactive oxygen species (7, 8). Additionally, midgut microbiota indirectly influence malaria parasite development by modulating the mosquito immune defenses (9, 10) or development of the peritrophic matrix (11, 12).

Meanwhile, host-derived factors also enter the mosquito midgut during natural malaria transmission, leading to complex interactions between the parasites, mosquito immune responses, symbiotic bacteria, and host-derived factors. Although previous studies have shown that gametocyte-targeted antibodies, leukocytes, and transforming growth factor (TGF)-β1 ingested by the mosquito into the midgut modulate malaria transmission (13–16), the influence of host-derived factors on parasite development remains largely unknown.

The complement system serves as a cornerstone of innate immunity, providing rapid defense through three activation pathways: classical, lectin, and alternative. The classical pathway is triggered by antigen–antibody complexes, whereas the lectin pathway initiates when mannose-binding lectin (MBL) or ficolins bind pathogen-associated carbohydrate

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The authors declare no competing interest.

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motifs. In contrast, the alternative pathway is constitutively activated by spontaneous hydrolysis of C3. All pathways converge at the cleavage of C3 into the opsonin C3b and the anaphylatoxin C3a, ultimately generating the membrane attack complex (MAC/C5b-9), which lyses pathogens (e.g., bacteria, viruses, parasites) via pore formation (17, 18). However, the role of host complement in malaria transmission is paradoxical. While ingested complement components in the mosquito midgut can reduce *Plasmodium* infection in rodent models (19), the parasite's gametes evade this defense by recruiting Factor H—a regulatory protein—to their surface, thereby inhibiting complement-mediated lysis (20). These conflicting findings underscore the need to define how host complement influences natural malaria transmission, particularly its dual capacity to both restrict and inadvertently aid parasite survival. Here, we report that host-derived complement C3, a critical host complement component, is utilized by malaria parasites to facilitate their transmission by modulating symbiont microbiota in the mosquito midgut.

Results

Host C3 Did Not Impact Parasite Development in the Host. Complement fixation is closely linked to the protective effects of antibodies against the blood-stage replication of malaria parasites (21); consequently, these parasites have evolved numerous strategies to evade complement attacks (22). However, the specific effects of complement on the sexual and asexual stages of malaria development within the host remain unclear. To address this, we first evaluated the impact of C3 on the growth of malaria parasites in the host, as varying levels of gametocytes could influence their infectivity to mosquitoes. Following infection with *Plasmodium yoelii* BY265-RFP (*P. yoelii*) or *Plasmodium berghei* (*P. berghei)*, complement C3 was rapidly activated, producing C3a in mice as early as 2 d postinfection (*SI Appendix*, Fig. S1 *A* and *B*). However, C3 did not significantly affect the growth of either parasite in mice, as parasitemia levels in C3-deficient mice were comparable to those in wild-type (WT) mice infected with either *P. yoelii* or *P. berghei* (*SI Appendix*, Fig. S1 *C* and *D*). Furthermore, the gametocytemia of *P. yoelii* and *P. berghei* in C3-deficient mice was similar to that in WT mice (*SI Appendix*, Fig. S1 *E* and *F*), with no significant differences observed in gametocyte development stages between the two groups (*SI Appendix*, Fig. S1 *G* and *H*). To further confirm the role of complement C3 in the sexual stage of *Plasmodium* development, we conducted in vitro cultures of ookinetes from *P. yoelii* or *P. berghei* collected from both infected WT and C3-deficient mice. The number of ookinetes produced by parasites from C3-deficient mice was comparable to those from WT mice (*SI Appendix*, Fig. S1 *I* and *J*). These findings suggest that C3 deficiency does not significantly impact the development or function of the sexual stages of *P. yoelii* or *P. berghei* in the host. Collectively, we demonstrate that C3 does not have a significant effect on the sexual or asexual stages of malaria development within the host.

Host Complement C3 Promoted Rodent Malaria Infection in Mosquitoes. Although we found that C3 had no significant effect on parasite growth in the host, it remains unclear whether C3 ingested into the mosquito midgut during feeding affects parasite infection in mosquitoes. To investigate this, we first examined whether host-derived C3 in the mosquito midgut would be activated by detecting the active cleavage product of complement C3, C3a. We detected C3a in the mosquito midgut as early as 30 min after mosquitoes fed on *P. yoelii*–infected mice; however, it disappeared by 10 h postfeeding (Fig. 1*A*). These results support the activation of host-derived C3 in the mosquito midgut.

Next, we aimed to determine whether host-derived C3 that enters the mosquito midgut affects malaria infection in mosquitoes. WT and C3-deficient mice were infected with an equivalent dose of *P. yoelii*–infected red blood cells (RBCs). *Anopheles stephensi (An. stephensi)* mosquitoes were then allowed to feed on the mice at various time points postinfection (*SI Appendix*, Fig. S2*A*). The infection intensity and prevalence were significantly reduced in mosquitoes that fed on C3-deficient mice compared to those that fed on WT mice infected with *P. yoelii* for 3 and 4 d (*SI Appendix*, Fig. S2*B*). The most pronounced inhibition occurred in mosquitoes that fed on C3-deficient mice infected for 4 d, with the number of salivary glands sporozoites reduced by over 99% compared to those from infected WT mice (Fig. 1 *B*–*D*). Additionally, the infection intensity and prevalence were significantly lower in mosquitoes that fed on C3-deficient mice infected with *P. berghei* for 2 d compared to those that fed on infected WT mice (Fig. 1*E*). To validate these findings, we evaluated the effect of C3 on malaria transmission using field-caught mosquitoes. *Anopheles sinensis (An. sinensis)*, the major malaria vector in China, were collected in Yingjiang, Yunnan, a border city with Myanmar, and then fed on *P. yoelii* -infected WT and C3-deficient mice. The malaria infection intensity and prevalence in *An. sinensis* were significantly lower in those that fed on the infected C3-deficient mice compared to those that fed on infected WT mice (Fig. 1 *F* and *G*). However, potential confounding factors in rodent malaria transmission should be considered in this field study, given the lack of life history data for field-caught adult mosquitoes.

These findings were further corroborated using cobra venom factor (CVF), which depletes host C3 (23), but without significantly affecting parasite growth in the host (*SI Appendix*, Fig. S3). Mosquitoes that fed on parasite-infected, C3-depleted mice exhibited significantly lower infection intensity compared to those that fed on control mice (Fig. 1*H*). To eliminate the possibility of indirect effects from C3 deficiency on malaria transmission, recombinant C3 protein (rC3) was injected into parasite-infected C3-deficient mice. The introduction of rC3 substantially increased the infection intensity in mosquitoes that fed on these C3-deficient mice (Fig. 1*I*). Collectively, these data indicate that host complement C3 plays a significant role in promoting rodent malaria infection in mosquitoes.

C3 Did Not Influence Mosquito Attraction to the Host or Their Fitness Cost. In addition to sexual development in the host, mosquitoes' acquisition of parasites from a host is another critical step in malaria transmission. C5a receptor (C5aR) deficiency alters the composition and diversity of skin microbiota (24), and the change of skin microbiota influences the attractiveness of *flavivirus*-infected hosts to *Aedes aegypti* (25). Therefore, we evaluated the effect of C3 deficiency on the attraction of *An. stephensi* to mice. However, C3 deficiency did not significantly affect mouse attraction, as the number of mosquitoes attracted to the infected C3-deficient mice was comparable to that of the infected WT mice at all time points (*SI Appendix*, Fig. S4*A*). Furthermore, the volume of blood ingested by mosquitoes feeding on parasite-infected WT and C3-deficient mice was similar (*SI Appendix*, Fig. S4*B*). Additionally, no significant differences were observed in fitness costs, including fecundity, hatching rate, and survival, between mosquitoes feeding on WT or C3-deficient mice (*SI Appendix*, Fig. S4 *C*–*E*). Thus, the reduced malaria transmission observed in C3-deficient mice cannot be attributed to altered mosquito attraction or fitness costs.

Host-Derived C3 Enhanced Rodent Malaria Transmission by Reducing *Elizabethkingia anophelis* Abundance in the Mosquito Midgut. Although gametes in the mosquito midgut have evolved to prevent the deposition of MAC on their surface by

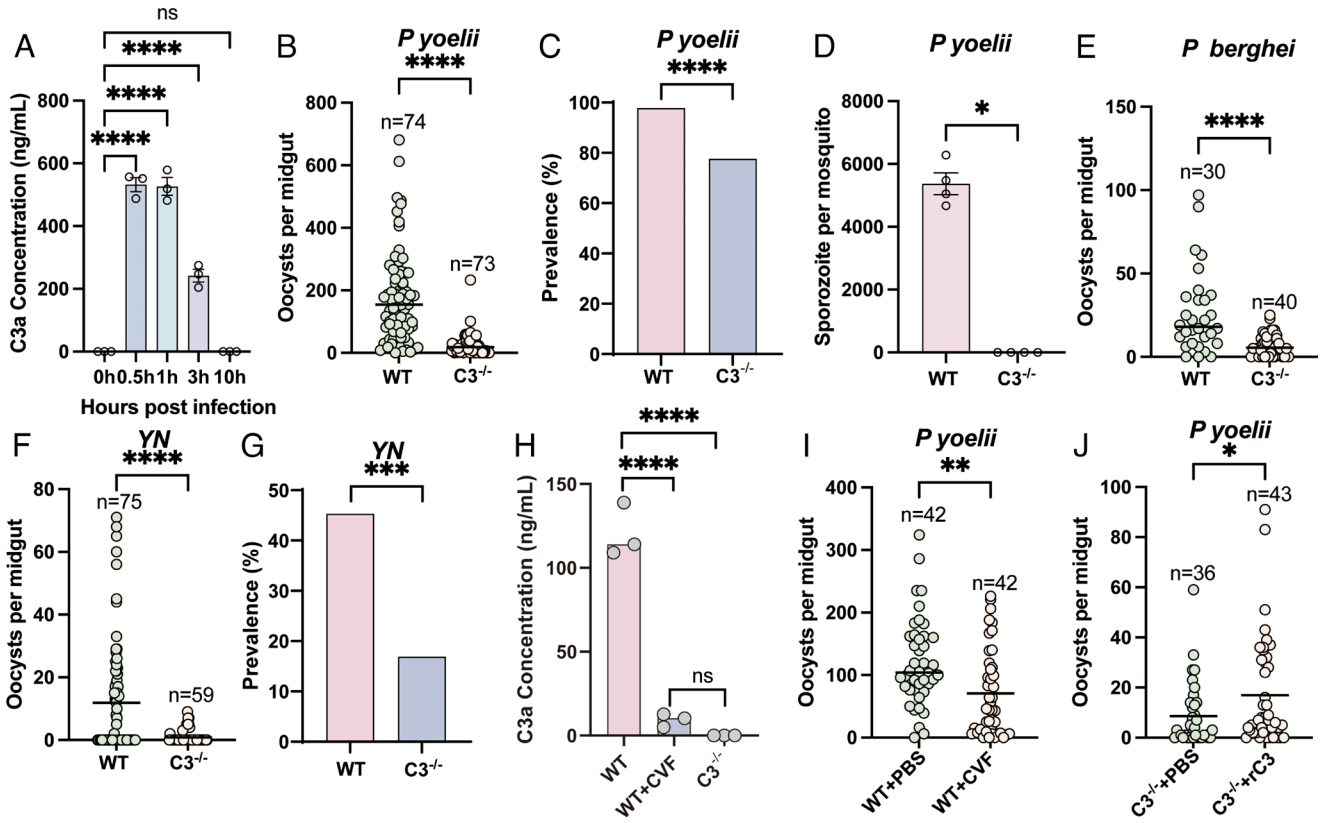

Fig. 1. Host complement C3 promotes rodent malaria transmission. (*A*) Concentration of C3a in the midgut of mosquitoes feeding on *P. yoelii*–infected WT mice were detected using ELISA. Twenty mosquito midguts were pooled to constitute one biological replicate (n = 3). (*B* and *C*) The oocyst number (*B*) and prevalence (*C*) in midgut of mosquitoes that fed on WT or $C3^{-/-}$ mice infected with *P. yoelii* for 4 d. (*D*) The number of sporozoites in the salivary glands of mosquitoes feeding on the infected WT and $C3^{-/-}$ mice. Ten mosquito salivary glands were pooled to constitute one biological replicate (n =4). (*E*) The number of oocyst in midgut of mosquitoes feeding on WT or $C3^{-/-}$ mice infected with *P. berghei* for 2 d. (*F* and *G*) The oocyst number (*F*) and prevalence (*G*) in midgut of field-caught *An. sinensis* feeding on infected WT and $C3^{-/-}$ mice. (*H*) The concentration of C3a in the serum of WT mice (n = 3) was determined via ELISA after 3 d of intraperitoneal injection of CVF compared to control and $C3^{-/-}$ mice. Data are presented as median, with dots indicating biological replicates. (*I*) After infection with *P. yoelii* for 4 d, WT mice were intraperitoneally injected daily with or without CVF for 3 d before being fed to mosquitoes. Seven days later, the number of oocysts in the mosquito midgut was compared. (*J*) Following infection with *P. yoelii* for 4 d, $C3^{-/-}$ mice were intravenously injected with or without rC3, and then were fed to mosquitoes. Seven days later, the number of oocysts in the mosquito midgut was compared. Two independent experiments were performed for each experiment with similar results. For (*B*, *E*, *F*, *I*, and *J*), the individual dot represents each mosquito, with the line indicating the median. Data are pooled and presented as mean ± SD for both (*A* and *D*). One-way ANOVA analysis (two-tailed) was applied for results (*A* and *H*); Mann–Whitney *U* test (two-tailed) for results (*B*, *E*, *F*, *I*, and *J*); Fisher's exact test (two-sided) for results (*C* and *G*); unpaired *t* test (two-tailed) for result (*D*). YN: *An. sinensis* caught in Yunnan Provence. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns: not statistically significant.

recruiting Factor H (20), Host-derived C3 has been shown to have detrimental effects on gametes (19, 26). Interestingly, C3 deficiency does not significantly impact sexual development in the host, host attraction, or mosquito fitness. This led to the hypothesis that the enhanced effect of host-derived C3 on malaria transmission was closely associated with its influence on the parasites in mosquitoes. Notably, the addition of human serum to in vitro cultured ookinetes resulted in MAC deposition on both immature retort-form ookinetes and mature ookinetes (*SI Appendix*, Fig. S5), highlighting the detrimental effect of C3 on the sexual stage of development. Consequently, one would expect that C3 deficiency would promote malaria infection; however, this contradicts our findings.

Growing evidence has revealed resident symbiont bacteria in the midgut of laboratory-reared and field-caught mosquitoes, with pivotal roles in modulating malaria transmission (7–9, 11, 27). Additionally, complement serves as the first line of the host's innate immune response against bacteria (17, 18), and host-derived C3 is taken into the mosquito midgut and activated post-blood-feeding (Fig. 1*A*). Based on this, we hypothesized that host complement C3 in the mosquito midgut may kill symbiotic bacteria, thereby indirectly influencing malaria transmission. To test this hypothesis, we compared the abundance of symbiotic bacteria in the midgut of mosquitoes that fed on *P. yoelii*–infected C3-deficient and WT mice using real-time PCR to detect genus-specific 16S ribosomal(r) DNA. As anticipated, the bacterial load was significantly higher in the midgut of mosquitoes that fed on infected C3-deficient mice compared to those that fed on infected WT mice at both 12 and 24 h post blood meal (Fig. 2*A*). Pretreating mosquitoes with antibiotics effectively eliminated the symbiotic bacteria in the midgut (Fig. 2*B*), which in turn abolished the differences in infection levels between mosquitoes feeding on infected C3-deficient and WT mice (Fig. 2*C*). These findings suggest that host complement C3 promotes malaria infection by modulating the symbiotic microbiota in the mosquito midgut.

Some symbiotic bacteria are known to influence malaria transmission by modulating the immune responses of mosquitoes against parasites (9). To investigate this, we compared RNA-seq data from mosquitoes that fed on *P. yoelii*–infected C3-deficient mice with those that fed on infected WT mice. Our analysis revealed that immune-related genes were not significantly upregulated in mosquitoes feeding on the infected C3-deficient mice compared to those feeding on infected WT mice (28) (*SI Appendix*, Fig. S6 *A*–*C*). Therefore, the changes in symbiotic bacteria did not appear to affect the immune responses of the mosquitoes.

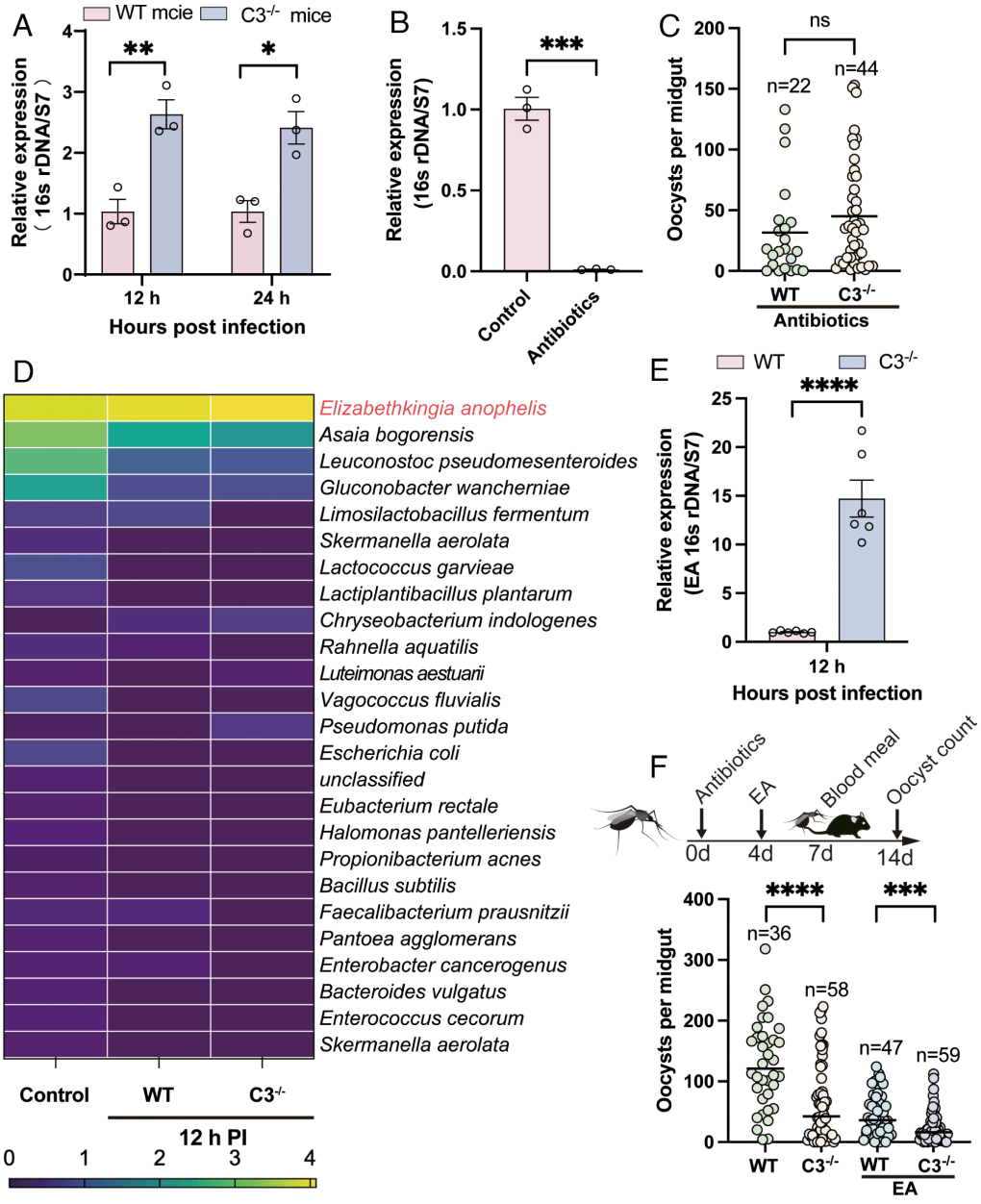

Fig. 2. Host-derived C3 promotes rodent malaria transmission by modulating *E. anophelis* abundance in the mosquito midgut. (*A*) The abundance of symbiotic bacteria in mosquitoes that fed on *P. yoelii*–infected WT or C3$^{-/-}$ mice at 12- and 24-h post-blood-feeding is expressed as the relative ratio of bacterial 16S rDNA to mosquito S7. Twenty mosquitoes were pooled to constitute one biological replicate (n = 3). (*B*) The abundance of symbiotic bacteria in the mosquito midgut before and after a 4-d antibiotic treatment. Twenty mosquitoes were pooled to constitute one biological replicate (n = 3). (*C*) The number of oocysts in antibiotic-pretreated mosquitoes that fed on the infected C3$^{-/-}$ and WT mice. (*D*) The relative abundance of symbiont microbiota in mosquito midgut 12 h postfeeding on infected WT and C3$^{-/-}$ mice. (*E*) Real-time PCR detection of *E. anophelis* abundance in the mosquito midgut at 12 h postfeeding on the infected WT and C3$^{-/-}$ mice, expressed as the relative ratio of *E. anophelis*-specific 16S rDNA to mosquito S7. Twenty mosquitoes were pooled to constitute one biological replicate (n = 6). (*F*) The number of oocysts in the midgut of mosquitoes pretreated with or without antibiotics to clear symbiont microbiota, colonized with *E. anophelis* in the midgut, and fed on the infected C3$^{-/-}$ and WT mice for 7 d. Each experiment was performed twice with similar results. Data are pooled and presented as mean ± SD for (*A*, *B*, and *E*). In (*C* and *F*), the individual dot represents each mosquito, and the total number of mosquitoes is noted as N in the panel with a line indicating the median. Statistical significance was determined using an unpaired *t* test (two-tailed) for results (*A*, *B*, and *E*) and Mann–Whitney *U* (two-tailed) test for results (*C* and *F*). PI: post infection; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns: not statistically significant.

Several studies have reported that specific symbiotic bacteria can directly kill sexual stage *Plasmodium* spp. (7, 8, 27). Thus, to identify the symbiont bacteria in the mosquito midgut that are modulated by host complement C3, 16S rDNA sequencing of the symbiont bacteria in the midgut of mosquitoes feeding on infected C3-deficient and WT mice was performed. *E. anophelis* was identified as the predominant species, which showed the most significant increase in relative abundance in mosquitoes feeding on parasite-infected C3-deficient mice compared to those feeding on infected WT mice (29) (Fig. 2*D*). These findings were further validated using real-time PCR (Fig. 2*E*). Next, we aimed to determine whether the increased relative abundance of *E. anophelis* in the mosquito midgut contributed to the reduced malaria infection observed in mosquitoes feeding on infected C3-deficient mice. To assess this, we first eliminated the endogenous microbiota in mosquitoes through antibiotic treatment. The mosquitoes were then provided with a sugar solution supplemented with isolated *E. anophelis* to facilitate its colonization of the midgut (*SI Appendix*, Fig. S7). Following this, the mosquitoes fed on parasite-infected C3-deficient and WT mice. The infection intensity in mosquitoes

colonized by *E. anophelis* that fed on infected C3-deficient mice was significantly lower than in those feeding on WT mice (Fig. 2*F*). These results indicate that complement C3 promotes malaria infection by reducing the abundance of *E. anophelis* in the mosquito midgut.

Symbiotic Bacteria *E. anophelis* Block the Zygote-to-Ookinete Transition. To determine whether *E. anophelis* could directly inhibit the sexual development of malaria in the mosquito midgut, mosquitoes were pretreated with antibiotics, colonized with *E. anophelis*, and then fed to infected WT mice. Following this, we observed a significantly lower number of ookinetes in the mosquitoes colonized with *E. anophelis* compared to those that were not colonized after feeding on parasite-infected WT mice (Fig. 3*A*). This finding demonstrates the inhibitory effect of *E. anophelis* on the sexual stage development of malaria parasites in the mosquito midgut.

To verify the inhibitory effect of *E. anophelis* on the generation of malaria ookinetes, *E. anophelis* was added to in vitro cultures of *P. yoelii* gametocytes. While *E. anophelis* did not significantly affect gametocyte survival, it notably suppressed ookinete generation from gametocytes in vitro (Fig. 3*B*), indicating that *E. anophelis* inhibits the transformation of gametocytes into ookinetes. Additionally, the Transwell assay showed that *E. anophelis* placed in the upper chamber significantly inhibited ookinete generation (Fig. 3*C*). Furthermore, the culture supernatant of *E. anophelis* (EAS) exhibited a marked inhibitory effect on oocyst formation in mosquitoes (Fig. 3*D*), suggesting that *E. anophelis* releases secreted components that inhibit ookinete generation. Subsequent filtration of the EAS revealed that the >3 kDa fraction had a significantly higher inhibitory capacity on ookinete formation compared to the <3 kDa fraction (Fig. 3*E*). However, heat inactivation of the EAS completely abolished its inhibitory effect (Fig. 3*F*), confirming that the active antimalarial components are proteins secreted by *E. anophelis*.

Next, we examined which stage of ookinete formation was inhibited by the secreted protein. To do this, the generation of ookinetes was similarly suppressed regardless of whether EAS was added at 0 h or 4 h in the in vitro ookinete cultures. Therefore, *E. anophelis* inhibited the transformation of zygotes into ookinetes by secreting an unidentified protein (Fig. 3*G*).

***E. anophelis* Was Directly Killed by Alternative Pathway-Activated Host C3.** Next, we aimed to determine whether host C3 could directly destroy *E. anophelis*. The addition of intact human serum, as opposed to heat-inactivated serum, completely inhibited the growth of *E. anophelis* in vitro (Fig. 4*A*). Furthermore, incubation with human serum resulted in the deposition of MAC on the surface of *E. anophelis* (Fig. 4*B*). Notably, significant MAC deposition was also observed on the surface of another antimalarial bacterium, *Serratia marcescens* (7), but not on the control bacterium *Asaia bogorensis* (Fig. 4*B*). These findings indicate that host-derived C3 can inhibit the growth of the antimalarial *E. anophelis* by depositing MAC on its surface.

Complement C3 is activated by the classical, lectin, and alternative pathways (30). The classical pathway is triggered by the formation of antibody–antigen complexes, which may occur during the sexual stage of malaria infection (31). Therefore, we investigated whether host C3 in the mosquito midgut was activated by antibodies binding to antigens in the sexually developed forms of the parasite. Parasite-specific IgG and IgM were detected in the serum of infected WT and $C3^{-/-}$ mice as early as 4 d post–*P. yoelii* infection. While IgM and IgG2b titers were significantly elevated in the serum of infected $C3^{-/-}$ mice, the level of IgG3 was significantly reduced compared to that in infected WT mice (*SI Appendix*, Fig. S8 *A–F*). Notably, C3 activation levels in the midgut of mosquitoes that fed on either parasite-infected WT or B cell–deficient (μMT) mice were comparable (*SI Appendix*, Fig. S8*G*), indicating that C3 activation occurs in an antibody-independent manner.

Host-derived C3 in mosquitoes may be activated through the alternative pathway (26). This pathway relies on the spontaneous hydrolysis of the internal C3 thioester group, which subsequently binds to Factor B. The resulting hydrolyzed C3 and Factor B complex cleaves additional C3 molecules into C3a and C3b, forming the alternative C3 convertase, C3bBb (32). To assess the role of the alternative pathway in C3 activation within the mosquito midgut, parasite-infected mice received oral gavage of the Factor B inhibitor LNP023 (33) prior to mosquito feeding. C3 activation was completely inhibited in mosquitoes that fed on LNP023-treated mice. Furthermore, we observed a significant increase in the abundance of symbiotic microbiota and a decrease in the number of oocysts and sporozoites compared to mosquitoes that fed on control mice (Fig. 4 *C–G*). These results indicate that host C3 in the mosquito midgut is activated via the alternative pathway, leading to the direct killing of *E. anophelis*.

Host-Derived Complement C3 Significantly Promoted *P. falciparum* Transmission. Next, we investigated whether the influence of C3 on rodent malaria transmission also applies to the human malaria parasite, *P. falciparum*. We used the standard membrane-feeding assay (SMFA) to feed in vitro-induced *P. falciparum* gametocytes to *An. stephensi* in the presence of either normal human serum (NHS) or heat-inactivated human serum (HIS). The infection intensity and prevalence in mosquitoes that fed on *P. falciparum* gametocytes with NHS were significantly higher than those in mosquitoes fed with HIS (Fig. 5 *A–C*). Additionally, the application of the Factor B inhibitor LNP023 markedly reduced both the infection intensity and prevalence in mosquitoes fed on *P. falciparum* gametocytes in NHS (Fig. 5 *D* and *E*). These findings strongly indicate that host-derived complement C3 enhances the transmission of *P. falciparum*.

To investigate whether the effect of host-derived C3 on *P. falciparum* transmission is dependent on the modulation of *E. anophelis*, we assessed the abundance of *E. anophelis*-specific 16S rDNA in the midguts of mosquitoes that fed on *P. falciparum* gametocytes in either NHS or HIS. We found that the abundance of *E. anophelis* was significantly lower in mosquitoes that fed on *P. falciparum* gametocytes in NHS compared to those in HIS (Fig. 5*F*). Subsequently, we eliminated the symbiotic bacteria in the mosquito midgut using antibiotics and then colonized the mosquitoes with *E. anophelis* before feeding them *P. falciparum* gametocytes. The infection intensity and prevalence in mosquitoes that fed on *P. falciparum* gametocytes in NHS were comparable to those in mosquitoes that fed on HIS (Fig. 5*G*). However, after colonization with *E. anophelis*, the infection intensity and prevalence in mosquitoes feeding on *P. falciparum* gametocytes in NHS were significantly higher than in those feeding on HIS (Fig. 5 *H* and *I*). These results confirm that host-derived complement C3 can significantly enhance *P. falciparum* transmission by modulating the abundance of *E. anophelis* in the mosquito midgut.

The Absence of Host C3 Significantly Augmented the Transmission-Blocking Efficacy of Anti-Pfs25. Malaria transmission-blocking vaccines are designed to induce antibodies against surface proteins on the sexual forms of the parasites, such as Pfs230 and Pfs25. While the transmission-blocking effect of anti-pfs230 is known to depend on complement activation (34, 35), the role of complement in the efficacy of anti-Pfs25 remains unclear. To

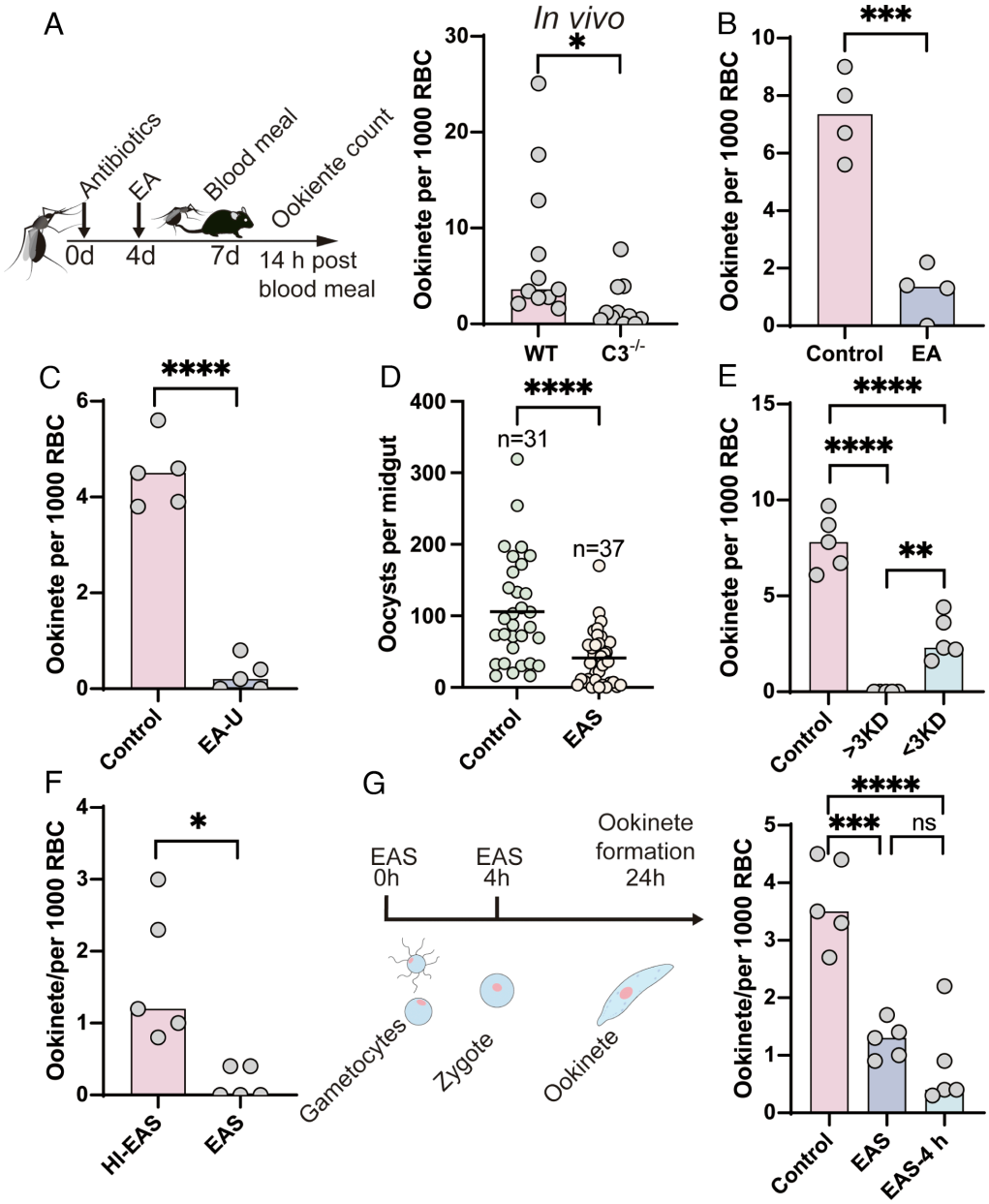

Fig. 3. Symbiotic bacteria *E. anophelis* directly inhibit malaria parasite sexual stage development in the mosquito midgut. (*A*) Mosquitoes were treated with antibiotics, colonized with or without *E. anophelis*, and then fed on *P. yoelii*–infected WT mice. 14 h later, the number of ookinetes per 1,000 RBCs in the midgut of mosquitoes, regardless of *E. anophelis* colonization, was determined. (*B*) The number of ookinetes generated per 1,000 RBCs in the blood of *P. yoelii*–infected WT mice (n = 4) was measured after in vitro culture with or without 10^6 *E. anophelis* for 24 h. (*C*) The number of ookinetes generated in the blood of *P. yoelii*–infected mice (n = 5) cultured in the bottom chamber of a Transwell system, with or without *E. anophelis* in the upper chamber (EA-U), for 24 h. (*D*) The number of oocysts in the midgut of mosquitoes prefed with or without the supernatant from in vitro cultured *E. anophelis* (EAS) and subsequently fed on *P. yoelii*–infected mice. Individual dot represents each mosquito, with a line indicating the median. The N in the panel is noted as the total number of mosquitoes. (*E*) The number of ookinetes generated in the in vitro cultured blood of *P. yoelii*–infected WT mice (n = 5) supplemented with EAS fractions greater than 3 kDa and less than 3 kDa for 24 h. (*F*) The number of ookinetes generated in the blood of *P. yoelii*–infected mice (n = 5) cultured in vitro for 24 h in the presence of heat-inactivated EAS or normal EAS (HI-EAS). (*G*) EAS was added to the in vitro cultured blood of *P. yoelii*–infected mice (n = 5) at 0 or 4 h, after zygote formation. Twenty-four hours later, the number of generated ookinetes was counted and compared. Two independent experiments were performed for each experiment with similar results. Data are presented as median with dots representing biological replicates. Unpaired Welch's correction *t* test (two-tailed) was applied for results (*A* and *F*); unpaired *t* test (two-tailed) was applied for results (*B* and *C*); one-way ANOVA analysis for results (*E* and *G*); Mann–Whitney *U* test (two-tailed) for result (*D*). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns: not statistically significant.

investigate this, we infected mice with Pfs25 transgenic *P. berghei* (*P. berghei*-pfs25) (*SI Appendix*, Fig. S9) and then inoculated WT and $C3^{-/-}$ mice with or without anti-Pfs25 prior to mosquito feeding. The infection intensity and prevalence in mosquitoes that fed on infected WT or $C3^{-/-}$ mice pretreated with anti-Pfs25 were markedly reduced compared to those that fed on control mice (Fig. 6*A*). However, mosquitoes that fed on infected $C3^{-/-}$ mice exhibited significantly lower infection intensity and prevalence than those that fed on infected WT mice, regardless of anti-Pfs25 pretreatment (Fig. 6*A*). Similar results were observed when infected WT mice were pretreated with the Factor B inhibitor LNP023 (Fig. 6*B*). Furthermore, we validated the inhibitory effect of C3 on the transmission-blocking activity of anti-Pfs25 against *P. falciparum*. Anti-Pfs25 demonstrated higher transmission-reducing activity (TRA%) and transmission-blocking activity (TBA%) when mosquitoes fed on *P. falciparum* gametocytes in HIS compared to NHS (Fig. 6*C*). In summary, the absence of host C3 significantly enhances the transmission-blocking efficacy of anti-Pfs25.

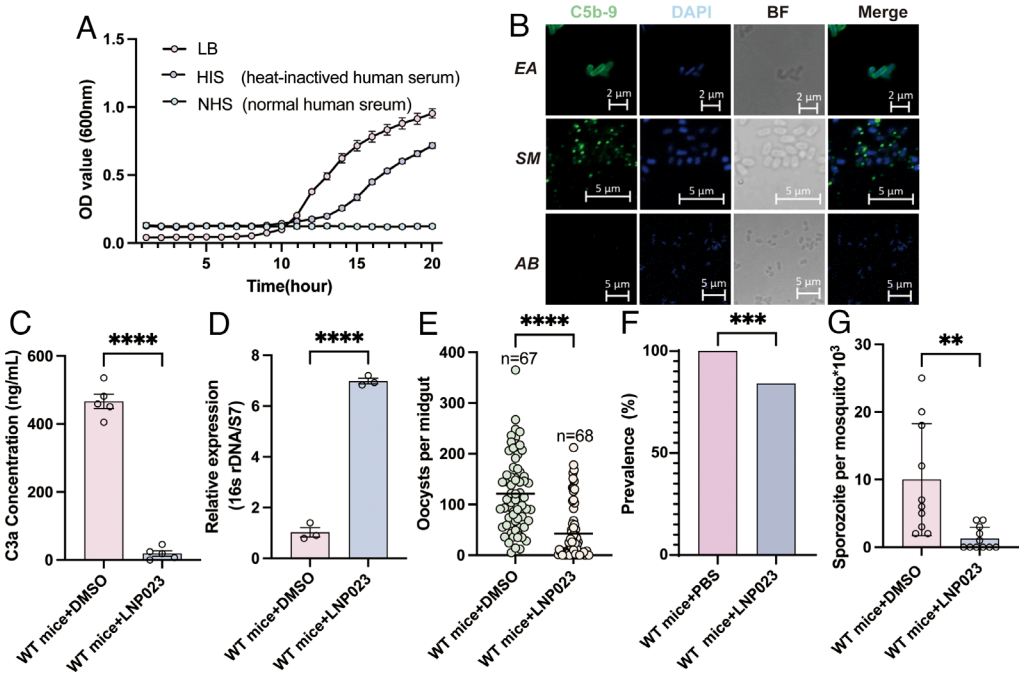

Fig. 4. *E. anophelis* was directly killed by alternative pathway-activated host C3. (*A*) The growth curve of *E. anophelis* (n = 5) incubated for 20 h with HIS, NHS, or LB (control). (*B*) Indirect immunofluorescence detection of C5b-9 (MAC) formation on the surface of *E. anophelis* (EA), *S. marcescens* (SM), or *Asaia bogorensis* (AB) after incubation with human serum. (*C*) C3a concentration in mosquito midgut was measured using ELISA 3 h after feeding on infected mice pretreated with dimethyl sulfoxide (DMSO) or Factor B inhibitor LNP023. Twenty mosquito midguts were pooled to constitute one biological replicate (n = 5). (*D*) The relative ratio of symbiont microbiota 16S rDNA to S7 at 12 h in the midgut of mosquitoes feeding on infected mice pretreated with or without LNP023. Twenty mosquito midguts were pooled to constitute one biological replicate (n = 3). (*E* and *F*) The number (*E*) and prevalence (*F*) of oocysts in the midgut of mosquitoes feeding on infected mice pretreated with or without LNP023. Individual dot represents each mosquito, with a line indicating median. The N in the panel is noted as total number of mosquitoes. (*G*) The number of sporozoites in the salivary gland of mosquitoes feeding on infected mice pretreated with or without LNP023. Ten salivary glands were pooled to constitute one biological replicate (n = 10). Two independent experiments were performed for each experiment with similar results. An unpaired *t* test (two-tailed) was applied for results (*C* and *D*); data presented as mean ± SD (*C*–*E*). An unpaired *t* test with Welch's correction (two-tailed) was used for result (*G*). Mann–Whitney *U* test (two-tailed) for result (*E*); and Fisher's exact test (two-sided) for result (*F*). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

After pretreatment with anti-Pfs25, the abundance of *E. anophelis* was significantly higher in mosquitoes that fed on infected $C3^{-/-}$ mice compared to those that fed on infected WT mice (Fig. 6*D*). Additionally, the elimination of symbiotic bacteria in the mosquito midgut using antibiotics resulted in similar TRA% and TBA% of anti-Pfs25 between mosquitoes feeding on infected WT and $C3^{-/-}$ mice. However, once the antibiotic-treated mosquitoes were recolonized with *E. anophelis*, the TRA% and TBA% of anti-Pfs25 were higher in those feeding on infected $C3^{-/-}$ mice than in those feeding on infected WT mice (Fig. 6*E*). Therefore, unlike Pfs230, C3 significantly diminished the transmission-blocking activity of anti-Pfs25 by influencing the abundance of *E. anophelis* in the mosquito midgut.

Discussion

In natural malaria transmission, both host serum components and parasite gametocytes are taken up into the mosquito midgut. In this context, we demonstrated that host complement C3 significantly enhances malaria transmission in both rodent and human models. Moreover, inhibiting C3 activation markedly increases the transmission-blocking efficacy of anti-Pfs25 by promoting the lysis of *E. anophelis* in the mosquito midgut. Mechanistically, once ingested into the mosquito midgut, host C3 targets and destroys symbiotic bacteria that hinder the sexual development of the malaria parasite. This action effectively promotes malaria infection and diminishes the transmission-blocking efficacy of anti-Pfs25. Our findings reveal a previously unrecognized strategy employed by malaria parasites, whereby they utilize host complement C3 to facilitate their transmission. This insight opens an avenue for enhancing the efficacy of anti-Pfs25 in blocking malaria transmission through the inhibition of host C3 activation.

As blood-stage malaria parasites have developed numerous strategies to evade complement attacks (36), it is not surprising that C3 deficiency did not significantly affect parasite growth in the host. Additionally, C3 deficiency had no notable impact on the sexual stages of malaria within the host, as demonstrated by the ookinete assay conducted in vitro. This finding suggests that gametocytes in the host may also evade complement-mediated attacks. While *P. falciparum* gametes in the mosquito midgut have been shown to prevent the deposition of the MAC on their surface by recruiting factor H (20, 37), similar research has not yet been reported for gametocytes within the host.

To determine whether malaria parasites can utilize host-derived C3 to enhance their transmission, we employed three approaches: using C3-deficient mice, depleting C3 with CVF, and rescuing $C3^{-/-}$ mice with rC3. Our results indicated that administering rC3 (20 μg) to C3-deficient mice did not fully restore infection intensity to levels observed in mosquitoes feeding on infected wild-type (WT) mice. This incomplete restoration may be attributed to the lower dosage of rC3 compared to the physiological levels of C3 typically found in mouse serum (38). Previous studies have shown that malaria transmission is enhanced when mosquitoes feed on *Plasmodium*-infected, C5-deficient DBA/2 mice compared to C5-competent DBA/1 mice (19). However, the distinct genetic backgrounds of the DBA/1 and DBA/2 strains complicate the interpretation, preventing us from attributing this phenotype solely to C5 deficiency (39). Thus, their findings do not contradict our results. Moreover, Simon et al. demonstrated that gametes can evade C3-mediated lysis by binding factor H to their surface in the midgut

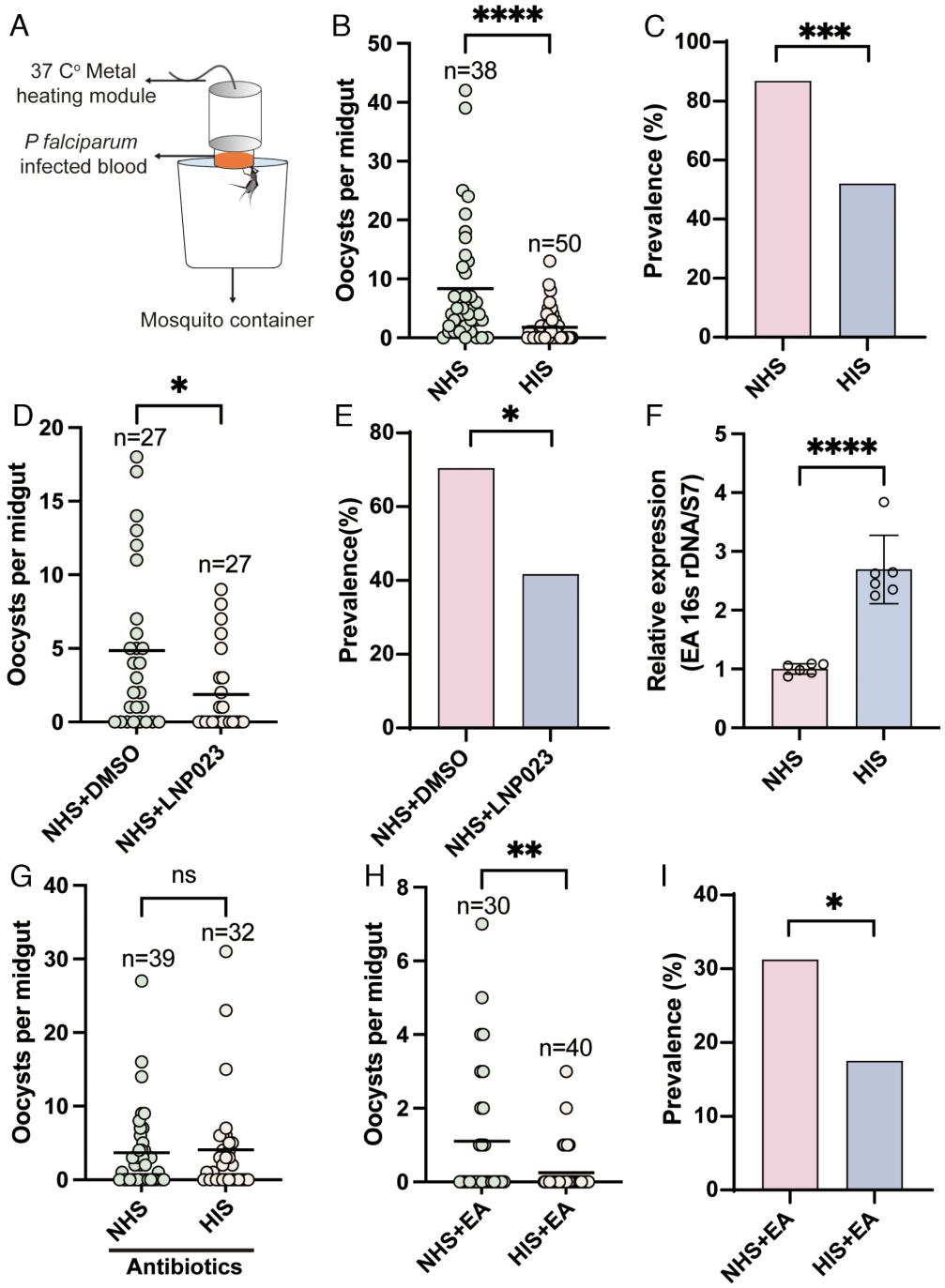

Fig. 5. Host-derived complement C3 significantly promotes *P. falciparum* transmission. (*A*) Schematic representation of the experimental procedure used to investigate the impact of host-derived C3 on *P. falciparum* transmission. (*B* and *C*) The number (*B*) and prevalence (*C*) of oocysts in the midgut of mosquitoes feeding on *P. falciparum* gametocytes resuspended in NHS or HIS were determined by SMFA. (*D* and *E*) The number (*D*) and prevalence (*E*) of oocysts in the midgut of mosquitoes feeding on *P. falciparum* gametocytes in the presence of DMSO or Factor B inhibitor LNP023. (*F*) The relative ratio of *E. anophelis*-specific 16S rDNA to S7 in the midgut of mosquitoes 12 h postfeeding on *P. falciparum* gametocytes in NHS or HIS. Data were presented as mean ± SD, with 20 mosquito midguts were pooled to create one biological replicate (n = 6). (*G*) The number of oocysts in the midgut of mosquitoes pretreated with antibiotics and fed *P. falciparum* gametocytes resuspended in NHS or HIS. (*H* and *I*) The number (*H*) of and prevalence (*I*) of oocysts in mosquitoes pretreated with antibiotics, colonized with *E. anophelis*, and fed on *P. falciparum* gametocytes resuspended in NHS or HIS. Two independent experiments were performed for each experiment with similar results. For (*B*, *D*, *G*, and *H*), individual dot represents each mosquito, with the line indicating the median. The N in the panel is noted as total number of mosquitoes. A Mann–Whitney *U* test (two-tailed) was applied for results (*B*, *D*, *G*, and *H*); unpaired *t* test (two-tailed) for result (*F*); and Fisher's exact test (two-sided) for results (*C*, *E*, and *I*). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; ns: not statistically significant.

of mosquitoes pretreated with antibiotics (20). Our study reveals that malaria parasites can similarly exploit the bactericidal effect of activated C3 against *E. anophelis*, a symbiotic bacterium that typically limits parasite development. Notably, both our work and that of Simon et al. suggest that disrupting C3 activation or blocking factor H binding significantly reduces malaria transmission. Together, these dual mechanisms may foster an environment conducive to transmission by diminishing bacterial competition and preventing direct immune attacks, showcasing the remarkable adaptive strategies employed by the pathogen.

Our study demonstrated that *E. anophelis* inhibits the zygote-to-ookinete transition in the mosquito midgut, aligning with

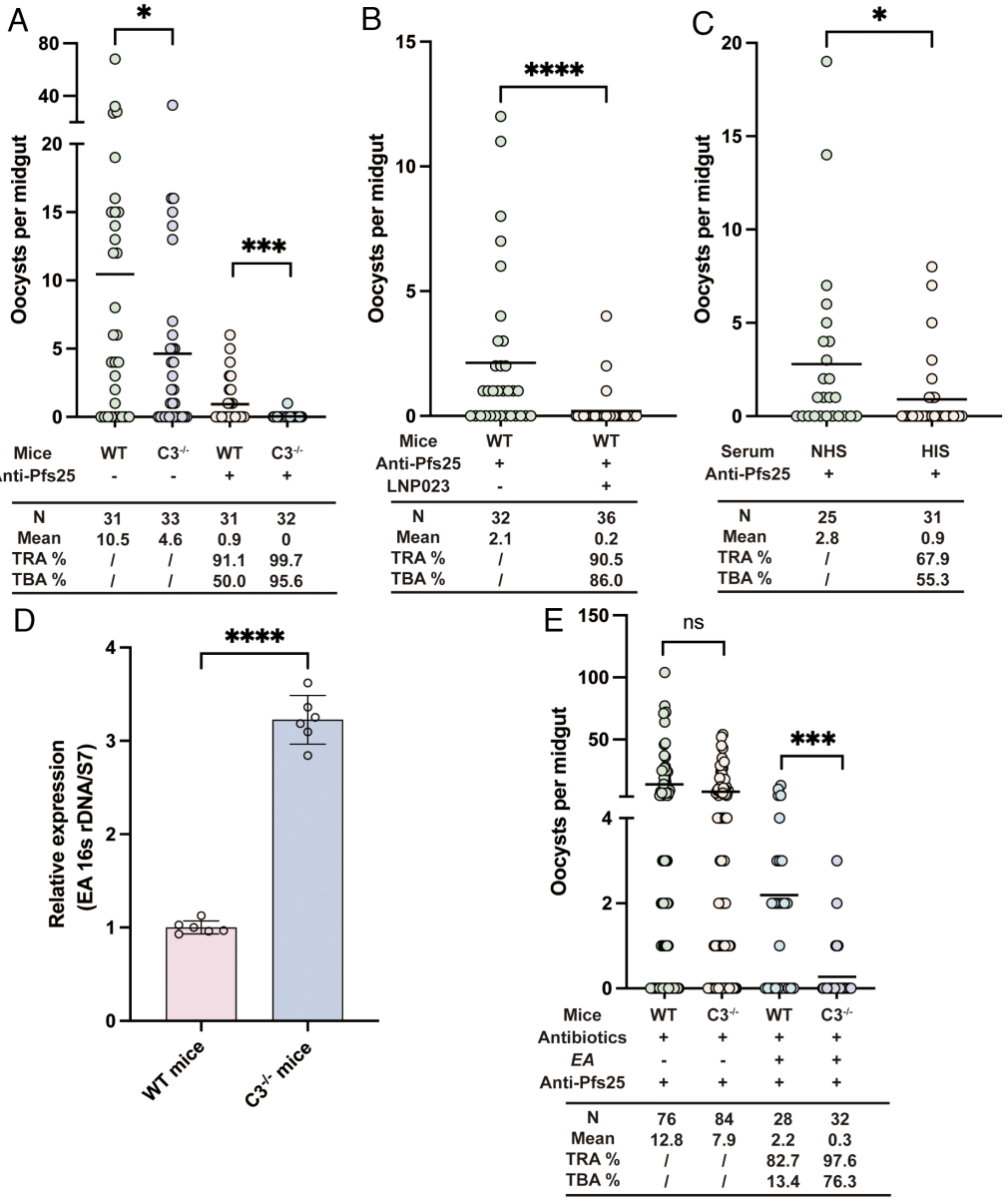

Fig. 6. Absence of host C3 significantly augments the transmission-blocking efficacy of anti-Pfs25. (*A*) The number of oocysts in the midgut of mosquitoes feeding on infected WT or $C3^{-/-}$ mice pretreated with or without anti-Pfs25. (*B*) The number of oocysts in the midgut of mosquitoes feeding on infected WT mice pretreated with anti-Pfs25 in the presence or absence of LNP023. (*C*) The number of oocysts in the midgut of mosquitoes feeding on *P. falciparum* gametocytes resuspended in NHS with anti-Pfs25 or HIS with anti-Pfs25. (*D*) The relative ratio of *E. anophelis*-specific 16S rDNA to S7 in the midgut of mosquitoes 12 h postfeeding on infected WT or $C3^{-/-}$ mice pretreated with anti-Pfs25. Data were presented as mean ± SD, with 20 mosquito midguts pooled to comprise one biological replicate (n = 6). (*E*) The number of oocysts in mosquitoes pretreated with antibiotics, recolonized with or without *E. anophelis,* and fed on infected-WT or $C3^{-/-}$ mice pretreated with anti-Pfs25. Two independent experiments were performed for each experiment with similar results. The total number of mosquitoes detected in the experiment is noted as N in the table below for panels (*A*–*C* and *E*), with a line indicating mean. A Mann–Whitney *U* test (two-tailed) was applied for results (*A*–*C* and *E*); unpaired *t* test (two-tailed) for result (*D*). *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$; ns: not statistically significant.

prior findings regarding its effects on gametocytes (40). The specific virulence factor responsible for this inhibition remains under investigation in our laboratory. Notably, recent research indicates that *E. anophelis* can eliminate other microbes by forming pores through the secretion of complement MAC-like factors (41). This finding not only clarifies why *E. anophelis* is the predominant symbiotic bacterium in the mosquito midgut but also lays the groundwork for exploring whether it blocks the zygote-to-ookinete transition through the secretion of these complement MAC-like factors.

In addition to *E. anophelis*, several other symbiotic bacteria in the mosquito midgut have been reported to influence malaria transmission (7, 8, 11, 27), suggesting that modulating these bacteria could be a promising strategy for reducing malaria transmission (27, 42). However, administering specific microorganisms or antimicrobial agents to mosquitoes in the field poses significant challenges. A more feasible approach may involve administering antimicrobial or other drugs to humans to modulate the symbiotic microbiota in the mosquito midgut. Unfortunately, while antibiotic exposure can alter the symbiont microbiota, it has also been shown to significantly increase malaria transmission (43). This method is further complicated by the emergence of antibiotic-resistant bacteria, such as *Elizabethkingia* (44) and *S. marcescens* (45), in the mosquito midgut. In contrast, complement has a broader spectrum of activity against the symbiotic microbiota in the mosquito midgut. Our findings indicate that the antimalarial bacteria *E. anophelis* and *S. marcescens* are sensitive to C3. Notably, administering a Factor B inhibitor LNP023 hindered malaria transmission by significantly increasing the abundance of the symbiotic bacterium *E.*

anophelis in *An. stephensi*. Interestingly, C3 deficiency has been shown to significantly reduce the development of experimental cerebral malaria (ECM) (46). Therefore, inhibiting host-derived C3 activation may represent a strategy for preventing ECM development in *P. falciparum*–infected patients and controlling *P. falciparum* transmission.

Although anti-Pfs230 relies on a complement-dependent mechanism to block malaria transmission (34, 35), our findings indicate that complement C3 actually reduces the effectiveness of anti-Pfs25 in this regard. This discrepancy may stem from the differing expression patterns of Pfs230 and Pfs25 on the sexual forms of malaria parasites. Specifically, Pfs230 is predominantly expressed by prefertilized forms, while Pfs25 is primarily expressed by postfertilized stages. In our study, we detected C3a in the mosquito midgut for only up to 3 h postblood meal, prior to the zygote-to-ookinete transition. Consequently, C3 in the mosquito midgut was degraded before ookinete formation, preventing any C3 activation by the classical complement pathway after anti-Pfs25 had bound to the ookinetes. However, host C3 could still become activated and lyse *E. anophelis* in the midgut during the first 3 h postfeeding, thereby promoting malaria transmission. Thus, complement C3 may diminish the efficacy of anti-Pfs25 in blocking malaria transmission. Conversely, the binding of anti-Pfs230 to prefertilized stages could activate C3 and lead to the destruction of the parasites. This effect might counteract the inhibitory influence of C3 on malaria transmission by reducing the abundance of *E. anophelis* in the mosquito midgut. Therefore, inhibiting C3 activation presents a promising strategy to enhance the efficacy of transmission-blocking vaccines targeting Pfs25.

In conclusion, the findings of this study suggest that malaria parasites can use host-derived C3 to enhance their transmission by modulating the symbiont microbiota within the mosquito midgut. This reveals a previously unrecognized strategy employed by malaria parasites. Therefore, inhibiting the activation of host-derived C3 could serve as an approach to prevent extrinsic transmission, reduce malaria transmission, and improve the effectiveness of anti-Pfs25 in blocking malaria transmission.

Materials and Methods

Ethics Statement. All animal protocols were reviewed and approved by the Animal Ethics Committee of the Army Medical University Institute of Medical Research (AMUWEC20218022). Anonymous human blood and serum were obtained from Chongqing Blood Bank; related experiments were reviewed and approved by the Ethics Committee of the Army Medical University Institute of Medical Research (AF/SC-08/1.0). Experiments involving *P. falciparum* were conducted in BSL-2+ laboratories.

Mosquito Rearing. *An. stephensi* (Hori strain) were maintained under controlled conditions (27 °C, 70 ± 10% RH, 12-h light:dark cycle with dark phase at 18:00) and provided ad libitum 10% sucrose.

Parasites and Mice. The rodent malaria parasites *P. yoelii*-BY265-RFP (47) and *P. berghei* ANKA were maintained in our laboratory through alternating passage between Kunming mice and mosquitoes. The *P. berghei*-pfs25 strain was created by replacing P25 (*PBANKA_0515000*) in *P. berghei* ANKA with Pfs25 (*PF3D7_1031000*) using CRISPR-Cas9. Kunming mice (female, 6 to 8 wk old) were obtained from the Laboratory Animal Center of the Army Medical University (Chongqing, China), while C57BL/6J mice (female, 6 to 8 wk old) were sourced from GemPharmat Animal Institute (Nanjing, China). $C3^{-/-}$ mice and B cell–deficient mice (μMT) on a C57BL/6J background were acquired from Jackson Laboratory (USA). All mice were housed in a temperature- and humidity-controlled environment with a 12-h light/dark cycle (lights off at 18:00).

Mice Infection. Female C57BL/6J mice or $C3^{-/-}$ mice aged 6 to 8 wk were intravenously infected with 1×10^6 parasitized RBCs (pRBCs) of *P. yoelii* or *P. berghei*. Parasitic levels and gametocyte counts were recorded daily by examining Giemsa-stained blood films under a light microscope.

Mosquito Infection. Female *An. stephensi* (3 to 6 d old) were starved for 24 h and then allowed to feed on *P. yoelii*- or *P. berghei*–infected WT or $C3^{-/-}$ mice for 30 min. Engorged mosquitoes were maintained under controlled conditions (species-specific temperatures: 19 to 20 °C for *P. berghei*; 23 to 24 °C for *P. yoelii*; 70 ± 10% RH; 12-h light/dark cycle) with ad libitum 10% sucrose. Midguts were dissected to quantify oocyst number at 7 d postinfection (*P. yoelii*) or 9 d (*P. berghei*). Salivary gland sporozoites were enumerated on day 18.

C3a Concentration Detected by ELISA, and Depletion and Rescue of Mouse C3. Serum was collected from WT and $C3^{-/-}$ mice at 0, 2, 4, and 6 d postinfection with *P. yoelii* or *P. berghei*. The concentration of C3a in the serum was measured using the ELISA kit (Cloud-Clone Corp, USA) following the manufacturer's instructions. The midguts of female *An. stephensi* mosquitoes were dissected between 0.5 and 10 h postfeeding, and the C3a concentrations in the midguts were also determined by ELISA. To deplete mouse C3 in serum, 25 μg CVF (Quidel, USA) was injected intraperitoneally into parasite-infected WT mice daily for 3 d before mosquito feeding. The efficacy of C3 depletion was determined based on the C3a concentration in mouse serum detected as above. For the C3 rescue assay, parasite-infected $C3^{-/-}$ mice were administered 20 μg of rC3 (Cloud-Clone Corp, USA) intravenously 30 min before mosquito feeding.

Quantitative Real-Time PCR. Total DNA was extracted from mosquitoes fed infected WT mice or $C3^{-/-}$ mice using a DNA extraction kit (Omega, USA) according to the manufacturer's instructions. The quality and concentration of each DNA sample were determined using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, 100 ng of DNA was used as a template for subsequent qPCR using the TB Green® Premix Ex TaqTMII (Tli RNaseH Plus, Takara Bio Inc, Japan). For quantifying the total bacterial load in the mosquito midgut, universal 16S rDNA primers were used (forward: 5′-ACTCCTACGGGAGGCAGCAG-3′; reverse: 5′-GGACTACHVGGGTWTCTAAT-3′). To measure the relative abundance of *E. anophelis*, specific 16S rDNA primers were employed (forward: 5′-CAAGCGGTGGAGCATGTGGTT-3′; reverse: 5′-ACGGCACGAGCTGACGACAA-3′). The assay was conducted on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA), and relative quantification results were normalized to the ribosomal protein S7 gene (internal control).

Antibiotic Treatment of Mosquitoes. Female mosquitoes aged 3 to 6 d were administered fresh filtered 10% sucrose supplemented with 10 U/mL penicillin (Sangon, China), 10 μg/mL streptomycin (Sangon, China), and 15 μg/mL gentamicin (Sangon, China) daily for 4 d. The bactericidal efficacy was assessed by detecting the bacterial 16S rDNA gene using real-time PCR.

DNA Extraction and 16S rDNA Amplicon Deep Sequencing. Mosquitoes were surface-sterilized with cold 75% ethanol and then washed three times with sterile 1× PBS. Each group consisted of 20 mosquito midguts, with three replicates for each group. Total bacterial DNA was extracted from the samples using the Power Fecal DNA Isolation Kit (QIAGEN, USA) following the manufacturer's protocol. The quality and quantity of the DNA were assessed using the 260 nm/280 nm and 260 nm/230 nm ratios. The V3–V4 region of the bacterial 16S rDNA gene was amplified using a universal primer pair along with adapter and barcode sequences. The PCR products from the first amplification were purified using VAHTS™ DNA Clean Beads. Subsequently, all PCR products were quantified with Quant-iT™ dsDNA HS Reagent and pooled together. High-throughput sequencing analysis of the bacterial rDNA genes was performed on the purified, pooled sample using the Illumina NovaSeq 600.

In Vitro Ookinete Culture. C57BL/6J mice were intraperitoneally injected with phenylhydrazine (2.5 mg/mouse). Three days later, mice were infected with 4×10^6 *P. yoelii*-RFP via intravenous injection. Blood samples were collected from the mice and immediately added to the ookinete culture medium, which consisted of RPMI-1640, 10% fetal calf serum, 100 μM xanthurenic acid (XA), 25 mM HEPES (pH 8.0), 100 μg/mL streptomycin, and 100 U/mL penicillin. The gametocytes were cultured at 22 °C for 24 h to allow gametogenesis, fertilization, and ookinete differentiation. The number of generated ookinetes was quantified per 1,000 RBCs in Giemsa-stained thin blood smears.

Effect of *E. anophelis* on Ookinete Generation In Vitro. To assess the inhibitory effect of *E. anophelis* on ookinete formation, 1×10^6/mL *E. anophelis* was added to the in vitro ookinete culture medium, and the ookinetes were counted after 24 h of incubation. To investigate whether *E. anophelis* secreted factors that inhibit ookinete formation, 200 μL of 1 OD *E. anophelis* was washed three times with PBS, resuspended

in 1 mL of ookinete culture medium, and then added to the upper chamber of a Transwell plate. The ookinete numbers were counted after 24 h of incubation.

Effect of In Vitro Cultured *E. anophelis* Supernatants on Ookinete Generation. *E. anophelis* was cultured overnight in LB medium (Sangon, China), washed, resuspended in M9 medium (Sigma, USA), and incubated at 28 °C with shaking at 200 rpm for 8 h. Cultures were centrifuged (3,000 rpm, 10 min), and supernatants were sterile-filtered (0.22 μm). For component analysis, supernatants were fractionated via 3 kDa centrifugal filters (Millipore). To assess whether the inhibitory activity was protein-mediated, supernatants were heat-treated (100 °C, 30 min). Subsequently, 200 μL aliquots of <3 kDa, >3 kDa, or heat-treated fractions were tested in ookinete cultures. To assess *E. anophelis*' inhibitory stage, supernatant was added to cultures either at initiation or 4 h postzygote formation. Ookinetes were quantified 24 h postincubation.

Gut Symbiont Isolation and Identification in *An. Stephensi*. Gut symbionts were isolated from 20 surface-sterilized (75% ethanol) *An. stephensi* females. Midguts were dissected, homogenized, and serially diluted (10-fold) on LB agar for 28 °C/24-h culture. Colonies underwent morphology-based classification (shape/color/size). Bacterial 16S rDNA was amplified via PCR (universal primers), purified, and sequenced (ABI 3730). Sequences were BLAST-aligned against GenBank for identification.

***E. anophelis* Growth Curve After Serum Incubation.** *E. anophelis* (2×10^3) were incubated with 200 μL of either intact or HIS for 30 min at 30 °C. The serum was removed by centrifugation at 3,000 rpm for 10 min, and 150 μL of fresh LB medium was added to each well. The 48-well plates were then placed on an orbital shaker set at 30 °C and 200 rpm for 20 h. The optical density at 600 nm (OD600) was measured using a microplate reader every hour.

Immunofluorescence Analysis. Bacteria were collected by centrifugation at 14,000 rpm for 10 min, washed three times with PBS, and then resuspended in an equal volume of PBS. The bacteria were incubated with human serum for 30 min at 28 °C. Ookinetes were harvested after 24 h of in vitro culture. All samples were washed and blocked with PBS/BSA (1:9, v/v), followed by incubation with primary anti-C5b-9 + C5b-8 antibodies (Abcam, USA) diluted 1:200 in PBS/BSA at 4 °C overnight. The samples were then washed 2 to 3 times with PBS and incubated for 2 h at room temperature (22 to 25 °C) with DyLight 488 secondary antibody (Abbkine, China) diluted 1:500 in PBS/BSA. Cell nuclei were counterstained with DAPI (Beyotime Biotech, China). Finally, the microscope slides were mounted using Dako Fluorescence Mounting Medium (Agilent Technologies, USA).

Inhibition of the Complement Alternative Pathway. Parasite-infected mice were administered an oral gavage of 60 mg/kg of the Factor B inhibitor LNP023 (MedChemExpress, USA) every 12 h for three doses prior to mosquito feeding. The alternative pathway inhibition efficiency was determined by measuring the C3a concentration in the midgut homogenate of mosquitoes fed on the parasite-infected mice using ELISA.

Rodent Malaria Transmission in Field-Caught Mosquitoes. *An. sinensis* mosquitoes were collected at Yingjiang, Dehong, Yunnan Province, China, using a mouth aspirator and were maintained at 26 °C and 70% RH. The mosquitoes were deprived of sugar solution for 2 d (as most mosquitoes caught in the field were engorged) before being fed on WT and $C3^{-/-}$ mice infected with *P. yoelii*-RFP for 4 d. Six days after the blood meal, the midguts were dissected, and the oocysts were counted under a phase contrast microscope.

***P. falciparum* Culture and Mosquito Infection.** The *P. falciparum* NF54 strain was used to culture mature gametocytes, as described previously (48). Briefly, the gametocyte culture was initiated at a parasitemia of 0.3 to 1% with a hematocrit of 4%. Culture medium (25 mM HEPES, 100 μg/mL hypoxanthine, 25 mM $NaHCO_3$, 5 μg/mL gentamicin, 10% human serum, pH 7.4) was refreshed daily at fixed times without fresh RBCs was added. Gametocyte development and contamination were monitored via blood smears every 2 d over 15 to 18 d. Mature gametocytes were diluted with fresh RBCs and mixed with 60% NHS or HIS (56 °C/45 min) to reconstruct blood for infection. All steps were conducted on a 37 °C heater to avoid temperature-induced preactivation and low infection rates.

SMFA was performed for mosquito infection, where 3 to 6-d-old female *Anopheles* mosquitoes were starved for 12 h before being fed on the infected blood. Mosquitoes were allowed to feed for 30 min through an artificial membrane feeding device. Unengorged mosquitoes were discarded. Mosquitoes were kept at 26 °C/70% RH under 12-h light/dark cycles for 9 d. Midguts were stained with 0.1% mercurochrome (10 min), and oocysts were quantified using phase-contrast microscopy.

Anti-Pfs25 Transmission Blocking Assay. The anti-Pfs25 antibody was expressed by GenScript (Shanghai, China). Briefly, the amino acid sequences for the variable light (VL) and heavy (VH) chains of anti-Pfs25 were obtained from the Protein Data Bank (PDB accession number: 6phc). The corresponding target DNA sequences were derived from the amino acid sequences, which were then optimized and synthesized. and the DNA sequence was optimized and synthesized. This DNA sequence was subcloned into pcDNA3.4 vectors, expressed upstream of the mouse CH1, Cκ, or Cλ regions. A transfection-grade plasmid was then maxi-prepared for expression in CHO cells. A transfection-grade plasmid was then maxi-prepared for expression in CHO cells. The filtered cell culture supernatant was loaded onto an affinity purification column. Following washing and elution, the eluted fractions were pooled, and the buffer was exchanged for the final formulation buffer. The purified protein was analyzed using SDS-PAGE and HPLC to assess molecular weight and purity, while the antibody concentration was determined using the bicinchoninic acid assay.

To evaluate the ability of anti-Pfs25 to block *P. berghei*-pfs25 transmission, WT or $C3^{-/-}$ mice were intravenously injected with 4×10^6 *P. berghei*-pfs25 pRBCs. Three days later, 20 μg of anti-Pfs25 was injected intravenously 1 h before mosquito feeding. Nine days after the feeding, the mosquitoes were dissected, and the oocysts were counted.

To assess the transmission-blocking activity of anti-Pfs25 against *P. falciparum*, 10 μg/mL of anti-Pfs25 was added to in vitro-cultured mature *P. falciparum* gametocytes, which were then fed to mosquitoes using a SMFA. Nine days later, mosquitoes were dissected, and TRA% and TBA% were determined by counting the oocysts in each mosquito.

Statistics. All analyses were conducted using Prism 10.0 (GraphPad Software, La Jolla, CA). When the number of replicates was less than 30, data were presented as the median with dots, unless it is pooled data. The statistical significance of differences in the number of oocysts between treatment groups and control group was assessed using the Mann–Whitney *U* test (two-tailed). Fisher's exact test (two-sided) was utilized to compare the prevalence of oocysts. Survival data for WT mice and $C3^{-/-}$ mice fed mosquitoes were analyzed using the log-rank (Mantel-Cox) test. Additional statistical significance was determined using either an unpaired *t* test (two-tailed) or one-way ANOVA analysis (two-tailed) after the Brown–Forsythe test, if equal variance is violated, then Welch's corrected *t* test or Welch's corrected one-way ANOVA were used instead; repeat measuring datasets were analyzed by using two-way ANOVA mix model. A $P < 0.05$ was considered statistically significant.

Data, Materials, and Software Availability. The 16S rDNA sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession number PRJNA1129287 (29). Additionally, the RNA sequencing datasets have been submitted to the NCBI Gene Expression Omnibus database under the accession number GSE271070 (28).

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