Trends in Parasitology

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Review

Two-Faced Immunity? The Evidence for Antibody Enhancement of Malaria Transmission

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Plasmodium **gametocytes can induce an immune response in humans that interferes with the development of sexual-stage parasites in the mosquito gut. Many early studies of the sexual-stage immune response noted that mosquito infection could be enhanced as well as reduced by immune sera. For *Plasmodium falciparum*, these reports are scarce, and the phenomenon is generally regarded as a methodological artefact. *Plasmodium* transmission enhancement (TE) remains contentious, but the clinical development of transmission-blocking vaccines based on sexual-stage antigens requires that it is further studied. In this essay, we review the early literature on the sexual-stage immune response and transmission-modulating immunity. We discuss hypotheses for the mechanism of TE, suggest experiments to prove or disprove its existence, and discuss its possible implications.**

Highlights

Individuals infected with *Plasmodium* can develop antigametocyte antibodies that are able to reduce or block the parasites onward transmission to mosquitoes, by inhibiting their development in the mosquito gut.

Enhancement of gametocyte transmission by immune factors is a common feature of early studies of the immune response to sexual-stage malaria parasites.

It is unclear if TE is a methodological artefact, or if it is mediated by specific antibody responses which may cause reduction at higher titres.

TE may affect the efficacy of transmission-blocking vaccines when antibody titres decline. Similarly, TE may contribute to recent observations that malaria transmission efficiency increases after successful control.

We review the literature on immune responses to *Plasmodium* sexual stages and suggest experiments to prove or disprove the existence of TE.

Antibodies and *Plasmodium* Transmission

A dominant role for specific antibodies in controlling malaria disease severity was first demonstrated in the 1960s by Cohen and McGregor [1,2]. IgG from immune adults was passively transferred to children with severe disease, rapidly reducing their parasite density and improving their symptoms. Anti-*Plasmodium* antibodies have since been shown to have multiple functions: preventing erythrocyte invasion by merozoites [3], activating complement [4], stimulating the neutrophil respiratory burst [5], opsonising infected cells for phagocytosis [6,7], reversing rosetting [6], preventing cells from binding to the microvasculature [8,9], and inhibiting sporozoite traversal or hepatocyte invasion [10,11]. Antibody responses against the transmissible **gametocyte** (see Glossary) stages of *Plasmodium* can also interrupt the parasite's life cycle by preventing it's sexual development in the mosquito midgut (Box 1). In short, the consequences of antibody responses to *Plasmodium* parasites appear overwhelmingly disadvantageous for their survival and transmission.

In other host–pathogen systems, parasite–antibody interactions may be more beneficial to the pathogen. In 1964 Hawkes showed that highly diluted antibodies increased the viral yields of flaviviruses, including West Nile virus and Japanese encephalitis virus [12]. Antibody-dependent enhancement (ADE) of infection has since been observed *in vitro* for many other viruses of medical and veterinary importance, including dengue virus (DENV), HIV, Zika fever virus, and foot-and-mouth disease virus (FMDV) [12,13]. Viruses with evidence for ADE share a few key features: all replicate inside macrophages, all show a degree of antigenic diversity, and all cause the production of partially neutralising antibodies [13]. For DENV, enhancement has been linked with severe clinical consequences during secondary, heterotypic infection in humans [12–18]. Halstead proposed that this was due to the opsonisation of DENV particles by cross-reactive

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Box 1. Immune Responses to Sexual-Stage *Plasmodium* sp.

During their replication in the blood, a minority of *Plasmodium* schizonts become committed to sexual development, producing merozoites that form gametocytes when they invade healthy red blood cells (RBCs). *P. falciparum* gametocytes develop in the bone marrow, and when almost mature they are released back into the blood where they may be ingested by blood-feeding mosquitoes. The infectiousness of gametocytes to mosquitoes is influenced by numerous factors, including gametocyte density [52–54,66,72], maturity [73], sex ratio [74], and human immune factors [75].

Human immunity may influence gametocyte transmission either by affecting gametocyte formation and survival in the blood, or by affecting the life stages that emerge after ingestion by mosquitoes. There is some evidence that inflammatory cytokines (TNF-α) induce cell-mediated killing of asexual parasites and gametocytes in hosts experiencing acute paroxysm [76,77]. However, cell-mediated gametocyte-specific killing in humans appears minimal or absent [78,79]. Because mature gametocytes lack the erythrocyte surface proteins of their asexual progenitors, antibody responses targeting gametocyte-infected erythrocytes are also either absent or difficult to detect [38,39,80,81]. Eventually though, all gametocytes not transmitted to mosquitoes break down in the blood, eliciting responses against gametocyte antigens that are inaccessible to antibodies whilst gametocytes are circulating in the blood stream. These gametocyte-specific antibodies may be ingested by mosquitoes alongside transmissible gametocytes, and if these antibodies interact with parasite proteins involved in gametocyte activation or gamete fertilisation they may inhibit the parasites' further development in the mosquito. In this way, exposure to the sexual stages of *Plasmodium*, or to specific sexual-stage antigens, can induce transmission-modulating [more commonly, transmission-reducing (TR)] immunity: an immunity elicited in the blood, which functions only in the mosquito.

IgG, which would bind the virus to Fc receptors on the macrophage surface, and possibly mediate immune suppression to further increase viral load [19,20].

For malaria parasites, there is sparse evidence of immune enhancement of asexual parasite infection; monoclonal antibodies (mAbs) to a *Plasmodium* asparagine-rich protein enhance invasion and growth of *in vitro* parasite cultures [21], and some sporozoite-specific antibodies, though inhibitory at high concentration, appear to enhance hepatocyte invasion when diluted [22]. For sexual-stage malaria parasites, immune TE is a common feature of the early literature in both humans [23–29] and animals [30,31]. In one of the most recent and comprehensive assessments of transmission-modulating immunity in humans, standard membrane feeding assays (SMFAs) showed that a significant proportion (7%) of 642 immune sera from gametocyte-positive individuals in Cameroon, Indonesia, and Tanzania enhanced the infectivity of gametocytes from culture by >20% [32]. Observations of antibody-mediated *Plasmodium* TE have been associated with low titres of **gamete**-specific antibodies – while high titres are associated with the more established and better quantified phenomenon of transmission reduction (TR). An untested hypothesis is that though low titres of antigamete antibodies may be unable to reduce transmission, their binding to proteins present on both male and female gametes may increase sexual interaction in the mosquito gut, increasing the likelihood of successful fertilisation [24,33].

Malaria control has entered a new era, in which declining global malaria incidence has made elimination a realistic prospect, with vaccines targeting sexual-stage parasites in development as part of the intervention arsenal [34]. The consequences of naturally acquired antigametocyte immunity for transmission efficiency are increasingly being studied [35,36]; TE as a possible counteracting immunological phenotype has not been examined in recent years. Moreover, malaria transmission-blocking vaccines (TBVs) are currently being assessed in human volunteers [37], and trials with transmission or incidence outcomes at the community level can be anticipated in the near future. As the efficacy of malaria TBVs depends on the dynamics of the immune response to sexual stage *Plasmodium* antigens, the evidence and potential mechanisms for antibody-mediated *Plasmodium* TE, however equivocal, require re-examination.

Glossary

Gametes: sexually dimorphic parasite forms that develop from gametocytes activating in the mosquito gut to undergo fertilisation. Female gametocytes give rise to a single female gamete, male gametocytes give rise to up to eight motile microgametes; each female gamete may be fertilised by a male microgamete.
Gametocytes: the sexual stages of the malaria parasite capable of reproduction in the mosquito. Female and male gametocytes circulate in the human peripheral blood, where they may be ingested by blood-feeding *Anopheles* mosquitoes and continue sexual development.
Mosquito feeding assay (MFA): xenodiagnostic assay used to determine the infectiousness of *Plasmodium* gametocytes to *Anopheles* mosquitoes. Mosquito feeding assay may refer to (i) skin feeding assays, in which mosquitoes are allowed to feed directly on a subject's skin, (ii) direct membrane feeding assays (DMFAs), in which mosquitoes feed on venous blood maintained at body temperature in a membrane feeding device, or (iii) standard membrane feeding assays (SMFAs), in which mosquitoes feed on cultured gametocytes in a membrane-based feeder system.
Prefertilisation antigens: antigens present during gametocyte development that are retained during gamete formation and may have important roles in gamete fertility. Naturally acquired transmission modulating immunity is due to exposure to prefertilisation antigens, including Pfs48/45 and Pfs230.
Relative infectivity: an alternative metric to TRA/% inhibition for transmission data, in which the mean oocyst intensity in test mosquitoes is presented as a value relative to the mean oocyst intensity in control mosquitoes. TRA and relative infectivity are both used in the literature; in this review we favour the use of relative infectivity (enhancement being positive, and reduction being negative).
Transmission-modulating immunity: if antibodies targeting *Plasmodium* proteins with a role in parasite development (e.g., Pfs48/45, Pfs230, and Pfs25) are ingested

Assessing Immune Modulation of *Plasmodium* Transmission

Assessing immune modulation of transmission requires measurement of gametocyte viability and infectiousness. *In vitro* assays can measure the interaction of immune factors with intraerythrocytic gametocytes [38,39], and assess their inhibition of gamete activity or the formation of postzygotic parasites [40,41]. The most comprehensive assays for assessing transmission modulation are **mosquito feeding assays**, in which mosquitoes are allowed to feed on potentially infectious blood, and transmission is later confirmed by the detection of *Plasmodium* oocysts in the mosquito gut or sporozoites in the salivary glands. The blood source can be either from naturally infected gametocyte carriers or from nonmalaria-exposed donor blood mixed with gametocytes from culture. In the former, transmission modulation by immune factors can be demonstrated with direct membrane feeding assays (DMFAs) by feeding infectious blood to mosquitoes separately with the donor's own (autologous) serum, or with the serum of an individual with no exposure to malaria [42]; higher relative infectivity with autologous serum would reflect serum-mediated TE, while the opposite would reflect TR [43,44]. The SMFA with cultured gametocytes allows for repeated measurements under controlled conditions [43], with transmission modulation by added immune factors measured against controls fed the same gametocyte-containing blood.

Using these assays, abundant evidence has accumulated that TR immunity exists in *Plasmodium*-exposed populations. Indirect evidence comes from studies showing that mosquito infection rates tend to increase in the field-based DMFA when autologous serum is replaced by naïve serum [25–27,45,46]. The use of the SMFA has formally demonstrated that whole serum and (now more common) purified IgG from malaria-exposed individuals can reduce mosquito infection rate and density [32,47,48]. The use of purified IgG has the advantage that the transmission-modulating effect of antibodies of this class of immunoglobulins can be examined independently of other serum components such as antimalarial drugs [49].

Evidence for Immune TR and TE

Animal Models

The existence of TR immunity was first definitively demonstrated in *Plasmodium gallinaceum*-infected chickens that had been immunised with inactivated gametocytes or gametes [40,50,51]. Antigamete antibodies appeared to be short-lived, but their titre was positively associated with gametocyte density and TR activity. Serum from the immunised birds retained TR activity in mosquito feeding assays for 1–2 months, at which point monitoring ceased. Antibodies that bound gamete surfaces were also observed in infected control birds immunised only with inactivated asexual stage parasites, indicating *de novo* antibody generation in response to live sexual-stage parasites [40,51]. TR immunity was subsequently demonstrated by similar methods in mice (*Plasmodium yoelii*) [52] and monkeys (*Plasmodium knowlesi*) [53,54]. Inoculations with high densities of *P. knowlesi* microgametes stimulated long-lived TR activity, which was successfully boosted by annual infection with blood-stage parasites and thus lasted the full 6 years of follow up in most animals [53].

Longitudinal observations of the immune response to viable infections were made from Rhesus macaques infected with *Plasmodium cynomolgi* (a close relative of *Plasmodium vivax*) [30]; Figure 1 is a graphic representation of antigamete antibody titres and infectivity to mosquitoes during these infections. Antigamete indirect immunofluorescence test (IFT) titres increased rapidly, in line with increasing parasite density. Relative infectivity in the DMFA was highest prior to peak parasitaemia, when antigamete titres were low and increasing. Peak parasitaemia coincided with the start of a decline into TR activity, which

by mosquitoes along with mature gametocytes in a blood meal, antibody interaction can prevent parasite development and cause mosquito transmission potential to be reduced or blocked. As described in this review, these or other immune components may also enhance immunity, by unknown mechanisms. Transmission-modulating immunity may be naturally acquired (see below, prefertilisation antigens) or elicited by vaccination.

Transmission-reducing activity (TRA)/% inhibition: TRA is the percent inhibition of infection (normally measured as the mean oocyst intensity) in a group of mosquitoes under test conditions, relative to a group of mosquitoes under control conditions. Test conditions may be the presence of a transmission-reducing drug or antibody in the infectious blood meal, while control conditions would indicate the absence of the antibody in the same blood meal, or more properly the presence of an antibody which has no effect on transmission.

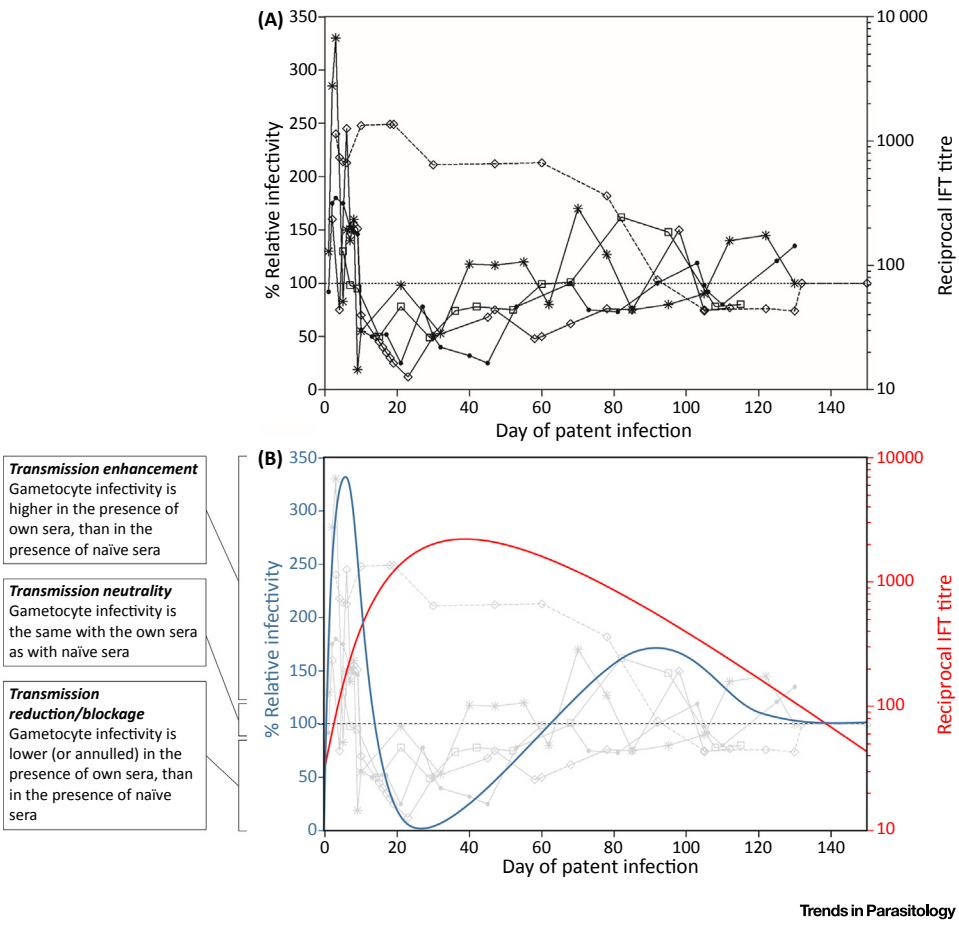

Figure 1. The Relationship between Antigamete Antibody Titre and Infectivity to Mosquitoes during Natural Infection. (A) Data from Naotunne *et al.* (1990) (Figure 3) [30] showing the relative infectivity of four toque monkeys (*Macaca sinica*) infected with *P. cynomolgi* to *Anopheles tessellatus* mosquitoes. Relative infectivity for each monkey is shown as symbols separated by unbroken lines. Relative infectivity was calculated as the geometric mean oocysts in mosquitoes after a blood meal containing each monkey's own serum, as a percentage of the geometric mean oocysts in mosquitoes after a blood meal in which the monkey's serum was removed and replaced with naïve (from an uninfected monkey) serum. The infectious blood meal was centrifuged and washed before resuspension in either autologous or nonimmune sera. The reciprocal immunofluorescence test (IFT) titre is given as reactivity to a gamete-enriched mixture of *P. cynomolgi* parasites for one monkey (*hollow diamond symbol), shown as symbols separated by broken lines. (B) Graphical representation of the same data, with explanation of transmission-modulating effects of the antigamete antibodies.

was strongest between 11 and 19 days after patency, when antigamete IFT titres peaked. As in chickens, antigamete antibodies appeared to have short half-lives. In monkeys, enhancement was again observed around 3 months after treatment during convalescence, when antibody titres were similar to the pre-peak period (<1:320 reciprocal titre). The authors reported that when total infectivity for each monkey was calculated as the sum of each day's mean oocyst count, 78–95% of the total infectivity between 0 and 150 days was during a period when the animal's sera resulted in enhancement of transmission. In separate experiments, transmission of *P. cynomolgi* from monkeys with prior *P. knowlesi* infection was enhanced threefold [31]. Here though, transmission modulation was not attributable to serum factors; sera from monkeys previously infected had no enhancing effect on gametocytes from monkeys with no prior infection.

Immune Enhancement and Reduction of Transmission to Mosquitoes in Natural Infections in Humans

Cross-Sectional Assessments

The first serological assessments of antigamete responses during naturally acquired human infections showed evidence of serum-mediated TR and TE [27]. Mendis *et al.* showed that Sri Lankan patients with acute *P. vivax* infections produced antibodies that bound *P. vivax* gamete proteins, and that their titre correlated with serum-mediated TR activity in the DMFA. Notably, gametocytes from 3 of the 40 patients studied were less infective to mosquitoes in the presence of naïve serum than autologous serum, suggestive of TE.

In 1988, Graves *et al.* published the first direct evidence of TR immunity in humans infected with *P. falciparum* [55], also demonstrating that malaria-exposed human sera recognised sexual-stage proteins Pfs230 and Pfs48/45 (Box 2). Among SMFA experiments that were duplicated, enhancement of infection (131–204% of the control) was observed in 6/33 individuals, the remainder showing variable levels of reduction (0.6–89% of the control). These data from an area of intense transmission were compared with an area of unstable transmission in Sri Lanka [25]. All Sri Lankan donors were *P. falciparum*-infected, and all infections were primary and symptomatic. TR activity, assessed by serum replacement DMFA, was observed in 23/41 individuals, while TE (relative infectivity between 125 and 400% of the controls) was observed in 13/41 individuals. Interestingly, immunoprecipitation of Pfs230 (in which the fluorescent conjugate recognised IgG only) correlated poorly with TR activity, while immunofluorescence assays (recognising IgG and IgM) correlated well.

In 1999 Healer *et al.* analysed TR immunity in 26 Gambian sera in SMFA experiments [28]. Again, both reduction (5/26) and enhancement (7/26) were observed, enhancement being up to 10 times higher than the control. High Pfs230 and Pfs48/45 Ab reactivity was associated with low relative infectivity in the SMFA; low reactivity had no clear association with infectivity. Importantly, both TR and TE were statistically significant and reproducible.

Box 2. Pfs48/45 and Pfs230

Early immunisation studies that stimulated interest in TR immunity [40,50,51] were followed quickly by others that identified Pfs48/45 and Pfs230 as immunodominant gamete surface proteins [82–84]. mAbs against Pfs48/45 protein are able to bind and neutralise gametes and have potent **transmission-reducing activity** in mosquito feeding assays [82], whereas mAbs specific to the larger Pfs230 lacked TR activity in primary tests [82]. It was shown elsewhere that the TR activity of α-Pfs230 mAbs was due to the antibodies' activation of complement-mediated gamete lysis [85–87]. The protein's presence in gametocytes is indicated by their recognition in malaria-endemic populations, and has been proven by proteomic analyses [88,89].

van Dijk *et al.* showed that Pfs48/45 was anchored to the gametocyte surface, and was essential for fertilisation [90]. When Pfs48/45 was knocked out, Pfs230 was not observed on the gamete surface, indicating that the protein was retained on the gamete surface only by its association with Pfs48/45. On the other hand, targeted disruption of Pfs230 also significantly inhibited oocyst production, indicating a central role in gamete fertility, possibly in the formation of exflagellation centres by male gametes [91,92].

Recognition of Pfs48/45 and Pfs230 in malaria-exposed individuals is often but not always associated with TR activity [28,45,59,61,75,93]. This has led to an assumption that other unknown gamete surface proteins may be jointly mechanistic in the development of antibody responses with TR activity. Recent data show empirically that naturally acquired human antibodies against Pfs48/45 and Pfs230 can reduce mosquito transmission, independent of other serum antibodies [36], and that immune sera with potent TR activity recognise unknown proteins on the surface of female gametes. Antibody responses to proteins other than Pfs48/45 and Pfs230 are associated with TR activity in the SMFA, and reduced transmission efficiency in the DMFA [36].

Other analyses of sexual-stage immunity with cross sectional or convenience sampling have generally restricted their analyses to individuals with observable gametocytes by microscopy. DMFA data from gametocyte carriers in high-endemic Yaoundé, Cameroon, showed that immune modulation occurred on a spectrum, with the majority of samples showing some level of reduction. Among the 65 gametocytaemic donors, TR (<50% of the control oocyst intensity, referred to as 'high' reduction) was common (29/65 sera), while very marginal higher infection (between 100 and 110% relative infectivity) was observed in 7/65 donors. [29]. Justifiably, the latter was dismissed as evidence of TE. In DMFA experiments with serum replacement, the transmission-modulating effect of Cameroonian and Gambian sera was observed to vary for autologous and nonautologous parasite isolates [56]. Of the 41 serum/isolate combinations tested, 16 blocked and 2 enhanced transmission; both enhancing sera blocked with different parasite isolates. Only one serum showed a consistent (blocking) effect for all parasite isolates, indicating significant variability due to gametocyte density, antibody titre, and/or antigenic polymorphism.

The most recent study with a specific focus on TE and TR immunity was by van der Kolk [32], using 642 sera from patent *P. falciparum* gametocyte carriers in Cameroon, Indonesia, and Tanzania. The authors concluded that TR immunity was more common than TE and had a larger effect size. Effect size was calculated as the relative infectivity/the standard deviation of oocyst intensities; TR (effect size >0.2) was present in 48% of sera, TE (effect size <0.2) in 7% of sera. Of 18 sera with TE in the primary experiment, six (33%, $P = 0.01$) retained their TE activity in a secondary feed. Of 175 sera with TR, 101 (58%, $P < 0.001$) retained TR in a second experiment. TR was associated with anti Pfs48/45 and Pfs230 seropositivity, whilst TE was not, that is, individuals with antibody titres over a defined cut-off were as common in the group that enhanced as in the group that had no effect on transmission. A more informative analysis would have assessed the association of specific antibody concentrations with ranked transmission modulation.

Longitudinal Assessments

A hypothesis that emerged from studies with animal models was that gamete antibodies might have both TE and TR properties, which manifest according to their concentration that varies over time (Figure 1) [30]. Such detailed assessments in humans may become more viable with controlled human malaria infections allowing gametocyte production [57,58], but existing data from naturally acquired malaria infections inevitably start from the point of patency or symptom presentation, excluding the assessment of transmission-modulation early in the infection during antibody proliferation.

Among *P. vivax* patients sampled by Mendis and colleagues, six patients were followed for 100 days after treatment and cure [26]. TR activity generally declined in line with antigamete Ab titres, which had a half-life of around 2 months. However, by 80 days post-treatment, serum from one individual was associated with TE eight times higher than the control. TR antibodies from these donors were later studied in the SMFA and compared with parallel dilutions of antigamete mAbs [24]. The results were noteworthy: at high dilutions/low antibody concentrations, TR serum promoted infection in mosquitoes feeding on blood that failed to infect mosquitoes in their absence.

Various studies have assessed TR activity longitudinally but did not report TE. Nonimmune Javanese migrants arriving in Indonesian Papua acquired antigamete Ab and TR immunity rapidly, and antibody titre appeared correlated with infection frequency [59]. Assessments in Tanzania showed inconsistent patterns of TR activity with age, but demonstrated the short-

lived nature of sexual-stage-specific antibodies [60,61]. The object of these studies was specifically to examine immune TR, so relative infectivity in the SMFA was capped at 100%, and TE was not reported.

mAbs Enhancing and Reducing Transmission to Mosquitoes

mAbs or polyclonal antibodies can be tested in DMFA or SMFA at a range of dilutions, allowing assessment of the relationship between antibody titre and transmission modulation. Most data available are for the transmission-modulating effect of P48/45 and P230 mAbs.

Pieiris and colleagues showed that when transmission-blocking *P. vivax* mAbs (targeting Pvs48/45) were diluted out in *P. vivax* gametocyte infected blood, the mAb TR activity declined until, at low titre, they gave rise to enhanced transmission [24]. Diluted still further, infection intensity returned to the same level as the control baseline. IgG purified from the hybridoma supernatants showed the same effect. As for the human sera from Sri Lanka described above, *vivax*-specific mAbs (diluted in naïve sera) were able to promote infection in serum replacement DMFA experiments in which gametocyte density was insufficient to cause infection alone.

Ponnudurai and colleagues investigated the impact of diluting *P. falciparum* gametocyte densities and mAb concentrations independently [62]. Unexpectedly, gametocyte dilution increased the mosquito infection rate in the presence of anti-Pfs48/45 mAbs, while decreasing infection rate in the presence of anti-Pfs25 mAbs. This difference may be due to increased fertilisation efficiency in parasites escaping reduction at low Pfs48/45 antibody concentrations. When both mAbs were diluted with static parasite densities, relative infectivity initially declined, then enhanced by 19.1–23% at low titre (0.01–0.02 mg/ml), before returning to baseline infectivity at the lowest tested titre (0.01–0 mg/ml). This variation was judged to be 'within normal range' relative to the control, and therefore 'in contrast to the enhancement of transmission by low antibody concentrations observed with *P. vivax*'. These conclusions precipitated a view that enhancement, if present, was lower in magnitude for *P. falciparum* than for other species combinations.

A recent assessment aimed to compare SMFA outputs between two laboratories, using the same mAbs and human sera [63] (Figure 2). Pfs48/45 mAb (85RF45.1) caused variable enhancement at the lowest tested concentration (1.2 μg/ml) in one laboratory (TropIQ, The Netherlands), and variable reduction in the second laboratory at the same concentration (LMVR, Bethesda, MD, USA). Further dilutions would be required to clarify the effect of low 85RF45.1 mAb titres. On the other hand, IgG from human serum caused enhancement at the lowest titre (23 μg/ml) at both laboratories: this across three replicates in each. Pfs25 mAbs (4B7) caused no enhancement in either laboratory, but the lowest dilution had not reached baseline in either laboratory.

Of note, mAbs against central peptides of the D2 region of gametocyte/gamete protein Pfs47 were recently shown to block transmission to mosquitoes, while mAbs against proteins at the N terminus of the same region were shown to double the mean oocyst density relative to controls [64]. These latest observations go against the hypothesis that TE may be due to nonantibody components of immune sera.

Testing Immune Transmission Modulation and the Mechanisms of Action

There are several reasons why historic evidence of immune-mediated *Plasmodium* TE needs to be interpreted with caution. Box 3 summarises the uncertainties that surround prior reporting on TE.

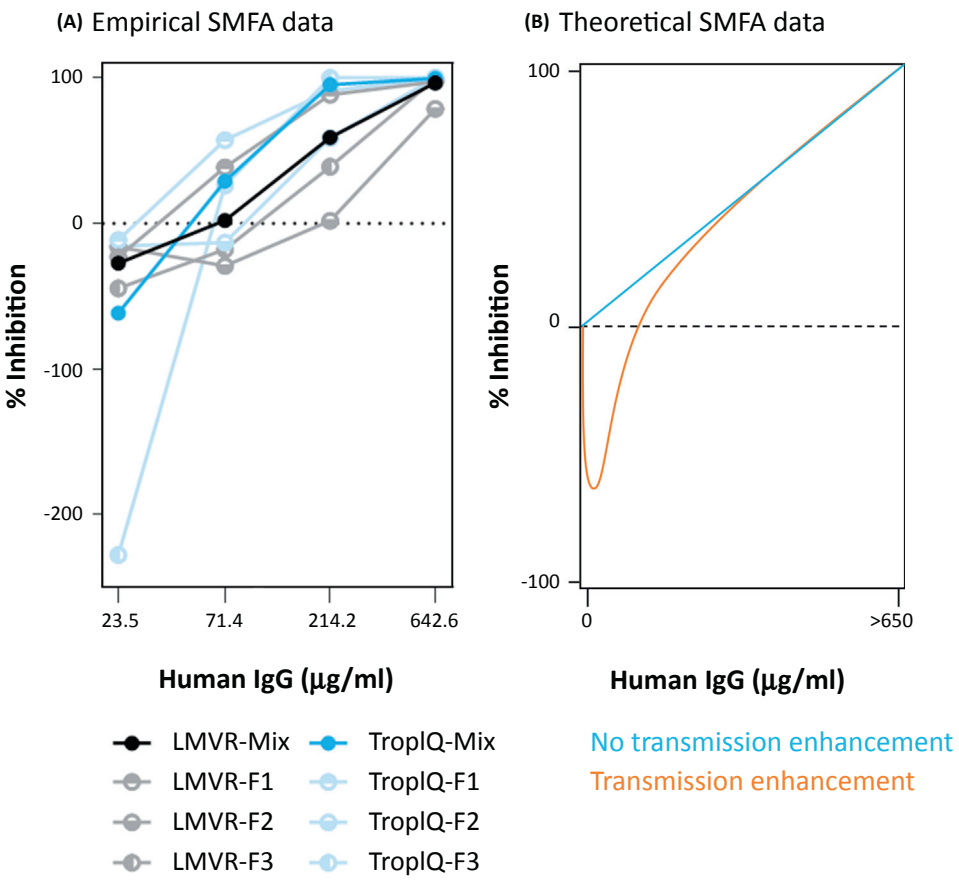

Figure 2. Serial Dilution of Actual (A) and Representative (B) Transmission-Blocking Human IgG in the Standard Membrane Feeding Assay (SMFA). (A) Transmission inhibition and titre of transmission-blocking human IgG from a Dutch expatriate, who had lived for many years in Cameroon and was gametocytaemic at the time of sampling – redrawn from the original data of Figure 4 of Miura *et al.* (2016) [63]. The sera were tested in triplicate SMFA at two independent institutions: TropIQ (Nijmegen, The Netherlands), and the Laboratory of Malaria and Vector Research (LMVR/NIH, Bethesda, MD, USA). Transmission inhibition (inhibition %) attributed to test antibodies was calculated as the percentage inhibition of mean oocyst density relative to isotypic controls (IgG from malaria-naïve donors). The exact transmission reduction (TR) from replicates is denoted as F1/2/3. Mix denotes the best estimate of the TR from the combined replicates, with 95% confidence intervals (CI). SMFA was performed as described above, and full details are in the paper in which these data were presented [63]. Average oocysts in the isotypic control experiments of LMVR-F1, -F2, -F3, TropIQ-F1, -F2 and -F3 experiments were 3.9, 60.3, 14.0, 16.9, 4.3, and 5.9, respectively. (B) Theoretical TR and transmission enhancement (TE) as a function of antibody titre, as might be apparent in a longer serial dilution of the same antibody as in panel A (reaching 0 µg/mL). The orange line represents IgG with enhancing and reducing properties; the blue line represents IgG with only reducing properties.

Despite these limitations, taken together, previous assessments provide tentative evidence for *Plasmodium* TE, suggesting that low titres of antibodies in gametocyte-exposed individuals may enhance transmission, while high titres of the same antibodies may reduce transmission (Figure 2B). Several possible mechanisms of action for TE have been proposed. As some gamete proteins are known to be present on both male and female gametes (Pfs48/45 and Pfs230), enhancement could feasibly occur if antibodies were able to bind simultaneously to proteins on gametes of both sexes [24]. With IgG, the presence of two binding sites makes this possible,

Box 3. Factors Influencing the Reliability of Observations of Plasmodium TE

Assay Performance

• The SMFA is optimised for assessment of strong TR

The SMFA has been optimised to achieve consistently high oocyst intensity and prevalence in control infections [94]. Though strong TR effects are detectable in these 'saturated' conditions, TE may be masked. There are similar concerns that because the SMFA does not produce naturalistic mosquito infections (ideally with the majority of mosquitoes harbouring one to five oocysts [95,96]), the assay may not do justice to the effects of intermediate TR/TE activity [67].

• The impact of nonspecific factors in blood meals is unknown

It is conceivable that higher nonspecific antibody content in a blood meal may be nutritive to parasites or mosquitoes, and that this could (directly or indirectly) benefit parasite survival. Most previous assessments of immune TR/TE have used isotypic controls to calculate relative infectivity (e.g., naïve serum vs test serum, nonspecific mAbs vs TR mAbs), but it has become commonplace to use nonisotopic human or foetal bovine serum as a control for feeds with additional purified antibodies. If any transmission modulation is due to nonspecific blood-meal components, the use of nonisotopic controls could give rise to apparent TR/TE where there is none.

Reporting

• TE is not reported

TE is often regarded as an artefact of the feeding system and not recorded. Relative infectivity is often floored at 100% (i.e., 0% TR activity) in published data. Artefact or otherwise, the true extent to which TE is observed is unlikely to be fully reflected in the literature.

Experimental Design

• Sample selection is biased toward TR

The majority of studies have focused on infectivity or TR activity, sampling only gametocyte-positive individuals to boost infectiousness in the DMFA, or 'to increase the chances of observing antigamete responses' [32]. Low sexual-stage antibody titre and TE may be most apparent at the start and end of an infection, at which times gametocytes are more likely to be subpatent [97]. Indiscriminate sampling or prospective longitudinal sampling may be more appropriate study designs to capture the full range of immune transmission modulation.

• Immune transmission modulation may vary between *Plasmodium* species

Parasite species and strains are used interchangeably to provide evidence for TE/TR, but differences in species gametocyte development may affect the kinetics of sexual-stage immunity.

• Is IgG purification appropriate for testing TE?

Assessments of transmission modulation have focused on the impacts of total IgG, but it is possible that other antibody classes (e.g., IgM), subclasses (e.g., IgG3), or as above, nonantibody factors, may have different transmission-modulating properties, and that such effects are generally missed.

though multiple gamete binding would potentially be more effective with multimeric IgM antibodies. Peiris and colleagues suggested alternatively that enhancement may occur if low level antibody binding were able to positively affect the conformation of proteins critical to gamete fertilisation, or that enhancement may be due to antibody-mediated prevention of inhibition by other human or mosquito factors [24]. The latter hypothesis would not be unique to transmission-stage parasites: Non-neutralising antibodies binding merozoite surface protein-1 (MSP-1) outside the MSP-1_{19} region appear to compete with anti-MSP-1_{19}-specific antibodies for its binding site during the parasite's erythrocytic cycle. Anti-MSP-1_{19} antibody binding results in the inhibition of MSP processing, which is required for cell invasion, whereas the binding of nonspecific MSP antibodies results in no such inhibition [65], thus enhancing infection rates.

de Arruda-Mayer suggested that TE of *P. cynomolgi* infection after exposure to *P. knowlesi* may be due to the absence of inhibitory serum factors during secondary infection rather than the presence of enhancing factors, though they could not prove this [31]. Da *et al.* showed that *Plasmodium berghei* infection was higher after dilution with uninfected blood, despite the resulting decrease in parasite density [66]. It is therefore possible that nonspecific factors may contribute to transmission modulation (either the presence of inhibitory factors during primary infections, or the absence of enhancing factors).

Several experiments can be proposed to confirm the existence of TE and elucidate its mechanism (Box 4).

Is Malaria TE Relevant?

As the sparse data described above suggest that there is some degree of TE for *Plasmodium*, the obvious question is how this might impact broader transmission dynamics. Modelling the impact of TE requires sensible parameterisation of its frequency and magnitude, both of which are unknown.

Epidemiology

When accurately quantified, there appears to be a relatively simple, saturating relationship between gametocyte density and mosquito infection rate [67]. In endemic populations, gametocyte density is generally low and overdispersed; surveys in Kenya and Burkina Faso, show that individuals who infect mosquitoes tend to infect few (2–23% infection rate, with median sample sizes between 17 and 91 mosquitoes) [99]. Based on the evidence we have described, TE appears to have a lower effect size than TR. However, as low gametocyte densities and low infection rates are the norm, even small increases in mosquito receptivity to parasite development could significantly affect population transmission potential. The relevance of intermediate TR activity on controlled transmission between rodents has been demonstrated, warning against a narrow focus on highly effective TR as the sole determinant of transmission efficiency [68]. Similar experiments with antibodies causing low and intermediate TE would be highly informative.

Box 4. Considerations for Testing *Plasmodium* TE

Does TE Occur, and Does It Occur as a Function of Serum Titre?

To determine if TE occurs at low serum/Ab titre, dilution series SMFA (with serum, purified serum Abs or mAbs) should be conducted, ensuring that total antibody content is consistent between feeds. Dilution should continue beyond the point at which relative infectivity reaches 100% (TR activity 0%); if TE occurs at low titres, further dilution would return infectivity to the level of the control (see Figure 2B in main text).

Is TE Due to Antigamete Antibodies, or Nonspecific Immune Factors?

SMFA could be conducted using whole sera, purified IgG (and other Ab classes/subclasses), and sera after extraction of antibodies to clarify the transmission-modulating effects of antibody and nonantibody serum factors; controls should be isotypic, that is, SMFA with whole endemic sera should use malaria-naïve sera as controls.

Does TE Occur with Antibodies Specifically Elicited by TBVs?

SMFA should include antibodies specific to both **prefertilisation antigens** (e.g., Pfs48/45 and Pfs230) and post-fertilisation antigens (e.g., Pfs25) to investigate mechanisms other than enhancement of gamete fertilisation (e.g., enhanced midgut homing/binding by ookinetes). SMFA should be conducted with and without complement; though some sexual-stage antibodies (α-Pfs230) are known to have complement-mediated TR activity [85], it is unclear whether the mechanisms leading to enhancement would be similarly dependent. Experiments should also include both functional (blocking) and nonfunctional mAbs, as it is currently unclear whether TE is due to Ab binding to TR epitopes, distinct non-TR epitopes, or whether any gamete binding is sufficient [64].

Is TE Due to Binding Antigens on Adjacent Gametes?

This hypothesis could be tested with bispecific antibodies: one fab region targeting a gamete antigen, the other targeting a nonmalaria-specific antigen (e.g., an HIV protein). If the presence of two binding sites is responsible for enhancement with IgG, dilution of bispecific antibodies will result in a linear decline of TR activity with Ab titre, while monospecific antibodies will cause enhancement at lower titres [98].

Do Different Antibody Classes/Subclasses Modulate Transmission Differently?

IgM has more binding sites than IgG, which increases the likelihood of binding different gametes. Each bond may have lower affinity, but multiple binding may result in a net increase in avidity. Purification of IgM from immune sera for the SMFA is therefore of significant interest for the assessment of transmission modulation. As antibody concentration, affinity, circulation time, and complement-activating activity could feasibly affect transmission modulating activity [86], assessments focused on antibody subclass would also be valuable.

Few studies have aimed to link transmission-modulating immunity with natural transmission rates in human populations. A recent study showed that high sexual-stage antibody titres were associated with significant TR in individuals with high gametocyte burdens, but not in individuals with submicroscopic infections [35]. These assessments modelled the impact of specific antibody responses (α-Pfs48/45 and α-Pfs230) on natural infectivity in the DMFA. The absence of transmission inhibition may be due only to the absence of reducing antibodies, but it is tempting to speculate that enhancement may be apparent in some of these individuals. There is evidence from longitudinal studies in Dielmo, Senegal, that the efficiency of malaria transmission increases as malaria is controlled. Between 1990 and 2007, slide prevalence of malaria parasites decreased from 68% to 30%, while over the same period the proportion of mosquitoes with sporozoites increased from 5% to 14% [69]. The increased transmission was linked to higher gametocyte biomass in infected individuals, which could occur if commitment rates were driven up by increased expression of the AP2-G protein [70]. The role of **transmission-modulating immunity** was not considered, but it is possible that the low antibody titres that result from infrequent parasite exposure (and thus immune boosting) have enhanced the efficiency of transmission from infected individuals gradually over time [69].

Vaccines

Trials to evaluate the safety and immunogenicity of Pfs25- and Pfs230-based TBVs in Malian adults are ongoing [37]. Such trials are welcome and long overdue, providing hope that these or other candidate TBVs close to clinical assessment [71] may soon be tested at the population level. If TE exists, and is associated with low or waning antibody titres, TBVs based on gametocyte proteins like Pfs230 could induce antibodies that initially cause transmission blockade but may be followed by a period of TE. The experiments suggested above will confirm if TE exists, and if it does, whether it is likely to be induced by current TBV candidates, or instead by a response to alternative epitopes within same protein, by a specific response to different (non-TR) proteins, or by nonspecific serum factors. In general, it is essential that the half-life of sexual-stage antibodies and the duration of their efficacy after exposure to natural gametocyte antigens or TBVs be determined. It would also be prudent to ensure that individual-based studies assessing the longevity of immune response to TBV candidates in phase I and II trials continue follow-up until, and for a short time after, antibody titres appear to return to baseline. Phase III trials, evaluated with transmission, infection, or clinical incidence outcomes, should incorporate longitudinal monitoring to rule out the possible effects of TE, and assess the association of antibody titre with immune boosting by reinfection.

Concluding Remarks

We have known for decades that antibodies with specificity for gametocyte proteins can inhibit *Plasmodium* establishment in the mosquito midgut. The knowledge that it could work both ways, inhibiting and enhancing, could change our understanding of natural malaria transmission and effect the development of vaccines based on sexual-stage proteins. At present, the evidence for TE in *P. falciparum* is incomplete whilst comparatively more evidence exists for *P. vivax*. If TE is proven to occur, several important questions will need to be answered to determine its relevance (see Outstanding Questions). If TE effects are reproducibly observed in malaria-exposed human sera, it will be of significant interest to determine its mechanism and interpret its role in natural malaria epidemiology; experiments to test its existence and mechanism are suggested in Box 4. The potential induction of TE by TBVs will also need to be investigated before it can be excluded.

Outstanding Questions

Does TE occur, and does it occur as a function of serum titre?

Does TE occur due to generic serum factors or specific antibody responses?

Are the causal responses, if antigen-specific, the same responses giving rise to transmission-reduction at higher titre?

Does TE occur with antibodies specifically elicited by malaria TBVs?

Is TE due to binding antigens on proximate gametes?

Do antibody classes/subclasses modulate transmission differently?

Is there variation in TE across *Plasmodium* host–parasite systems?

Is TE common across endemic settings?

How is the frequency and intensity of TE in endemic populations associated with gametocyte exposure and antigametocyte immunity?

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Disclaimer Statement

The authors declare that they have no conflicts of interest.

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