Immune tolerance caused by repeated *P. falciparum* infection against SE36 malaria vaccine candidate antigen and the resulting limited polymorphism

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Keywords: malaria, immune tolerance, vaccine, *Plasmodium falciparum*, serine repeat antigen, immune evasion

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Abstract (≤ 250 words)

The call for second generation malaria vaccines needs not only the identification of novel candidate antigens or adjuvants but also a better understanding of immune responses and the underlying protective processes. *Plasmodium* parasites have evolved a range of strategies to manipulate the host immune system to guarantee survival and establish parasitism. These immune evasion strategies hamper efforts to develop effective malaria vaccines. In the case of a malaria vaccine targeting the N-terminal domain of *P. falciparum* serine repeat antigen 5 (SE36), now in clinical trials, we observed reduced responsiveness (lowered immunogenicity) which may be attributed to immune tolerance/immune suppression. Here, immunogenicity data and insights into the immune responses to SE36 antigen from epidemiological studies and clinical trials are summarized. Documenting these observations is important to help identify gaps for SE36 continued development and engender hope that highly effective blood-stage/multi-stage vaccines can be achieved.

1. Introduction

Malaria, due to *Plasmodium falciparum*, disproportionately affects sub-Saharan African children, pregnant women especially the primigravidae, as well as those immunocompromised in malaria endemic areas [1]. The African region accounts for 95% of cases and 96% of malaria deaths worldwide. After more than 30 years of research and clinical trials, the World Health Organization (WHO) approved the world's first malaria vaccine in 2021 [2]. RTS,S/AS01 (Mosquirix®) targets the pre-erythrocytic stage of *P. falciparum.* Based on phase 3 and large pilot implementation programs the vaccine is recommended as a 4-dose schedule for children from 5 months of age living in moderate to high transmission areas [3]. The modest and short-lived efficacy of RTS,S/AS01, however, highlights that new, more efficacious vaccines be sought. Another anti-sporozoite vaccine, R21/Matrix-M, has recently reported a 77% protective efficacy in a phase 1/2b clinical trial [4], has an on-going phase 3 trial and follow-up study (ClinicalTrials.gov: NCT04704830), and is fast tracked in its regulatory approval for use in Ghana, Nigeria and Burkina Faso [5]. Similar to RTS,S, R21 contains recombinant particles of the central repeat and C-terminal circumsporozoite protein (CSP) fused to Hepatitis B surface antigen (HBsAg) but with improved protective efficacy due to a higher density of the CSP antigen on the VLP surface and formulation with a new, potent saponin-containing adjuvant [4]. Missing from the arsenal is a blood-stage vaccine to confer protection against disease and death. Moreover, long term studies of children vaccinated with RTS,S and living in areas of moderate

transmission showed a significant increase in rebound episodes of clinical malaria 3 to 6 years after the primary trial [6]. Thus, the availability of more than one vaccine that targets other life stages of the parasite, either as a stand-alone or combination/multi-stage vaccine, has a public health value and would be preferable for risk mitigation.

The complex parasite life cycle, genetic diversity (high polymorphism and allele-specific variations), and the various immune escape mechanisms of the *Plasmodium* parasite are among the reasons why there is limited success for most candidate malaria vaccines tested to date [7-10]. Furthermore, humoral (antibody) responses have often been harnessed for the evaluation of vaccines, but a thorough understanding of the dynamic interplay of the host immune response, the parasites immune evasion mechanisms, and what level of antibody response can be sufficient to confer protection is lacking. There are differences in the immune response between malaria naïve individuals and those in endemic areas (malaria exposed) [11]. We summarize below the immunogenicity observations with the blood-stage vaccine antigen, SE36, based on serine repeat antigen 5 (SERA5). The findings from other vaccine studies that suggest instances where humoral and cellular responses appear to be compromised/suppressed are also presented. This review highlights the diversity of immune evasion mechanisms that malaria parasites use to gain an edge in the host-parasite relationship, their implications for

vaccine-based strategies, and allows us to identify gaps in developing highly effective, long-lasting malaria vaccines.

2. Epidemiological studies on SERA5

SERA5 is a highly conserved [12, 13], essential [14] and abundant asexual blood stage antigen, expressed specifically during late trophozoite and schizont stages [15] (Fig. 1A). The protein is a vaccine candidate based on (a) epidemiological studies that show a strong correlation between high antibody titers and protection from malaria symptoms and severe disease [15-17]; (b) *in vitro* studies of sero-positive sera that demonstrate parasite growth inhibition [15, 18-20]; and (c) non-human primate challenge studies demonstrating protection against parasite challenge [20-22].

In sero-epidemiological studies from populations residing in malaria holoendemic areas, IgG antibodies were detected against the amino terminal part of SERA5 using either the N-terminal 47 kDa domain with serine repeats (*i.e.*, SE47') or without the serine repeats (SE36) (Fig. 1B). Humoral responses were evaluated in Ugandan adults and children [15-17]; pregnant women and newborns [23]. High titers of IgG anti-SERA5 were associated with protection from severe malaria [16,17,24] or absence of placental parasitemia, and babies delivered with normal birthweights [23]. Children with uncomplicated malaria have significantly higher median titers

142 of anti-SE36 IgG than age-matched children who experience severe malaria [17].

143 In 2 to 70 year-old Guadalcanal, Solomon Island residents, a significant negative
144 correlation was observed between high parasite densities and those positive for anti-SE36 [21].
145 Seropositive individuals had low parasite burden or were in the non-infected group while
146 seronegative individuals bore high parasite densities. The observed association of parasite
147 density with anti-SE36 antibody titer was specific and not due to non-specific
148 immunosuppression driven by severe infection since people with high parasite densities showed
149 no downregulation of anti-polio antibody titers. The proportion of seropositive individuals were
150 generally lower compared to individuals positive for merozoite surface protein-1 (MSP-1)
151 [17,23], or other erythrocyte stage proteins (rhoptry proteins, exported proteins, etc) [25]. Using
152 40 adult sera from residents in Atopi Parish (a malaria holoendemic area in Uganda), high
153 responders (ELISA titer >1000) to SE47' and $MSP1_{19}$ were 38 and 80%, respectively [15].
154 Sero-positivity to SE36 also showed a clear age-dependency in the Solomon Islands, although
155 as noted above, the rate of seropositivity does not exceed >50% of the population [21].

156 **3. Assessing the vaccine response to SE36**

157 A recombinant form of SERA5 N-terminal domain (SE36) was selected for clinical
158 development, prepared under Good Manufacturing Practice (GMP) standards and formulated
159 with aluminum hydroxide gel (AHG) to yield BK-SE36 (100 µg/mL SE36 protein and 1 mg/mL

aluminum) [21]. AHG was first chosen as the standard vaccine adjuvant because of the proven safety profile and production of primarily humoral immune and Th2 biased cellular responses. However, as it became clear in recent years that alum is not sufficient to induce effective immune responses for malaria vaccines [10,26,27], a second formulation/generation of SE36 was developed. BK-SE36 was administered concomitantly with another adjuvant containing unmethylated cytosine guanine (CpG) oligonucleotide (ODN) motifs to yield BK-SE36/CpG (100 µg/mL SE36 protein, 1 mg/mL aluminum and 1 mg/mL CpG ODN K3). CpG-induced activation of innate immunity has been reported and in initial studies, CpG ODN (code name: K3) efficiently induced Th1 response, selectively promoting cellular and humoral immune responses [22]. So far, all clinical studies reported to date show that both vaccine formulations have acceptable reactogenicity and have no unexpected safety signals [21, 28-30].

3.1 Early vaccination studies in non-human primates. SE36/AHG was immunogenic in chimpanzees, and squirrel monkeys [21]. In all three immunized chimpanzees, antibody titers increased 2 weeks after the first administration, peaked 2 weeks after the second administration and were maintained for more than 40 weeks. Squirrel monkeys vaccinated two or three times were protected against high parasitemia after parasite challenge. The challenge with *P. falciparum*-infected red blood cells elevated the antibody titer and protected squirrel monkeys against high parasitemia.

178 Cynomolgus monkeys administered with SE36/AHG/CpG had approximately 10 times
179 greater serum anti-SE36 IgG antibody levels and induced mixed Th1/Th2 responses compared
180 to those administered with SE36/AHG alone [22]. However, in squirrel monkeys, it was
181 surprising that although protection that correlates with decreased parasite density in the *P.*
182 *falciparum* challenge study was observed, administration of SE36/AHG/CpG did not result in
183 higher antibody titers when compared to those vaccinated with SE36/AHG alone suggesting
184 additional or independent immune response(s) with the use of CpG (*e.g.* T cell-mediated cellular
185 immunity).

186 ***3.2 First-in-human trials (Japanese adults)***. Phase 1a trials for BK-SE36 and BK-
187 SE36/CpG were conducted in malaria naïve Japanese adults. Seroconversion was 100% after
188 two vaccinations of full-dose BK-SE36 given 21 days apart [21]. When BK-SE36/CpG was
189 used, immunogenicity assessments showed high antibody titers with accelerated seroconversion
190 [29]. A 100% seroconversion was achieved in malaria naïve adults with one full-dose of BK-
191 SE36/CpG *vs*. 2 full-doses of BK-SE36. The full-dose group had significantly higher titers than
192 half-dose ($p = 0.002$ Student's t-test) and remained above baseline even after 12 months post-
193 Dose 2. BK-SE36/CpG formulation induced > 3-fold higher antibody titer than BK-SE36.

194 ***3.3 Clinical trial: Uganda***. In sharp contrast to phase 1a in Japan, the phase 1b trial
195 in Uganda showed low seroconversion (25%) in most vaccinated adults (21–32 years-old) [28].

196 Those who were administered with BK-SE36 were categorized as either responder (*i.e.* those
197 whose fold-change in anti-SE36 antibody titer from baseline was ≥ 2-fold after vaccination) or
198 non-responder (those whose fold change in anti-SE36 antibody titer from baseline was <2-fold).
199 All seropositive subjects (except one), did not have any obvious increase in antibody
200 titers (non-responders) 21 days post Dose 2. However, notably, not all seronegative subjects also
201 had a significant change from baseline antibody titers: more than half (55%) of seronegative
202 adults (21-32 years-old) were classified as non-responders, while only 45% showed a 2-fold
203 higher change in antibody titer from baseline confirming the low seroconversion to SERA5
204 observed in epidemiological studies [21]. In 16–20 years-old, 11–15 years-old and 6–10 years-
205 old, the proportion of subjects with ≥ 2-fold increase in antibody titers after 2 full-dose
206 vaccinations were 27, 27 and 73%, respectively [28] (Fig. 2A). When vaccinated with full-dose,
207 the change in antibody titers before vaccination to 21 days after Dose 2 was significant in 6–10
208 years-old (p=0.01) and 11-15 years-old (p=0.02) but not in the 16-20 years-old cohort.
209 The low seroconversion observed in Ugandan adults was unexpected when compared
210 to the 100% seroconversion in Japanese adults after either half- (containing 50 µg/mL SE36
211 protein and 0.5 mg/mL aluminum) or full-dose [21]. There was no indication of general immune
212 suppression correlated to host genetic background. Analysis of the allelic polymorphism of
213 human leukocyte antigen (HLA)-DRB1 alleles found that age rather than a particular DRB1

allele was associated with antibody response to vaccination [31].

The follow-up study demonstrated boosting of vaccine-induced immune response as a result of natural infection [32]. Children whose antibody titers against SE36 increased by ≥2-fold after vaccination and had high antibody titers throughout the follow-up (*i.e*, those with a geometric mean (GM) of 314 arbitrary units (AU), 21 days post vaccination to 102 AU at Day 365) did not experience malaria infection (defined as any parasitemia ≥ 100 parasites/μL). Responders who had GM <100 AU experienced 1-2 episodes of natural *P. falciparum* infection during a year of follow-up. Responders had significantly decreased odds to reinfection: the percentage of children who experienced more than one infection in the responder group was 18% *vs* 46% in non-responders; and 55% in the control. The observed association of fewer reinfections in responders was robust and was not influenced by age (p=0.175), antibody titer after infection (p=0.156) or parasitemia levels (p=0.091). GMs of anti-SE36 antibody titers were significantly different among responder, non-responder and those in the control. The 3.3-fold increase from baseline antibody titer after the first infection in the responder group was 1.5x higher than non-responders and 2.3x higher than the control group, suggesting immunological memory. Thus, in vaccinated subjects, natural infection can boost the immune response. There is little evidence of boosting in non-responder and control groups which may reflect the inherent low immunogenicity of SE36 during natural infections [32].

3.4 Clinical trial: Burkina Faso. BK-SE36 was immunogenic in 12–60 month-old Burkinabe children using either intramuscular or subcutaneous route of administration [30]. Seroconversion was not markedly different after two full-dose vaccinations in 25–60 month-old (83%) *vs* 12–24 month-old (79%) (Fig. 2A). A third dose, 22 weeks after the second dose (*i.e.*, 6 months from Dose 1), resulted in higher immune response and increased the proportion of children with >2-fold increase in antibody titer 4 weeks after vaccination (89% for 25–60 month-old; and 97% for 12 –24 month-old children) (Fig. 2A). Dose 3 successfully raised anti-SE36 antibody titer to levels higher than after primary vaccination (Dose 1 and 2). This kinetics of immune response after primary vaccination is a characteristic response akin to immunological memory [33]. Of interest, it was in contrast to the observed antibody response after three vaccine doses of BK-SE36 in a phase 1a study [21]. When given at 21-days interval, the induced antibody titer did not differ significantly between Dose 2 and Dose 3 in Japanese adults. Delaying the timing of the third dose appeared to have contributed to improved immunogenicity in Burkinabe children [30]. A delayed third dose is likely recommended to boost memory responses and keep antibody titer high in malaria endemic areas.

The youngest cohort (12–24 month-old) had 2- and 4-fold higher anti-SE36 antibody titers after 2 and 3 doses of BK-SE36 compared to the 25–60 month-old cohort [30]. The 25–60 month-old children are presumed to have a longer history of exposure to repeated malaria

250 infections and, within this cohort, a subgroup of children may also have a lower SE36
251 responsiveness. Indeed, more children are infected in this cohort than their younger counterparts
252 during recruitment and vaccination day (52.8% *vs*. 19.4% in 12–24 month-old, $p = 0.003$). It
253 was noted that concurrent infection (defined as any parasitemia > 0 by microscopy) during
254 vaccination days resulted in lower SE36 antibody titer levels in vaccinees [34]. Study
255 participants who were not infected during vaccination days had the highest GMT one month
256 post Dose 3. The difference in antibody titers between uninfected and infected subjects was
257 statistically significant. By multivariate analysis, a negative correlation between parasite density
258 and anti-SE36 antibody GMT was observed: there was a decrease in antibody GMT for every
259 1000 parasites/μL increase in *P. falciparum* density after adjustment for baseline antibody titer,
260 age and interaction between age and baseline antibody titer.
261 With BK-SE36, titers dropped to near pre-vaccination titers 5 months after Dose 2,
262 but was boosted, at Dose 3 as noted above [30]. Data on what level of anti-SE36 antibody titer
263 can be considered protective is limited at the moment. Using the high-titer pooled serum as
264 standard (*i.e.,* from Ugandan adults with naturally acquired titers where the positive standard
265 was calculated at 5000), the average antibody titer observed in BK-SE36 vaccinees was 10×
266 less. The plateau level of SE36-specific antibody titers achieved in chimpanzees [21] was also
267 10× higher than the average titer obtained in all BK-SE36 trials reported to date. Learning from

268 the experience of RTS,S, a combination of adjuvants or choice of a multi-adjuvant approach
269 may be ideal to improve the level and duration of antibody response [35]. This was the
270 motivation for the improved formulation of BK-SE36 using the CpG ODN (K3) adjuvant. The
271 vaccine formulation was tested in Burkina Faso for three age groups [36] following the success
272 of a phase 1a trial in Japanese adults [29]. CpG ODN (K3) improved vaccine immunogenicity,
273 as seen by the high titers and high seroconversion rates in vaccinated participants aged 21-45
274 years, 5-10 years, and 12-24 months [36].
275
276 **4. Observations from other malaria vaccine trials**
277 Clues on hyporesponsiveness largely stem from observations on the heterogeneity of immune
278 response in malaria endemic areas. As most trials that have progressed to late-stage clinical
279 evaluation targets the pre-erythrocytic stage, Table 1 highlights observations from these
280 advanced vaccine candidates. Both the controlled human malaria infection (CHMI) and clinical
281 trials in malaria endemic areas report observations of immune suppression in vaccinated
282 volunteers. In a number of blood-stage vaccine trials, similar observations were reported (Table
283 2).
284 ***4.1 Repeated infection in malaria endemic areas overshadowed the inductive***
285 ***capacity of candidate vaccines in clinical trials.*** Repeated malaria infections tailor the host's

immune response, making the parasite less recognizable by the immune system. In vaccine trials of merozoite surface protein3-long synthetic peptide (MSP3-LSP), high baseline antibody levels (as a result of natural infection/high transmission intensity) in semi-immune adults were presumed to have overshadowed the inductive capacity of the vaccine [52,53]. In 18–40 year-old Burkinabe subjects, although there was some indication of cell-mediated immune response (increase in lymphocyte proliferation index and IFN-γ), there was no detectable humoral immune response from MSP3-LSP vaccination when compared to children [52]. The highly variable individual antibody titers to another blood-stage vaccine candidate, the FMP1 vaccine (consisting of the 42-kDa carboxy-terminal 392 amino acids of MSP-1 and 17 non-MSP-1 amino acids encoding a 6-histidine tag plus linking sequence), in 18–55 year-old Malian adults was also attributed to the variation in background immunity [50]. When the baseline GMT for MSP-1_{42} titer was < 3,000 there was an 8-fold or greater increase in titer after vaccination; but when the baseline titer is > 20,000, the observed increase was only < 2-fold. This was similar in 18–55 year-old Kenyan adults [51]: the highest rise in antibody responses was seen in individuals with low pre-existing antibody titers. In Mali, antibody titers rose from < 6,000 to a peak of nearly 38,000 *vs* an increase from 17,000 to 46,000 in Kenya [50]. Regression lines fitted using data from Day 0, 75 and 90 in the two study sites, showed similar rates of increase in vaccine-induced antibody responses to the 3D7 alleles of MSP-1_{42} and MSP-1_{19} with only

304 pre-existing antibody titers as the difference.

305 Valuable clues can be obtained in younger age groups. In a multi-site (Burkina Faso,
306 Gabon, Ghana and Uganda) phase 2 children (12–60 months-old) trial of a recombinant vaccine
307 with glutamate-rich protein and MSP3 antigens (GMZ2), there was a greater increase in anti-
308 GMZ2 antibodies in children 1–2 years-old compared to children 3–4 years-old (14-fold
309 increase, 95% CI 8.7, 23 *vs* 5.7-fold, 95% CI 4.0, 8.2; respectively) [55]. Children with low
310 baseline antibody titers to GMZ2 responded strongly to vaccination, whereas those with more
311 exposure to *P. falciparum* infection showed a smaller boost in anti-GMZ2 IgG titers.

312 In a recent report of an RH5 phase 1b trial in Bagamoyo, Tanzania with 3 age cohorts
313 (18-35 years, 1-6 years and 6-11 months) higher anti-RH5 IgG antibody was induced in
314 pediatric groups (6 to 10-fold higher) compared to the adults [56]. ChAd63-MVA RH5 is
315 another blood-stage vaccine based on the reticulocyte-binding protein homolog 5 (RH5)
316 formulated with recombinant replication-deficient chimpanzee adenovirus serotype 64
317 (ChAd63) and the attenuated orthopoxvirus modified vaccinia virus Ankara (MVA). Authors
318 determined that there was no significant correlation between existing anti-vector immunity at
319 baseline and the humoral immunogenicity obtained 14 days after vaccination. The pre-existing
320 anti-ChAd63 antibody was found to be unlikely the reason for improved immunogenicity and
321 the high titers in younger age groups was attributed to greater B cell immunogenicity and/or

relatively higher vaccine dose per unit body mass in infants and children [56].

4.2 Parasitemia on vaccination day was associated with reduced humoral immunogenicity/immunosuppression.

Low response to vaccination in infected individuals has been reported in pre-erythrocytic vaccine trials (Table 1). Also, as mentioned for BK-SE36, reduced response in Burkinabe children was associated with concomitant infection at the time of administration [34]. The effect of parasite clearance with sulphadoxine-pyrimethamine (SP) a week before vaccination in 5–9 years-old Papua New Guinean children was assessed in a study using Combination B vaccine (composed of three blood-stage antigens: ring-infected erythrocyte surface antigen and MSP1 and MSP2) [49]. Interestingly, concurrent *P. falciparum* infection and SP pre-treatment at the time of vaccination did not alter the antibody response to this blood-stage vaccine candidate. It is, however, noted that IFN-γ response to MSP1 was substantially lowered in the vaccine group who had received SP before vaccination. Further work on the possible influence of concomitant *P. falciparum* infection is important in vaccine trials conducted in malaria endemic areas.

5. Host vitronectin and immunogenicity to SE47/SE36 molecule

Just as the host has developed several defenses against pathogens, pathogens have evolved a variety of immune evasion mechanisms: *e.g.*, antigenic variation, latency/sequestration, antigen

capping, antigenic disguise, molecular mimicry/molecular smokescreen, and immune suppression (inhibition of host factors, evasion of complement-mediated killing, B cell manipulation, etc.) [57,58]. Indeed, these various strategies, using one or more in combination, contribute to poor immunogenicity or the lack of effective vaccines for several viruses (*e.g.* herpes simplex virus, human immuno-deficiency virus), bacteria (*Mycobacterium tuberculosis*, *Helicobacter pylori*), and parasites (*Leishmania*, *Trypanosoma*) [59].

5.1 Fooling the host: recognition of "non-self to self". Some examples of pathogen subterfuge can be seen from *Schistosoma mansoni* and *S. haematobium* where a protein with 98% identical nucleotide is shared with the human ortholog complement C2 receptor inhibitory trispannin gene; *Taenia solium* uses the parasite protein paramyosin to inhibit complement proteins that in turn binds to another complement to inhibit the membrane attack complexes (MAC); *Brugia malayi* generates a protein similar to host keratinocytes periphilin-1 protein [58,60]. Being recognized as "self", the parasites camouflage themselves to avoid recognition by the host immune system. *In silico* genome-wide identification in *P. falciparum* has identified several var family members of erythrocyte membrane protein 1 (PfEMP1) having a stretch of 13 to 16 amino acids identical to the heparin-binding domain in human vitronectin [60]. The candidate mimicry motif in vitronectin is in the N-terminal half and in PfEMP1 the motif lies in the extracellular part of the protein, close to the predicted transmembrane domain. A mimicked

structure was also found in TRAP and CSP.

Vitronectin has been implicated as one of the serum proteins that function for the adhesion of parasites to endothelial receptors and is selectively internalized and associated with malaria pathogenicity [61]. Known as the glue protein, it is reported to promote cytoadherence, tissue regeneration, cell colonization, stabilization of plasminogen activator inhibitor 1, and inhibition of the formation of the pore-forming MAC of the complement system [61-68]. The protein is abundant in the extracellular matrix of different tissues and in the serum.

5.2 SE36 molecule tightly binds to vitronectin. In further efforts to elucidate the role of SE36 and understand the heterogeneity in immunological responses in vaccinated subjects, proteins binding to SE36 were elucidated. Using SE36-immobilized column, Tougan et al. [69] demonstrated that while vitronectin in naive human serum and Ugandan high titer serum equally bound to SE36 even in the presence of other serum proteins (Fig. 2B), no clear direct binding was observed for complement factors (C5, C7, C8, C9, and H), apolipoproteins (ApoAI, HDL, and LDL), thrombin, clusterin, fibronectin, serum albumin, CD5L, or CD14. Purified vitronectin, not human serum albumin, bound to SE36 in a concentration dependent manner. Binding of SE36 to vitronectin occurred even on commercially available vitronectin lacking somatomedin-B motif and Hemopexin domain 4, suggesting that both domains may not be essential for binding. The study also showed that since the recombinant *E. coli*-produced

vitronectin is not glycosylated, the glycosylation moiety may also not be necessary.

Vitronectin was internalized before DNA replication when SERA5 was not yet expressed [69,70]. At the trophozoite stage, vitronectin colocalizes with SERA5; and with the processed 47-kDa fragment during the schizont stage and on the merozoite surface [69]. The binding (dimer bound as predicted by surface plasmon resonance) site was mapped to 18 residues (NH2-Tyr-Lys-Tyr-Leu-Ser-Glu-Asp-Ile-Val-Ser-Asn-Phe-Lys-Glu-Ile-Lys-Ala-Glu-COOH) in the C-terminal region of SE36. This site is predicted to form an α-helix structure [20] and was conserved in 445 geographically distributed *P. falciparum* parasites [13,71]. The binding was tight (equilibrium dissociation constant, K_{D1} = 3.7 x 10^{-9} M), concentration-dependent and specific, observed even in the presence of other serum proteins or under the presence of naturally acquired anti-SE36 IgG [69].

5.3 ***SE47-vitronectin complex camouflage merozoites.*** To elucidate the role of vitronectin on the merozoite surface, SE36 beads acted as merozoite models in a phagocytosis assay with and without vitronectin [69]. IgG-independent phagocytosis was demonstrated using IgG purified from naïve human serum and Ugandan high anti-SE36 IgG titer serum. Without vitronectin, it was demonstrated that SE36-beads were engulfed by THP-1 cells in an antibody-independent manner. When vitronectin was bound to SE36 beads, engulfment by THP-1 monocytes was inhibited. Several other host proteins (e.g. thrombin; antithrombin III;

complements C9, C7) were significantly recruited on the merozoite surface. Unlike vitronectin, these host proteins do not have the motif or structural similarity to facilitate direct binding to SE36. Moreover, the specificity of inhibition was confirmed when inhibition was partially recovered using vitronectin-depleted sera. The binding of several other host proteins is consistent with vitronectin acting as a glue or bridging molecule [61,63,65,68]. Interestingly, although *in silico* analysis revealed that most var family members of PfEMP1, TRAP and CSP had a stretch of 13-16 amino acids identical to the heparin-binding domain (HB1) in vitronectin acting as a mimicry motif [60], the binding site of vitronectin to SE36 was demonstrated at the hemopexin domain (with remarkable binding in regions between hemopexin motifs 2 and 3; near HB2) [69]. Binding to hemopexin-type repeats in human vitronectin was first reported in *Streptococcus pyrogenes* [72].

In different pathogens the vitronectin-binding molecules interact with a conserved region in the host vitronectin molecule to regulate the complement mediated lysis [64-68]. *Streptococcus pneumoniae* [66] engage vitronectin to bind various oligosaccharides and complement inhibitor Factor H for stronger adhesion and effective bacterial colonization to host cells. In *Haemophilus influenzae*, a gram-negative pathogen, the binding of vitronectin acted as an intermediate bridging molecule to form a multicomplex of bacterial and human proteins that served for adherence to host cells as well as inhibited the host immune response by inactivating

412 the formation of MAC [67]. The binding of vitronectin on the surface of *Helicobacter pylori*
413 (specifically sulfated polysaccharides), in the presence of complement, was shown to inhibit
414 phagocytosis by macrophages [64]. The resulting vitronectin-C5b-7 complex cannot be inserted
415 into the cell membrane [65]; blocks the membrane binding site of Cb5-7 and the
416 deposition/polymerization of C9 effectively inhibiting MAC formation [68]. In malaria
417 parasites, MAC formation can lyse sporozoites, merozoites and gametes and its formation was
418 reportedly controlled by CD59, clusterin and vitronectin [73]. Although further studies are
419 needed to understand the multifaceted profile of vitronectin (to prevent attack by phagocytes
420 and/or evasion by direct lysis by complement) and how it is exploited by malaria parasites, the
421 binding to SE36 was shown to aid in the binding of other host factors which in turn camouflages
422 the merozoite contributing to evasion from the host immune response.

423 **5.4** ***Limited polymorphism of SE36.*** Antigenic polymorphism is well documented as
424 one of the most difficult hurdles for the development of effective malaria vaccines, especially for
425 those targeting the blood-stages [7,9,74]. Added to the list of mechanisms by which SERA5/SE36 is
426 protected from the host immune response (aside from functional redundancy and expansion of
427 family members) would be molecular camouflage. As shown above, the presentation of vitronectin-
428 bound-SE36, as a result of infection, was exploited by the parasite to modulate immunity such that
429 SE36 disguises itself avoiding host clearance leading to the gradual acquisition of immune tolerance.

Immune tolerance may explain why adults or those with high baseline antibody titers before vaccination were low/non-responders and as a whole had low seroconversion compared to subjects with low baseline antibody titers. Indeed, young children or individuals with limited malaria infection history would respond better to BK-SE36 vaccination (Fig. 2A) similar to malaria naïve Japanese adults. This response was seen in all BK-SE36 clinical trials reported to date: individuals with high pre-existing anti-SE36 antibodies had markedly lower antibody response [28,30].

The observed immune tolerance from the host may explain why SERA5 is less likely to be under substantial immune selection pressure compared to other blood-stage malaria vaccine antigens such as AMA1 and CSP. Indeed, *ama1* and *csp* show high nucleotide diversity and significant levels of positive selection (dN>dS) in contrast to *sera5* [71]. The nucleotide diversity of non-repeat regions in the vaccine candidate SE36 was comparable to the housekeeping genes of P-type Ca2+-ATPase and adenylosuccinate lyase (Fig 2C) [13,71]. In a sero-epidemiological study in the Solomon Islands, <50% of adults and <10% of children under 10 years were seropositive to anti-SE36, although higher seropositivity to MSP-1 was observed in the population [21]. The low immunogenicity would mean consequently limited immune pressure for SERA5 which would suggest a limited need for the parasite to acquire mutations to escape the host immune response. From pre-clinical studies, polymorphism in SERA5 may not hamper the potency of SE36 since mouse, rat, or monkey antibodies raised against SE36 cross-react to all the parasite lines so far

examined. Mouse and rat antibodies against SE36 have been shown to inhibit the intraerythrocytic proliferation of parasites *in vitro* [20].

From our studies, SERA5 polymorphism is largely confined to the repeat regions of the gene [13,71,75-77]. There were variations in the number of octamer repeats and serine repeat regions near the N-terminal region of SERA5. Polymorphic sites in the non-repeat regions was confined to 24 nucleotides, and there was no strong signature of positive selection. Sequence analyses performed on strains collected from the two clinical trial/follow-up studies, and cross-sectional studies in Africa did show a consensus sequence with African-specific polymorphisms [77]. It was however, reassuring to note that despite mismatches with the BK-SE36 sequence (based on Honduras 1 strain) in the octamer repeat, serine repeat and flanking regions, and single-nucleotide polymorphisms in non-repeat regions, these polymorphisms did not compromise vaccine response and the observed promising effectiveness based on phase 1 trials [28,30,36]. Of note, sequence analysis of 445 geographically distributed *P. falciparum* parasites showed one genetic polymorphism, "Asn" to "Lys" at the 11th residue, in the 18 residues implicated for vitronectin binding [69]. It is suggested that the binding property of vitronectin is almost conserved in worldwide *P. falciparum* parasites.

6. Conclusion and perspective

Immune evasion strategies in malaria contribute to parasite persistence and immune

466 dysregulation making it difficult to develop effective vaccines. To achieve a robust immune
467 response and consequently high protective efficacy, it will be ideal to overcome some factors
468 which limit the host's ability to respond efficiently to vaccine administration. There are
469 differences in how vaccines may work in malaria naïve adults and in immunized African
470 infants/children and adults. We have observed variations in vaccine responsiveness in our
471 clinical trials which is highlighted for further studies. So far clinical trials for SE36 do provide
472 valuable positive clues: acceptable reactogenicity, absence of unexpected safety signals,
473 favorable immunogenicity profile (immune response can be boosted by natural infection,
474 absence of allele-specific immune response), and an immune response across an HLA diverse
475 population. Some bridging studies and practical strategies may aim to circumvent the immature
476 immune system in infants, *e.g*. presumably postponing vaccination similar to vaccination
477 recommendations for measles and yellow fever. Immune tolerance from previous
478 exposure/immune suppression may evaluate the vaccination schedule, the intervention of
479 chemoprophylaxis/drug pre-treatment or the use of potent novel adjuvants. Current trials do
480 show that delaying the third dose is beneficial. The lack of data on cell-mediated immunity in
481 SE36 and a robust functional assay are some limitations that need to be addressed. Although a
482 few cytokine analyses and complement assays have been done, streamlining and standardization
483 of these assays could prove valuable. Investigation of the underlying mechanisms of

suppression of vaccine responses can reveal novel insights into the capabilities and limitations of human immunity and enhance vaccine effectiveness. So far a hypothesis of immune tolerance/immune suppression has been suggested but the mechanism has not been thoroughly elucidated.

Declaration of interest: TH is the inventor of BK-SE36; TH, KJI, and TT are inventors of BK-SE36/CpG. NP and TH are both supported by a research fund from Nobelpharma Co., Ltd (NPC), the clinical trial sponsor of Burkina Faso trials. These involvements did not influence the writing of this review and the decision to submit the article for publication.

Acknowledgements: Authors gratefully acknowledges the funding support from NPC.

Funding: The writing of this review did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions:

NP and TH drafted the review; NA created Fig. 2C. All authors read, edited and approved the manuscript.

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Figure 1. Representation of serine repeat antigen (SERA5), SE47' and the recombinant vaccine candidate, SE36. **A.** Full-length *Plasmodium falciparum* SERA5 (orange) [13,21]. In a highly regulated protease cascade, the secreted 120 kDa (~100–130 kDa) precursor in the parasitophorous vacuole is processed to P47 (green) and P73 (blue) upon merozoite egress. The P47 is linked to the C-terminal P18 via disulfide bond, another cleavage site in P73. These two fragments are subsequently processed into smaller fragments (P47, P50, P6 and P18) [21]. **B.** The N-terminal fragment, SE47'. Antibodies against this fragment are elicited in immune individuals and is the basis of the blood-stage malaria vaccine, BK-SE36. SE36, the recombinant N-terminal domain without polyserine repeats, is based on Honduras 1 strain and expressed in *Escherichia coli* for mass production and purification under GMP conditions.

Figure 2. Clues from BK-SE36 clinical trials. **A**. Percentage of subjects with >2-fold increase in antibody titers. Data from Uganda [28] and Burkina Faso [30] clinical trials show that younger age group remarkably respond to BK-SE36 vaccination. Green bars, after two full doses of BK-SE36; yellow bars, after booster dose (Dose 3). A full dose contains 100 µg/mL SE36 protein and 1 mg/mL aluminum. A booster dose was done only in younger cohorts (1-5 years-old). **B**. Molecular camouflage of *P. falciparum* merozoites utilizing host vitronectin [69]. SE36 covers the whole merozoite surface and host vitronectin tightly binds to SE36. Vitronectin in turn binds to other host serum proteins to camouflage the merozoite from host immune attacks. Majority of bound proteins are blood coagulation proteins, apolipoproteins and proteins that belong to the complement system. This molecular camouflage is an immune evasion strategy that contributes to parasite persistence and being recognized as "self" the parasites protect themselves from the host immune system by having a host immune steady state, and may explain why there is lesser immune pressure (and thus, limited polymorphism) for SERA5. **C**. Comparison of sequence diversities in some *P. falciparum* antigen (*ama1, csp, sera* {SE36 region} and housekeeping (*serca* + *adsl*) genes [77]. Immune tolerance may explain the low polymorphism of SERA5. Nucleotide diversity (π, grey bars), the number of synonymous substitutions per synonymous site (dS, pink bars), and non-synonymous substitutions per non-synonymous site (dN, blue bars) are shown. The sequence length and the number of sequences used for each gene are reflected: *ama1*, apical membrane protein 1 (n = 241, 1866 bp); *csp*, circumsporozoite protein (n = 238,

681 bp); *se36*, serine repeat antigen (n = 314, 741 bp); and housekeeping genes *serca*, P-type Ca2+-ATPase; *adsl*, adenylosuccinate lyase (n = 292, 5043bp). Data of *ama1*, *csp* and *serca*+*adsl* are from three parasite populations from Africa (Uganda, Tanzania and Ghana). Data using the SE36 region are from four parasite populations (Uganda, Tanzania, Ghana and Burkina Faso): π was analyzed by DnaSP6; dS and dN by MEGA. Excluded from the analyses were: NANP repeat region in *ama1*, eight-mer amino acid repeat units in *csp*; the octamer region, 13-mer insertion/deletion region, serine repeat regions, and the 17-mer dimorphic region in *SE36* vaccine; and the asparagine repeat region in *adsl* + *serca*. When dN>dS, immune pressure/positive selection is inferred.

956 Table 1. Immunogenicity and hyporesponsiveness in pre-erythrocytic vaccine trials.

Vaccine	Study population	Observation	Authors note	Reference
PfSPZ	Tanzanian (20-35 years old) and Dutch adults (19-30 years old) in CHMI trials	Before CHMI: Tanzanian adults have higher baseline antibody titers for AMA-1, EXP-1, and CSP; although both populations had comparable IFN-γ responses. Post-CHMI: cellular recall responses were significantly increased in Dutch volunteers. Tanzanians showed lower lymphocyte IFN-γ production. Immunosuppression was still present 1-month post-CHMI	While the influence of genetic background cannot be excluded, the lack of increased proliferative Th1 responses in Tanzanian volunteers could be partially due to immunosuppression following exposure to blood-stage parasites during CHMI.	[37]
PfSPZ	11 clinical trials in Germany, US and Africa (5-month to 61 years-old)	Females ≥ 11 years of age had significantly higher levels of antibodies to PfCSP than males, but with no evidence of improved protection	Antibodies to PfCSP (and PfSPZ) primarily correlate with other potentially protective immune mechanisms (e.g. antibody dependent and antibody independent cellular responses in the liver)	[38]

		Individuals with prior malaria exposure, such as African adults, have significantly lower antibody responses to PfCSP than malaria naïve adults	Attributed to immune dysregulation due to (a) lifelong exposure to malaria parasites; (b) elimination of the PfSPZ by naturally acquired adaptive immune responses; and/or (c) immunosuppression due to concomitant coinfections	
		In African infants and children, there was a negative correlation between age and antibodies to PfCSP, with the highest antibody levels observed in infants and young children	Antibody levels in these children was comparable to responses seen with malaria-naive adults.	
PfSPZ-CVac (PfSPZ vaccination with concurrent antimalarial chemoprophylaxis)	CHMI trial in the US (18-45 years-old)	Vaccine efficacy was variable (0 to 75% efficacy with same dose but different dosing schedule: with a 7-day administration schedule the vaccine had no efficacy *vs* 75% efficacy on a 5-day dosing schedule)	In the 7-day dosing schedule, the second and third vaccine administration coincided with the period of blood-stage parasitemia from the first vaccination, demonstrating the absence of sterile protective immunity	[39]

Whole Sporozoite and CSP sub-unit vaccine	phase 1 and 2 clinical trials	Compared to malaria naïve adults, individuals with prior malaria exposure have significantly lower antibody responses to CSP (*e.g.* adult males from Equatorial Guinea reported lower antibody responses to PfSPZ compared to US adults; PfSPZ efficacy was greatly reduced in a site with seasonal transmission [30% protection at 6 months in Mali adults *vs* 64% protection in malaria naïve individuals]) Malaria blood-stage infection downregulate pre-erythrocytic stage immunity.	Protection was seen in vaccinated subjects living in malaria endemic areas, despite the observed several-fold lower antibody and cellular immune responses obtained from malaria naïve adults in Germany or the US. A fine, yet inadequately described balance between innate and adaptive immune responses is required for protection	[40] (radiation-attenuated sporozoites) [41] (whole-sporozoite vaccine)
RTS,S/AS01	Phase 2b data (2 sites in Mozambique, 1-4 years-old)	Induction of functional antibodies was lower among children with higher malaria exposure.	High baseline antibody titers obtained either through repeated malaria infection or vaccination impacts B cells, CD_{4+} T cells and innate immune cell phenotypes	[42]

RTS,S/AS01	Phase 2 and 3 trials in 5-17 month-old children	Younger age at time of vaccination (5-11 months *vs* 12-17 months) and high transmission setting were significantly associated with higher anti-CS antibody response	Anti-CS antibody titers after vaccination supports a short-lived and long-lived component of the humoral response; with a higher proportion of the long-lived response noted after Dose 4.	[43]
RTS,S/AS01	Phase 3 data (11 sites, 5-17 months and 6-12 week-old children)	In 6–12 week-old children, high baseline anti-circumsporozoite antibody titers were associated with low anti-circumsporozoite antibody titers after vaccination Within the 5–17 month age group, younger children had higher anti-circumsporozoite antibody titers after vaccination	Maternal antibodies or fetal exposure to malaria parasites inhibit immunogenicity	[44]
RTS,S/AS01	Immuno-informatic tools were used to compare T helper	CSP component of the RTS,S vaccine exhibited a low degree of T-cell epitope relatedness to circulating variants: The prevalence of epitopes restricted by specific HLA-	Reduction in CD4+ T-cell (reduced T-cell help, low T-cell epitope content, reduced presentation of T-cell epitopes by prevalent HLA-DRB1, high human-cross reactivity of T-cell epitopes and	[45]

	epitopes in RTS,S vaccine antigens *vs* 57 CSP variants isolated from infected individuals in Malawi	DRB1 alleles was inversely associated with prevalence of the HLA-DRB1 allele in the Malawi study population T-cell epitope content shared between the vaccine and the Malawian CSP variants was only 34%.	polymorphism of CSP in circulating strains) contributed to tolerance/immune camouflage and have overshadowed the protective efficacy of RTS,S	
ME-TRAP	Kilifi District, 1-6 years-old: one group vaccinated *vs* naturally exposed group	Parasitemia immediately before vaccination suppressed the acquisition of T cell responses (by 15-25%, as measured by IFN-γ production). Parasitemia immediately after vaccination did not suppress T cell response. Concurrent parasitemia did not influence T cell response.	Parasitemia influences initial priming but not subsequent recall and/or boosting of T cell responses. In 1-6 years old, age appears to be a less important consideration; likewise, mild and moderate malnutrition does not appear to reduce	[46]

			immunogenicity	
ChAd63 MVA ME-TRAP	Phase 2 (5-17 month-old Burkinabe children)	Anti-TRAP IgG titers were significantly lower in the parasite positive group compared to the parasite negative group. Anti-AMA titers were significantly higher in the positive parasitemia group at vaccination; but negatively correlated with peak T cell response	Using anti-AMA-1 titers as surrogate marker for prior exposure, acute malaria infection could have reduced humoral immunity to vaccination with ChAd63 MVA ME-TRAP, whilst chronic parasite exposure may have an impact on cell mediated immunity	[47]

968 Table 2. Vaccine immunogenicity and high baseline antibody titers in blood-stage vaccine candidate trials.

Vaccine	Study population	Observation and author's notes	Reference
MSP1, MSP2 and RESA in Montanide ISA720	Phase 1 18-50 years-old East Sepik Province, Papua New Guinea	Humoral response was significantly boosted by the vaccine in individuals with the lowest MSP1 titer at baseline. No change noted for MSP2 and RESA. The lack of boosting of humoral responses was attributed to the high concentration of antibodies prior to vaccination.	[48]
Combination B: MSP1, MSP2 and RESA in Montanide ISA720	Phase 1-11b 5-9 years-old	Vaccine immunogenicity was neither impaired by circulating parasites nor increased after pre-treatment with sulphadoxine-pyrimethamine.	[49]
FMP1/AS02A	Phase 1 18-55 years-old Bandiagara, Mali	Response to vaccine varied and was attributed to the variation in background immunity: in 6 participants that developed high antibody titers (≥ 8-fold increase in antibody titer) the baseline GMT for $MSP1_{42}$ was <3,000 *vs*. those with baseline GMT of >20,000, a <2-fold rise was observed.	[50]
FMP1/AS02	Phase 1	Highest rise in antibody responses was seen in those with low pre-existing antibody titers prior	[51]

	18-55 years-old Kombewa Division, Western Kenya	to immunization. However based on anti-MSP1$_{42}$ antibody titers at Day 90, FMP1/AS02S vaccinees with low and high pre-existing titers were boosted after 3 doses.	
MSP3-LSP	Phase 1b 18-40 years-old Balonghin, Burkina Faso	Total IgG, IgG subclasses and IgM to MSP3 and MSP3-LSP were similar in vaccinees and control (vaccinated with tetanus toxoid vaccine). The absence of response to vaccination was attributed to high pre-existing antibody levels.	[52]
MSP3-LSP	Phase 1b 12-24 months old Balonghin, Saponé, Burkina Faso	IgG1 and IgG3 responses to MSP3-LSP were higher post vaccination than at baseline. Immunogenicity in young children with limited exposure to natural *P. falciparum* infection contrast with [52].	[53]
GMZ2/Al (OH)	(serum samples from 3 phase 1 clinical trials obtained on day 0 and 4 weeks after the last vaccination): German, Gabonese adults and children	GMZ2 vaccination elicited increase in geometric mean antibody titers: 2.8-fold in Gabonese adults; 290-fold in Gabonese children and German adults compared to day 0 (D0). At D0, Gabonese adults have 50-fold higher anti-GMZ2 IgG than Gabonese children and 63.5-fold higher anti-GMZ2 IgG than German adults. There was a strong correlation between antibody titers after vaccination and pre-vaccination level.	[54]

GMZ2	Phase 2 12-60 months old Burkina Faso, Gabon, Ghana and Uganda	Higher baseline level of naturally acquired antibodies in 3-4 years-old resulted to 5.7-fold increase in anti-GMZ antibodies *vs*. 14-fold increase in 1-2 years old	[55]

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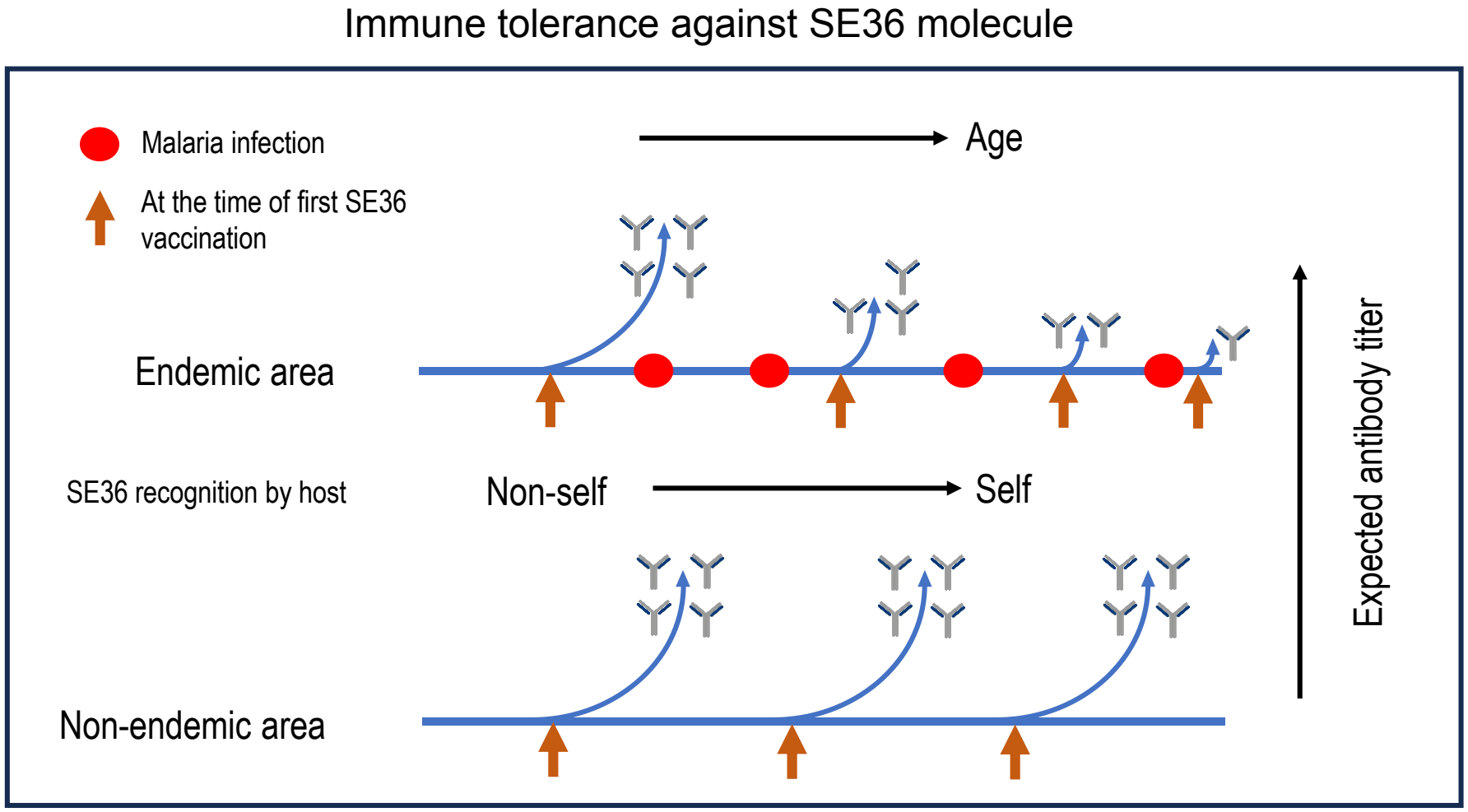
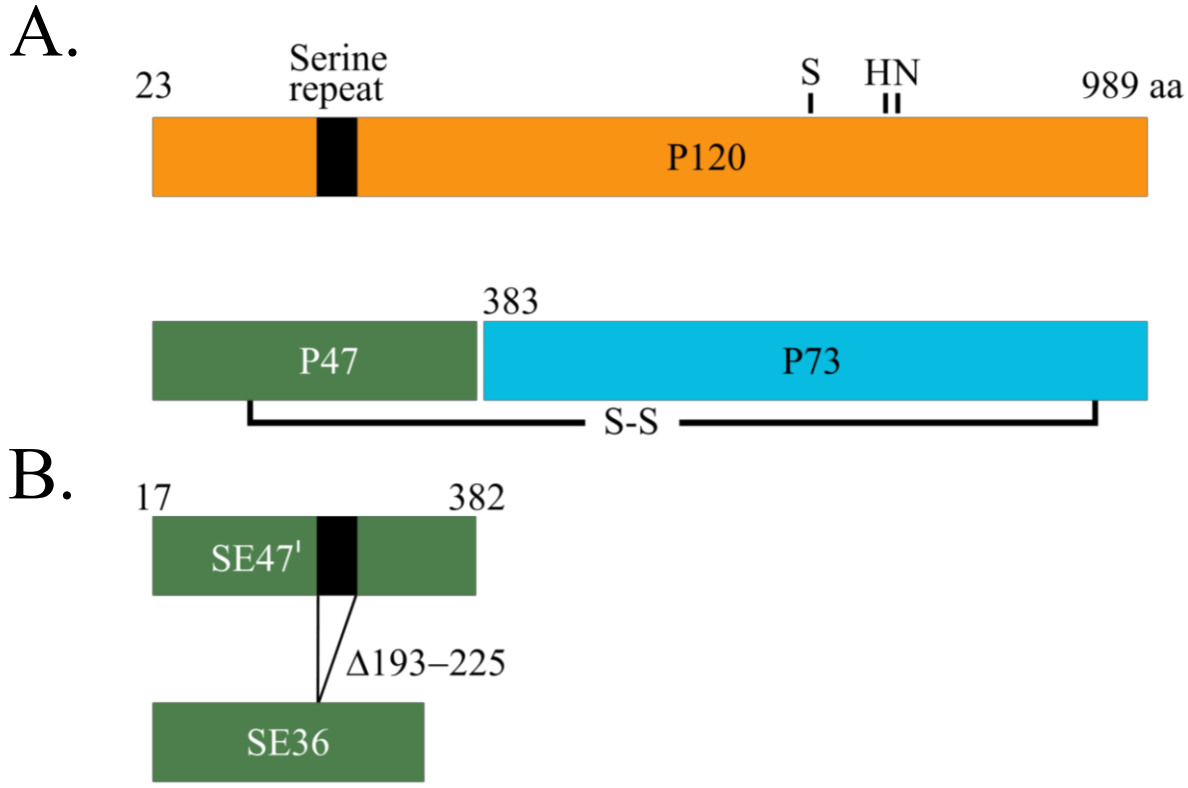
Highlights

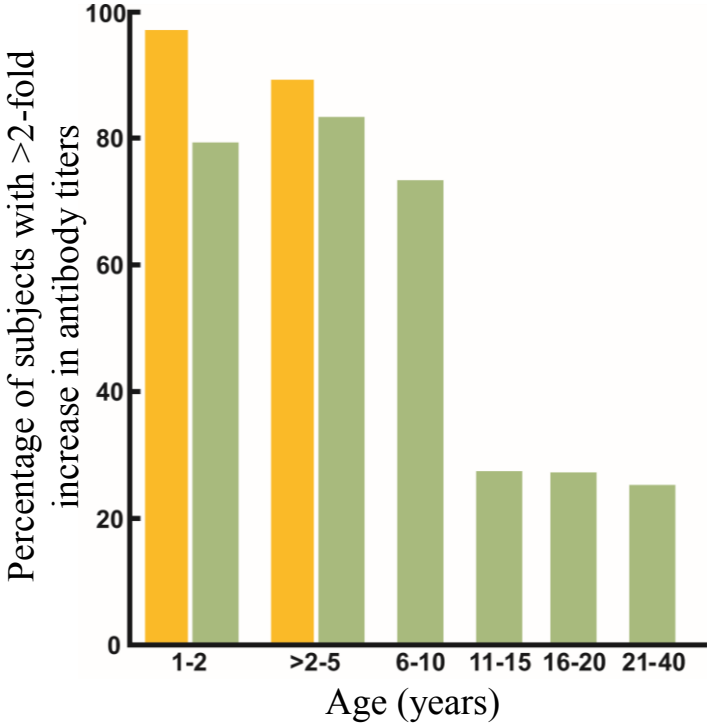
- Clinical trials show that SE36 antigen is a promising blood-stage vaccine candidate
- Repeated malaria infection lowers the humoral response in some SE36 vaccinees
- Concurrent parasitemia during vaccination resulted to reduced immunogenicity
- SE36 binds to host vitronectin akin to molecular camouflage
- Immune tolerance may explain the limited observed polymorphism of SE36

Graphical Abstract





A.


Percentage of subjects with >2-fold increase in antibody titers
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80
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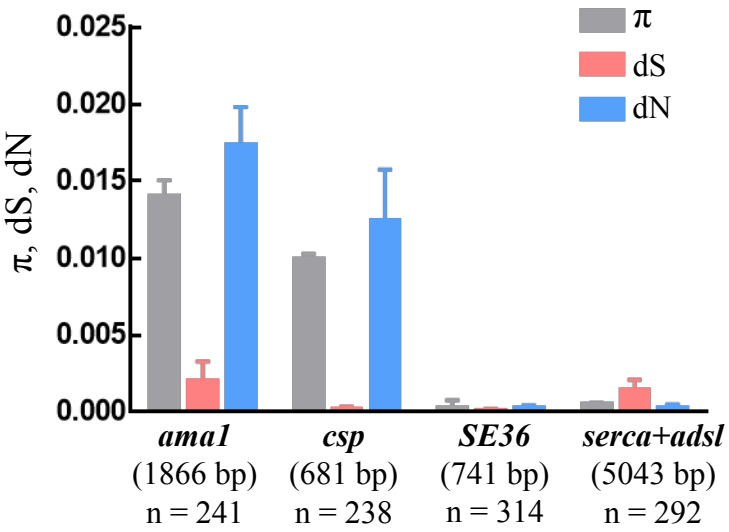
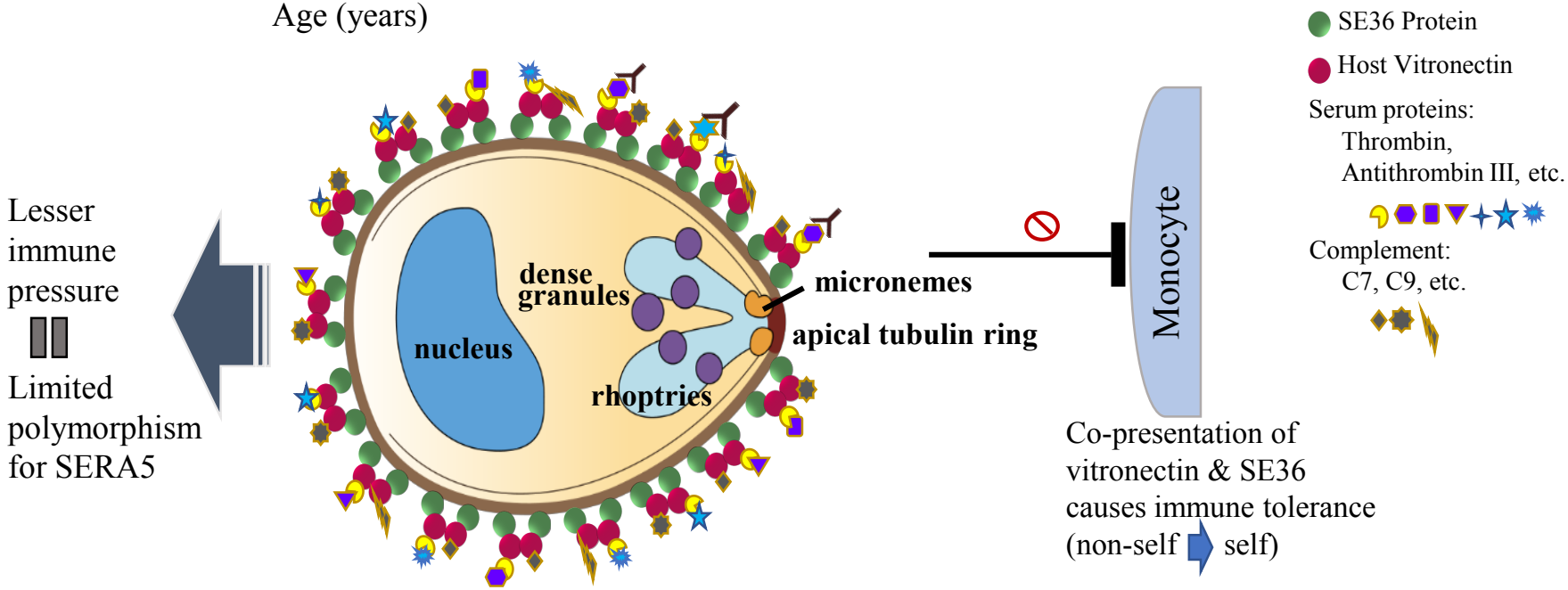
40
20
0
1-2
>2-5
6-10
11-15
16-20
21-40
Age (years)

C.


π
dS
dN
π, dS, dN
0.025
0.020
0.015
0.010
0.005
0.000
ama1 (1866 bp) n = 241
csp (681 bp) n = 238
SE36 (741 bp) n = 314
serca+adsl (5043 bp) n = 292

B.


Lesser immune pressure
Limited polymorphism for SERA5
nucleus
dense granules
micronemes
apical tubulin ring
rhoptries
Monocyte
SE36 Protein
Host Vitronectin
Serum proteins:
Thrombin,
Antithrombin III, etc.
Complement:
C7, C9, etc.
Co-presentation of vitronectin & SE36 causes immune tolerance (non-self self)

Immune tolerance caused by repeated *P. falciparum* infection against SE36 malaria vaccine candidate antigen and the resulting limited polymorphism

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Keywords: malaria, immune tolerance, vaccine, *Plasmodium falciparum*, serine repeat antigen, immune evasion

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Abstract (≤ 250 words)

The call for second generation malaria vaccines needs not only the identification of novel candidate antigens or adjuvants but also a better understanding of immune responses and the underlying protective processes. *Plasmodium* parasites have evolved a range of strategies to manipulate the host immune system to guarantee survival and establish parasitism. These immune evasion strategies hamper efforts to develop effective malaria vaccines. In the case of a malaria vaccine targeting the N-terminal domain of *P. falciparum* serine repeat antigen 5 (SE36), now in clinical trials, we observed reduced responsiveness (lowered immunogenicity) which may be attributed to immune tolerance/immune suppression. Here, immunogenicity data and insights into the immune responses to SE36 antigen from epidemiological studies and clinical trials are summarized. Documenting these observations is important to help identify gaps for SE36 continued development and engender hope that highly effective blood-stage/multi-stage vaccines can be achieved.

1. Introduction

Malaria, due to *Plasmodium falciparum*, disproportionately affects sub-Saharan African children, pregnant women especially the primigravidae, as well as those immunocompromised in malaria endemic areas [1]. The African region accounts for 95% of cases and 96% of malaria deaths worldwide. After more than 30 years of research and clinical trials, the World Health Organization (WHO) approved the world's first malaria vaccine in 2021 [2]. RTS,S/AS01 (Mosquirix®) targets the pre-erythrocytic stage of *P. falciparum.* Based on phase 3 and large pilot implementation programs the vaccine is recommended as a 4-dose schedule for children from 5 months of age living in moderate to high transmission areas [3]. The modest and short-lived efficacy of RTS,S/AS01, however, highlights that new, more efficacious vaccines be sought. Another anti-sporozoite vaccine, R21/Matrix-M, has recently reported a 77% protective efficacy in a phase 1/2b clinical trial [4], has an on-going phase 3 trial and follow-up study (ClinicalTrials.gov: NCT04704830), and is fast tracked in its regulatory approval for use in Ghana, Nigeria and Burkina Faso [5]. Similar to RTS,S, R21 contains recombinant particles of the central repeat and C-terminal circumsporozoite protein (CSP) fused to Hepatitis B surface antigen (HBsAg) but with improved protective efficacy due to a higher density of the CSP antigen on the VLP surface and formulation with a new, potent saponin-containing adjuvant [4]. Missing from the arsenal is a blood-stage vaccine to confer protection against disease and death. Moreover, long term studies of children vaccinated with RTS,S and living in areas of moderate

transmission showed a significant increase in rebound episodes of clinical malaria 3 to 6 years after the primary trial [6]. Thus, the availability of more than one vaccine that targets other life stages of the parasite, either as a stand-alone or combination/multi-stage vaccine, has a public health value and would be preferable for risk mitigation.

The complex parasite life cycle, genetic diversity (high polymorphism and allele-specific variations), and the various immune escape mechanisms of the *Plasmodium* parasite are among the reasons why there is limited success for most candidate malaria vaccines tested to date [7-10]. Furthermore, humoral (antibody) responses have often been harnessed for the evaluation of vaccines, but a thorough understanding of the dynamic interplay of the host immune response, the parasites immune evasion mechanisms, and what level of antibody response can be sufficient to confer protection is lacking. There are differences in the immune response between malaria naïve individuals and those in endemic areas (malaria exposed) [11]. We summarize below the immunogenicity observations with the blood-stage vaccine antigen, SE36, based on serine repeat antigen 5 (SERA5). The findings from other vaccine studies that suggest instances where humoral and cellular responses appear to be compromised/suppressed are also presented. This review highlights the diversity of immune evasion mechanisms that malaria parasites use to gain an edge in the host-parasite relationship, their implications for

124 vaccine-based strategies, and allows us to identify gaps in developing highly effective, long-
125 lasting malaria vaccines.
126
127 **2. Epidemiological studies on SERA5**
128 SERA5 is a highly conserved [12, 13], essential [14] and abundant asexual blood stage antigen,
129 expressed specifically during late trophozoite and schizont stages [15] (Fig. 1A). The protein is
130 a vaccine candidate based on (a) epidemiological studies that show a strong correlation between
131 high antibody titers and protection from malaria symptoms and severe disease [15-17]; (b) *in*
132 *vitro* studies of sero-positive sera that demonstrate parasite growth inhibition [15, 18-20]; and
133 (c) non-human primate challenge studies demonstrating protection against parasite challenge
134 [20-22].
135 In sero-epidemiological studies from populations residing in malaria holoendemic
136 areas, IgG antibodies were detected against the amino terminal part of SERA5 using either the
137 N-terminal 47 kDa domain with serine repeats (*i.e.*, SE47') or without the serine repeats (SE36)
138 (Fig. 1B). Humoral responses were evaluated in Ugandan adults and children [15-17]; pregnant
139 women and newborns [23]. High titers of IgG anti-SERA5 were associated with protection from
140 severe malaria [16,17,24] or absence of placental parasitemia, and babies delivered with normal
141 birthweights [23]. Children with uncomplicated malaria have significantly higher median titers

142 of anti-SE36 IgG than age-matched children who experience severe malaria [17].

143 In 2 to 70 year-old Guadalcanal, Solomon Island residents, a significant negative
144 correlation was observed between high parasite densities and those positive for anti-SE36 [21].
145 Seropositive individuals had low parasite burden or were in the non-infected group while
146 seronegative individuals bore high parasite densities. The observed association of parasite
147 density with anti-SE36 antibody titer was specific and not due to non-specific
148 immunosuppression driven by severe infection since people with high parasite densities showed
149 no downregulation of anti-polio antibody titers. The proportion of seropositive individuals were
150 generally lower compared to individuals positive for merozoite surface protein-1 (MSP-1)
151 [17,23], or other erythrocyte stage proteins (rhoptry proteins, exported proteins, etc) [25]. Using
152 40 adult sera from residents in Atopi Parish (a malaria holoendemic area in Uganda), high
153 responders (ELISA titer >1000) to SE47' and $MSP1_{19}$ were 38 and 80%, respectively [15].
154 Sero-positivity to SE36 also showed a clear age-dependency in the Solomon Islands, although
155 as noted above, the rate of seropositivity does not exceed >50% of the population [21].

156 **3. Assessing the vaccine response to SE36**

157 A recombinant form of SERA5 N-terminal domain (SE36) was selected for clinical
158 development, prepared under Good Manufacturing Practice (GMP) standards and formulated
159 with aluminum hydroxide gel (AHG) to yield BK-SE36 (100 µg/mL SE36 protein and 1 mg/mL

160 aluminum) [21]. AHG was first chosen as the standard vaccine adjuvant because of the proven
161 safety profile and production of primarily humoral immune and Th2 biased cellular responses.
162 However, as it became clear in recent years that alum is not sufficient to induce effective
163 immune responses for malaria vaccines [10,26,27], a second formulation/generation of SE36
164 was developed. BK-SE36 was administered concomitantly with another adjuvant containing
165 unmethylated cytosine guanine (CpG) oligonucleotide (ODN) motifs to yield BK-SE36/CpG
166 (100 µg/mL SE36 protein, 1 mg/mL aluminum and 1 mg/mL CpG ODN K3). CpG-induced
167 activation of innate immunity has been reported and in initial studies, CpG ODN (code name:
168 K3) efficiently induced Th1 response, selectively promoting cellular and humoral immune
169 responses [22]. So far, all clinical studies reported to date show that both vaccine formulations
170 have acceptable reactogenicity and have no unexpected safety signals [21, 28-30].
171 ***3.1 Early vaccination studies in non-human primates***. SE36/AHG was
172 immunogenic in chimpanzees, and squirrel monkeys [21]. In all three immunized chimpanzees,
173 antibody titers increased 2 weeks after the first administration, peaked 2 weeks after the second
174 administration and were maintained for more than 40 weeks. Squirrel monkeys vaccinated two
175 or three times were protected against high parasitemia after parasite challenge. The challenge
176 with *P. falciparum*-infected red blood cells elevated the antibody titer and protected squirrel
177 monkeys against high parasitemia.

178 Cynomolgus monkeys administered with SE36/AHG/CpG had approximately 10 times
179 greater serum anti-SE36 IgG antibody levels and induced mixed Th1/Th2 responses compared
180 to those administered with SE36/AHG alone [22]. However, in squirrel monkeys, it was
181 surprising that although protection that correlates with decreased parasite density in the *P.*
182 *falciparum* challenge study was observed, administration of SE36/AHG/CpG did not result in
183 higher antibody titers when compared to those vaccinated with SE36/AHG alone suggesting
184 additional or independent immune response(s) with the use of CpG (*e.g*. T cell-mediated cellular
185 immunity).

186 ***3.2 First-in-human trials (Japanese adults)***. Phase 1a trials for BK-SE36 and BK-
187 SE36/CpG were conducted in malaria naïve Japanese adults. Seroconversion was 100% after
188 two vaccinations of full-dose BK-SE36 given 21 days apart [21]. When BK-SE36/CpG was
189 used, immunogenicity assessments showed high antibody titers with accelerated seroconversion
190 [29]. A 100% seroconversion was achieved in malaria naïve adults with one full-dose of BK-
191 SE36/CpG *vs*. 2 full-doses of BK-SE36. The full-dose group had significantly higher titers than
192 half-dose ($p = 0.002$ Student's t-test) and remained above baseline even after 12 months post-
193 Dose 2. BK-SE36/CpG formulation induced > 3-fold higher antibody titer than BK-SE36.

194 ***3.3 Clinical trial: Uganda***. In sharp contrast to phase 1a in Japan, the phase 1b trial
195 in Uganda showed low seroconversion (25%) in most vaccinated adults (21–32 years-old) [28].

196 Those who were administered with BK-SE36 were categorized as either responder (*i.e.* those
197 whose fold-change in anti-SE36 antibody titer from baseline was ≥ 2-fold after vaccination) or
198 non-responder (those whose fold change in anti-SE36 antibody titer from baseline was <2-fold).
199 All seropositive subjects (except one), did not have any obvious increase in antibody
200 titers (non-responders) 21 days post Dose 2. However, notably, not all seronegative subjects also
201 had a significant change from baseline antibody titers: more than half (55%) of seronegative
202 adults (21-32 years-old) were classified as non-responders, while only 45% showed a 2-fold
203 higher change in antibody titer from baseline confirming the low seroconversion to SERA5
204 observed in epidemiological studies [21]. In 16–20 years-old, 11–15 years-old and 6–10 years-
205 old, the proportion of subjects with ≥ 2-fold increase in antibody titers after 2 full-dose
206 vaccinations were 27, 27 and 73%, respectively [28] (Fig. 2A). When vaccinated with full-dose,
207 the change in antibody titers before vaccination to 21 days after Dose 2 was significant in 6–10
208 years-old (p=0.01) and 11-15 years-old (p=0.02) but not in the 16-20 years-old cohort.
209 The low seroconversion observed in Ugandan adults was unexpected when compared
210 to the 100% seroconversion in Japanese adults after either half- (containing 50 µg/mL SE36
211 protein and 0.5 mg/mL aluminum) or full-dose [21]. There was no indication of general immune
212 suppression correlated to host genetic background. Analysis of the allelic polymorphism of
213 human leukocyte antigen (HLA)-DRB1 alleles found that age rather than a particular DRB1

214 allele was associated with antibody response to vaccination [31].

215 The follow-up study demonstrated boosting of vaccine-induced immune response as a
216 result of natural infection [32]. Children whose antibody titers against SE36 increased by ≥2-
217 fold after vaccination and had high antibody titers throughout the follow-up (*i.e*, those with a
218 geometric mean (GM) of 314 arbitrary units (AU), 21 days post vaccination to 102 AU at Day
219 365) did not experience malaria infection (defined as any parasitemia ≥ 100 parasites/μL).
220 Responders who had GM <100 AU experienced 1-2 episodes of natural *P. falciparum* infection
221 during a year of follow-up. Responders had significantly decreased odds to reinfection: the
222 percentage of children who experienced more than one infection in the responder group was
223 18% *vs* 46% in non-responders; and 55% in the control. The observed association of fewer
224 reinfections in responders was robust and was not influenced by age (p=0.175), antibody titer
225 after infection (p=0.156) or parasitemia levels (p=0.091). GMs of anti-SE36 antibody titers were
226 significantly different among responder, non-responder and those in the control. The 3.3-fold
227 increase from baseline antibody titer after the first infection in the responder group was 1.5x
228 higher than non-responders and 2.3x higher than the control group, suggesting immunological
229 memory. Thus, in vaccinated subjects, natural infection can boost the immune response. There is
230 little evidence of boosting in non-responder and control groups which may reflect the inherent
231 low immunogenicity of SE36 during natural infections [32].

3.4 Clinical trial: Burkina Faso. BK-SE36 was immunogenic in 12–60 month-old Burkinabe children using either intramuscular or subcutaneous route of administration [30]. Seroconversion was not markedly different after two full-dose vaccinations in 25–60 month-old (83%) *vs* 12–24 month-old (79%) (Fig. 2A). A third dose, 22 weeks after the second dose (*i.e.*, 6 months from Dose 1), resulted in higher immune response and increased the proportion of children with >2-fold increase in antibody titer 4 weeks after vaccination (89% for 25–60 month-old; and 97% for 12 –24 month-old children) (Fig. 2A). Dose 3 successfully raised anti-SE36 antibody titer to levels higher than after primary vaccination (Dose 1 and 2). This kinetics of immune response after primary vaccination is a characteristic response akin to immunological memory [33]. Of interest, it was in contrast to the observed antibody response after three vaccine doses of BK-SE36 in a phase 1a study [21]. When given at 21-days interval, the induced antibody titer did not differ significantly between Dose 2 and Dose 3 in Japanese adults. Delaying the timing of the third dose appeared to have contributed to improved immunogenicity in Burkinabe children [30]. A delayed third dose is likely recommended to boost memory responses and keep antibody titer high in malaria endemic areas.

The youngest cohort (12–24 month-old) had 2- and 4-fold higher anti-SE36 antibody titers after 2 and 3 doses of BK-SE36 compared to the 25–60 month-old cohort [30]. The 25–60 month-old children are presumed to have a longer history of exposure to repeated malaria

250 infections and, within this cohort, a subgroup of children may also have a lower SE36
251 responsiveness. Indeed, more children are infected in this cohort than their younger counterparts
252 during recruitment and vaccination day (52.8% *vs*. 19.4% in 12–24 month-old, $p = 0.003$). It
253 was noted that concurrent infection (defined as any parasitemia > 0 by microscopy) during
254 vaccination days resulted in lower SE36 antibody titer levels in vaccinees [34]. Study
255 participants who were not infected during vaccination days had the highest GMT one month
256 post Dose 3. The difference in antibody titers between uninfected and infected subjects was
257 statistically significant. By multivariate analysis, a negative correlation between parasite density
258 and anti-SE36 antibody GMT was observed: there was a decrease in antibody GMT for every
259 1000 parasites/μL increase in *P. falciparum* density after adjustment for baseline antibody titer,
260 age and interaction between age and baseline antibody titer.
261 With BK-SE36, titers dropped to near pre-vaccination titers 5 months after Dose 2,
262 but was boosted, at Dose 3 as noted above [30]. Data on what level of anti-SE36 antibody titer
263 can be considered protective is limited at the moment. Using the high-titer pooled serum as
264 standard (*i.e.,* from Ugandan adults with naturally acquired titers where the positive standard
265 was calculated at 5000), the average antibody titer observed in BK-SE36 vaccinees was 10×
266 less. The plateau level of SE36-specific antibody titers achieved in chimpanzees [21] was also
267 10× higher than the average titer obtained in all BK-SE36 trials reported to date. Learning from

the experience of RTS,S, a combination of adjuvants or choice of a multi-adjuvant approach may be ideal to improve the level and duration of antibody response [35]. This was the motivation for the improved formulation of BK-SE36 using the CpG ODN (K3) adjuvant. The vaccine formulation was tested in Burkina Faso for three age groups [36] following the success of a phase 1a trial in Japanese adults [29]. CpG ODN (K3) improved vaccine immunogenicity, as seen by the high titers and high seroconversion rates in vaccinated participants aged 21-45 years, 5-10 years, and 12-24 months [36].

4. Observations from other malaria vaccine trials

Clues on hyporesponsiveness largely stem from observations on the heterogeneity of immune response in malaria endemic areas. As most trials that have progressed to late-stage clinical evaluation targets the pre-erythrocytic stage, Table 1 highlights observations from these advanced vaccine candidates. Both the controlled human malaria infection (CHMI) and clinical trials in malaria endemic areas report observations of immune suppression in vaccinated volunteers. In a number of blood-stage vaccine trials, similar observations were reported (Table 2).

4.1 Repeated infection in malaria endemic areas overshadowed the inductive capacity of candidate vaccines in clinical trials.

Repeated malaria infections tailor the host's

286 immune response, making the parasite less recognizable by the immune system. In vaccine trials
287 of merozoite surface protein3-long synthetic peptide (MSP3-LSP), high baseline antibody levels
288 (as a result of natural infection/high transmission intensity) in semi-immune adults were
289 presumed to have overshadowed the inductive capacity of the vaccine [52,53]. In 18–40 year-
290 old Burkinabe subjects, although there was some indication of cell-mediated immune response
291 (increase in lymphocyte proliferation index and IFN-γ), there was no detectable humoral
292 immune response from MSP3-LSP vaccination when compared to children [52]. The highly
293 variable individual antibody titers to another blood-stage vaccine candidate, the FMP1 vaccine
294 (consisting of the 42-kDa carboxy-terminal 392 amino acids of MSP-1 and 17 non-MSP-1
295 amino acids encoding a 6-histidine tag plus linking sequence), in 18–55 year-old Malian adults
296 was also attributed to the variation in background immunity [50]. When the baseline GMT for
297 MSP-1_{42} titer was < 3,000 there was an 8-fold or greater increase in titer after vaccination; but
298 when the baseline titer is > 20,000, the observed increase was only < 2-fold. This was similar in
299 18–55 year-old Kenyan adults [51]: the highest rise in antibody responses was seen in
300 individuals with low pre-existing antibody titers. In Mali, antibody titers rose from < 6,000 to a
301 peak of nearly 38,000 *vs* an increase from 17,000 to 46,000 in Kenya [50]. Regression lines
302 fitted using data from Day 0, 75 and 90 in the two study sites, showed similar rates of increase
303 in vaccine-induced antibody responses to the 3D7 alleles of MSP-1_{42} and MSP-1_{19} with only

304 pre-existing antibody titers as the difference.

305 Valuable clues can be obtained in younger age groups. In a multi-site (Burkina Faso,
306 Gabon, Ghana and Uganda) phase 2 children (12–60 months-old) trial of a recombinant vaccine
307 with glutamate-rich protein and MSP3 antigens (GMZ2), there was a greater increase in anti-
308 GMZ2 antibodies in children 1–2 years-old compared to children 3–4 years-old (14-fold
309 increase, 95% CI 8.7, 23 *vs* 5.7-fold, 95% CI 4.0, 8.2; respectively) [55]. Children with low
310 baseline antibody titers to GMZ2 responded strongly to vaccination, whereas those with more
311 exposure to *P. falciparum* infection showed a smaller boost in anti-GMZ2 IgG titers.

312 In a recent report of an RH5 phase 1b trial in Bagamoyo, Tanzania with 3 age cohorts
313 (18-35 years, 1-6 years and 6-11 months) higher anti-RH5 IgG antibody was induced in
314 pediatric groups (6 to 10-fold higher) compared to the adults [56]. ChAd63-MVA RH5 is
315 another blood-stage vaccine based on the reticulocyte-binding protein homolog 5 (RH5)
316 formulated with recombinant replication-deficient chimpanzee adenovirus serotype 64
317 (ChAd63) and the attenuated orthopoxvirus modified vaccinia virus Ankara (MVA). Authors
318 determined that there was no significant correlation between existing anti-vector immunity at
319 baseline and the humoral immunogenicity obtained 14 days after vaccination. The pre-existing
320 anti-ChAd63 antibody was found to be unlikely the reason for improved immunogenicity and
321 the high titers in younger age groups was attributed to greater B cell immunogenicity and/or

relatively higher vaccine dose per unit body mass in infants and children [56].

4.2 Parasitemia on vaccination day was associated with reduced humoral immunogenicity/immunosuppression.

Low response to vaccination in infected individuals has been reported in pre-erythrocytic vaccine trials (Table 1). Also, as mentioned for BK-SE36, reduced response in Burkinabe children was associated with concomitant infection at the time of administration [34]. The effect of parasite clearance with sulphadoxine-pyrimethamine (SP) a week before vaccination in 5–9 years-old Papua New Guinean children was assessed in a study using Combination B vaccine (composed of three blood-stage antigens: ring-infected erythrocyte surface antigen and MSP1 and MSP2) [49]. Interestingly, concurrent *P. falciparum* infection and SP pre-treatment at the time of vaccination did not alter the antibody response to this blood-stage vaccine candidate. It is, however, noted that IFN-γ response to MSP1 was substantially lowered in the vaccine group who had received SP before vaccination. Further work on the possible influence of concomitant *P. falciparum* infection is important in vaccine trials conducted in malaria endemic areas.

5. Host vitronectin and immunogenicity to SE47/SE36 molecule

Just as the host has developed several defenses against pathogens, pathogens have evolved a variety of immune evasion mechanisms: *e.g.*, antigenic variation, latency/sequestration, antigen

capping, antigenic disguise, molecular mimicry/molecular smokescreen, and immune suppression (inhibition of host factors, evasion of complement-mediated killing, B cell manipulation, etc.) [57,58]. Indeed, these various strategies, using one or more in combination, contribute to poor immunogenicity or the lack of effective vaccines for several viruses (*e.g.* herpes simplex virus, human immuno-deficiency virus), bacteria (*Mycobacterium tuberculosis*, *Helicobacter pylori*), and parasites (*Leishmania*, *Trypanosoma*) [59].

5.1 Fooling the host: recognition of "non-self to self". Some examples of pathogen subterfuge can be seen from *Schistosoma mansoni* and *S. haematobium* where a protein with 98% identical nucleotide is shared with the human ortholog complement C2 receptor inhibitory trispannin gene; *Taenia solium* uses the parasite protein paramyosin to inhibit complement proteins that in turn binds to another complement to inhibit the membrane attack complexes (MAC); *Brugia malayi* generates a protein similar to host keratinocytes periphilin-1 protein [58,60]. Being recognized as "self", the parasites camouflage themselves to avoid recognition by the host immune system. *In silico* genome-wide identification in *P. falciparum* has identified several var family members of erythrocyte membrane protein 1 (PfEMP1) having a stretch of 13 to 16 amino acids identical to the heparin-binding domain in human vitronectin [60]. The candidate mimicry motif in vitronectin is in the N-terminal half and in PfEMP1 the motif lies in the extracellular part of the protein, close to the predicted transmembrane domain. A mimicked

358 structure was also found in TRAP and CSP.

359 Vitronectin has been implicated as one of the serum proteins that function for the
360 adhesion of parasites to endothelial receptors and is selectively internalized and associated with
361 malaria pathogenicity [61]. Known as the glue protein, it is reported to promote cytoadherence,
362 tissue regeneration, cell colonization, stabilization of plasminogen activator inhibitor 1, and
363 inhibition of the formation of the pore-forming MAC of the complement system [61-68]. The
364 protein is abundant in the extracellular matrix of different tissues and in the serum.

365 ***5.2 SE36 molecule tightly binds to vitronectin.*** In further efforts to elucidate the role
366 of SE36 and understand the heterogeneity in immunological responses in vaccinated subjects,
367 proteins binding to SE36 were elucidated. Using SE36-immobilized column, Tougan et al. [69]
368 demonstrated that while vitronectin in naive human serum and Ugandan high titer serum equally
369 bound to SE36 even in the presence of other serum proteins (Fig. 2B), no clear direct binding
370 was observed for complement factors (C5, C7, C8, C9, and H), apolipoproteins (ApoAI, HDL,
371 and LDL), thrombin, clusterin, fibronectin, serum albumin, CD5L, or CD14. Purified
372 vitronectin, not human serum albumin, bound to SE36 in a concentration dependent manner.
373 Binding of SE36 to vitronectin occurred even on commercially available vitronectin lacking
374 somatomedin-B motif and Hemopexin domain 4, suggesting that both domains may not be
375 essential for binding. The study also showed that since the recombinant *E. coli*-produced

vitronectin is not glycosylated, the glycosylation moiety may also not be necessary.

Vitronectin was internalized before DNA replication when SERA5 was not yet expressed [69,70]. At the trophozoite stage, vitronectin colocalizes with SERA5; and with the processed 47-kDa fragment during the schizont stage and on the merozoite surface [69]. The binding (dimer bound as predicted by surface plasmon resonance) site was mapped to 18 residues (NH2-Tyr-Lys-Tyr-Leu-Ser-Glu-Asp-Ile-Val-Ser-Asn-Phe-Lys-Glu-Ile-Lys-Ala-Glu-COOH) in the C-terminal region of SE36. This site is predicted to form an α-helix structure [20] and was conserved in 445 geographically distributed *P. falciparum* parasites [13,71]. The binding was tight (equilibrium dissociation constant, K_{D1} = 3.7 x 10^{-9} M), concentration-dependent and specific, observed even in the presence of other serum proteins or under the presence of naturally acquired anti-SE36 IgG [69].

5.3 ***SE47-vitronectin complex camouflage merozoites*****.** To elucidate the role of vitronectin on the merozoite surface, SE36 beads acted as merozoite models in a phagocytosis assay with and without vitronectin [69]. IgG-independent phagocytosis was demonstrated using IgG purified from naïve human serum and Ugandan high anti-SE36 IgG titer serum. Without vitronectin, it was demonstrated that SE36-beads were engulfed by THP-1 cells in an antibody-independent manner. When vitronectin was bound to SE36 beads, engulfment by THP-1 monocytes was inhibited. Several other host proteins (e.g. thrombin; antithrombin III;

394 complements C9, C7) were significantly recruited on the merozoite surface. Unlike vitronectin,
395 these host proteins do not have the motif or structural similarity to facilitate direct binding to
396 SE36. Moreover, the specificity of inhibition was confirmed when inhibition was partially
397 recovered using vitronectin-depleted sera. The binding of several other host proteins is
398 consistent with vitronectin acting as a glue or bridging molecule [61,63,65,68]. Interestingly,
399 although *in silico* analysis revealed that most var family members of PfEMP1, TRAP and CSP
400 had a stretch of 13-16 amino acids identical to the heparin-binding domain (HB1) in vitronectin
401 acting as a mimicry motif [60], the binding site of vitronectin to SE36 was demonstrated at the
402 hemopexin domain (with remarkable binding in regions between hemopexin motifs 2 and 3;
403 near HB2) [69]. Binding to hemopexin-type repeats in human vitronectin was first reported in
404 *Streptococcus pyrogenes* [72].

405 In different pathogens the vitronectin-binding molecules interact with a conserved
406 region in the host vitronectin molecule to regulate the complement mediated lysis [64-68].
407 *Streptococcus pneumoniae* [66] engage vitronectin to bind various oligosaccharides and
408 complement inhibitor Factor H for stronger adhesion and effective bacterial colonization to host
409 cells. In *Haemophilus influenzae*, a gram-negative pathogen, the binding of vitronectin acted as
410 an intermediate bridging molecule to form a multicomplex of bacterial and human proteins that
411 served for adherence to host cells as well as inhibited the host immune response by inactivating

412 the formation of MAC [67]. The binding of vitronectin on the surface of *Helicobacter pylori*
413 (specifically sulfated polysaccharides), in the presence of complement, was shown to inhibit
414 phagocytosis by macrophages [64]. The resulting vitronectin-C5b-7 complex cannot be inserted
415 into the cell membrane [65]; blocks the membrane binding site of Cb5-7 and the
416 deposition/polymerization of C9 effectively inhibiting MAC formation [68]. In malaria
417 parasites, MAC formation can lyse sporozoites, merozoites and gametes and its formation was
418 reportedly controlled by CD59, clusterin and vitronectin [73]. Although further studies are
419 needed to understand the multifaceted profile of vitronectin (to prevent attack by phagocytes
420 and/or evasion by direct lysis by complement) and how it is exploited by malaria parasites, the
421 binding to SE36 was shown to aid in the binding of other host factors which in turn camouflages
422 the merozoite contributing to evasion from the host immune response.

423 **5.4** ***Limited polymorphism of SE36.*** Antigenic polymorphism is well documented as
424 one of the most difficult hurdles for the development of effective malaria vaccines, especially for
425 those targeting the blood-stages [7,9,74]. Added to the list of mechanisms by which SERA5/SE36 is
426 protected from the host immune response (aside from functional redundancy and expansion of
427 family members) would be molecular camouflage. As shown above, the presentation of vitronectin-
428 bound-SE36, as a result of infection, was exploited by the parasite to modulate immunity such that
429 SE36 disguises itself avoiding host clearance leading to the gradual acquisition of immune tolerance.

Immune tolerance may explain why adults or those with high baseline antibody titers before vaccination were low/non-responders and as a whole had low seroconversion compared to subjects with low baseline antibody titers. Indeed, young children or individuals with limited malaria infection history would respond better to BK-SE36 vaccination (Fig. 2A) similar to malaria naïve Japanese adults. This response was seen in all BK-SE36 clinical trials reported to date: individuals with high pre-existing anti-SE36 antibodies had markedly lower antibody response [28,30].

The observed immune tolerance from the host may explain why SERA5 is less likely to be under substantial immune selection pressure compared to other blood-stage malaria vaccine antigens such as AMA1 and CSP. Indeed, *ama1* and *csp* show high nucleotide diversity and significant levels of positive selection (dN>dS) in contrast to *sera5* [71]. The nucleotide diversity of non-repeat regions in the vaccine candidate SE36 was comparable to the housekeeping genes of P-type Ca2+-ATPase and adenylosuccinate lyase (Fig 2C) [13,71]. In a sero-epidemiological study in the Solomon Islands, <50% of adults and <10% of children under 10 years were seropositive to anti-SE36, although higher seropositivity to MSP-1 was observed in the population [21]. The low immunogenicity would mean consequently limited immune pressure for SERA5 which would suggest a limited need for the parasite to acquire mutations to escape the host immune response. From pre-clinical studies, polymorphism in SERA5 may not hamper the potency of SE36 since mouse, rat, or monkey antibodies raised against SE36 cross-react to all the parasite lines so far

examined. Mouse and rat antibodies against SE36 have been shown to inhibit the intraerythrocytic proliferation of parasites *in vitro* [20].

From our studies, SERA5 polymorphism is largely confined to the repeat regions of the gene [13,71,75-77]. There were variations in the number of octamer repeats and serine repeat regions near the N-terminal region of SERA5. Polymorphic sites in the non-repeat regions was confined to 24 nucleotides, and there was no strong signature of positive selection. Sequence analyses performed on strains collected from the two clinical trial/follow-up studies, and cross-sectional studies in Africa did show a consensus sequence with African-specific polymorphisms [77]. It was however, reassuring to note that despite mismatches with the BK-SE36 sequence (based on Honduras 1 strain) in the octamer repeat, serine repeat and flanking regions, and single-nucleotide polymorphisms in non-repeat regions, these polymorphisms did not compromise vaccine response and the observed promising effectiveness based on phase 1 trials [28,30,36]. Of note, sequence analysis of 445 geographically distributed *P. falciparum* parasites showed one genetic polymorphism, "Asn" to "Lys" at the 11th residue, in the 18 residues implicated for vitronectin binding [69]. It is suggested that the binding property of vitronectin is almost conserved in worldwide *P. falciparum* parasites.

6. Conclusion and perspective

Immune evasion strategies in malaria contribute to parasite persistence and immune

466 dysregulation making it difficult to develop effective vaccines. To achieve a robust immune
467 response and consequently high protective efficacy, it will be ideal to overcome some factors
468 which limit the host's ability to respond efficiently to vaccine administration. There are
469 differences in how vaccines may work in malaria naïve adults and in immunized African
470 infants/children and adults. We have observed variations in vaccine responsiveness in our
471 clinical trials which is highlighted for further studies. So far clinical trials for SE36 do provide
472 valuable positive clues: acceptable reactogenicity, absence of unexpected safety signals,
473 favorable immunogenicity profile (immune response can be boosted by natural infection,
474 absence of allele-specific immune response), and an immune response across an HLA diverse
475 population. Some bridging studies and practical strategies may aim to circumvent the immature
476 immune system in infants, *e.g*. presumably postponing vaccination similar to vaccination
477 recommendations for measles and yellow fever. Immune tolerance from previous
478 exposure/immune suppression may evaluate the vaccination schedule, the intervention of
479 chemoprophylaxis/drug pre-treatment or the use of potent novel adjuvants. Current trials do
480 show that delaying the third dose is beneficial. The lack of data on cell-mediated immunity in
481 SE36 and a robust functional assay are some limitations that need to be addressed. Although a
482 few cytokine analyses and complement assays have been done, streamlining and standardization
483 of these assays could prove valuable. Investigation of the underlying mechanisms of

suppression of vaccine responses can reveal novel insights into the capabilities and limitations of human immunity and enhance vaccine effectiveness. So far a hypothesis of immune tolerance/immune suppression has been suggested but the mechanism has not been thoroughly elucidated.

Declaration of interest: TH is the inventor of BK-SE36; TH, KJI, and TT are inventors of BK-SE36/CpG. NP and TH are both supported by a research fund from Nobelpharma Co., Ltd (NPC), the clinical trial sponsor of Burkina Faso trials. These involvements did not influence the writing of this review and the decision to submit the article for publication.

Acknowledgements: Authors gratefully acknowledges the funding support from NPC.

Funding: The writing of this review did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions:

NP and TH drafted the review; NA created Fig. 2C. All authors read, edited and approved the manuscript.

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Figure 1. Representation of serine repeat antigen (SERA5), SE47' and the recombinant vaccine candidate, SE36. **A.** Full-length *Plasmodium falciparum* SERA5 (orange) [13,21]. In a highly regulated protease cascade, the secreted 120 kDa (~100–130 kDa) precursor in the parasitophorous vacuole is processed to P47 (green) and P73 (blue) upon merozoite egress. The P47 is linked to the C-terminal P18 via disulfide bond, another cleavage site in P73. These two fragments are subsequently processed into smaller fragments (P47, P50, P6 and P18) [21]. **B.** The N-terminal fragment, SE47'. Antibodies against this fragment are elicited in immune individuals and is the basis of the blood-stage malaria vaccine, BK-SE36. SE36, the recombinant N-terminal domain without polyserine repeats, is based on Honduras 1 strain and expressed in *Escherichia coli* for mass production and purification under GMP conditions.

Figure 2. Clues from BK-SE36 clinical trials. **A**. Percentage of subjects with >2-fold increase in antibody titers. Data from Uganda [28] and Burkina Faso [30] clinical trials show that younger age group remarkably respond to BK-SE36 vaccination. Green bars, after two full doses of BK-SE36; yellow bars, after booster dose (Dose 3). A full dose contains 100 µg/mL SE36 protein and 1 mg/mL aluminum. A booster dose was done only in younger cohorts (1-5 years-old). **B**. Molecular camouflage of *P. falciparum* merozoites utilizing host vitronectin [69]. SE36 covers the whole merozoite surface and host vitronectin tightly binds to SE36. Vitronectin in turn binds to other host serum proteins to camouflage the merozoite from host immune attacks. Majority of bound proteins are blood coagulation proteins, apolipoproteins and proteins that belong to the complement system. This molecular camouflage is an immune evasion strategy that contributes to parasite persistence and being recognized as "self" the parasites protect themselves from the host immune system by having a host immune steady state, and may explain why there is lesser immune pressure (and thus, limited polymorphism) for SERA5. **C**. Comparison of sequence diversities in some *P. falciparum* antigen (*ama1, csp, sera* {SE36 region} and housekeeping (*serca* + *adsl*) genes [77]. Immune tolerance may explain the low polymorphism of SERA5. Nucleotide diversity (π, grey bars), the number of synonymous substitutions per synonymous site (dS, pink bars), and non-synonymous substitutions per non-synonymous site (dN, blue bars) are shown. The sequence length and the number of sequences used for each gene are reflected: *ama1*, apical membrane protein 1 (n = 241, 1866 bp); *csp*, circumsporozoite protein (n = 238,

681 bp); *se36*, serine repeat antigen (n = 314, 741 bp); and housekeeping genes *serca*, P-type Ca2+-ATPase; *adsl*, adenylosuccinate lyase (n = 292, 5043bp). Data of *ama1*, *csp* and *serca*+*adsl* are from three parasite populations from Africa (Uganda, Tanzania and Ghana). Data using the SE36 region are from four parasite populations (Uganda, Tanzania, Ghana and Burkina Faso): π was analyzed by DnaSP6; dS and dN by MEGA. Excluded from the analyses were: NANP repeat region in *ama1*, eight-mer amino acid repeat units in *csp*; the octamer region, 13-mer insertion/deletion region, serine repeat regions, and the 17-mer dimorphic region in *SE36* vaccine; and the asparagine repeat region in *adsl* + *serca*. When dN>dS, immune pressure/positive selection is inferred.

956 Table 1. Immunogenicity and hyporesponsiveness in pre-erythrocytic vaccine trials.

Vaccine	Study population	Observation	Authors note	Reference
PfSPZ	Tanzanian (20-35 years old) and Dutch adults (19-30 years old) in CHMI trials	Before CHMI: Tanzanian adults have higher baseline antibody titers for AMA-1, EXP-1, and CSP; although both populations had comparable IFN-γ responses. Post-CHMI: cellular recall responses were significantly increased in Dutch volunteers. Tanzanians showed lower lymphocyte IFN-γ production. Immunosuppression was still present 1-month post-CHMI	While the influence of genetic background cannot be excluded, the lack of increased proliferative Th1 responses in Tanzanian volunteers could be partially due to immunosuppression following exposure to blood-stage parasites during CHMI.	[37]
PfSPZ	11 clinical trials in Germany, US and Africa (5-month to 61 years-old)	Females ≥ 11 years of age had significantly higher levels of antibodies to PfCSP than males, but with no evidence of improved protection	Antibodies to PfCSP (and PfSPZ) primarily correlate with other potentially protective immune mechanisms (e.g. antibody dependent and antibody independent cellular responses in the liver)	[38]

		Individuals with prior malaria exposure, such as African adults, have significantly lower antibody responses to PfCSP than malaria naïve adults	Attributed to immune dysregulation due to (a) lifelong exposure to malaria parasites; (b) elimination of the PfSPZ by naturally acquired adaptive immune responses; and/or (c) immunosuppression due to concomitant coinfections	
		In African infants and children, there was a negative correlation between age and antibodies to PfCSP, with the highest antibody levels observed in infants and young children	Antibody levels in these children was comparable to responses seen with malaria-naive adults.	
PfSPZ-CVac (PfSPZ vaccination with concurrent antimalarial chemoprophylaxis)	CHMI trial in the US (18-45 years-old)	Vaccine efficacy was variable (0 to 75% efficacy with same dose but different dosing schedule: with a 7-day administration schedule the vaccine had no efficacy *vs* 75% efficacy on a 5-day dosing schedule)	In the 7-day dosing schedule, the second and third vaccine administration coincided with the period of blood-stage parasitemia from the first vaccination, demonstrating the absence of sterile protective immunity	[39]

Whole Sporozoite and CSP sub-unit vaccine	phase 1 and 2 clinical trials	Compared to malaria naïve adults, individuals with prior malaria exposure have significantly lower antibody responses to CSP (*e.g.* adult males from Equatorial Guinea reported lower antibody responses to PfSPZ compared to US adults; PfSPZ efficacy was greatly reduced in a site with seasonal transmission [30% protection at 6 months in Mali adults *vs* 64% protection in malaria naïve individuals]) Malaria blood-stage infection downregulate pre-erythrocytic stage immunity.	Protection was seen in vaccinated subjects living in malaria endemic areas, despite the observed several-fold lower antibody and cellular immune responses obtained from malaria naïve adults in Germany or the US. A fine, yet inadequately described balance between innate and adaptive immune responses is required for protection	[40] (radiation-attenuated sporozoites) [41] (whole-sporozoite vaccine)
RTS,S/AS01	Phase 2b data (2 sites in Mozambique, 1-4 years-old)	Induction of functional antibodies was lower among children with higher malaria exposure.	High baseline antibody titers obtained either through repeated malaria infection or vaccination impacts B cells, CD_{4+} T cells and innate immune cell phenotypes	[42]

RTS,S/AS01	Phase 2 and 3 trials in 5-17 month-old children	Younger age at time of vaccination (5-11 months *vs* 12-17 months) and high transmission setting were significantly associated with higher anti-CS antibody response	Anti-CS antibody titers after vaccination supports a short-lived and long-lived component of the humoral response; with a higher proportion of the long-lived response noted after Dose 4.	[43]
RTS,S/AS01	Phase 3 data (11 sites, 5-17 months and 6-12 week-old children)	In 6–12 week-old children, high baseline anti-circumsporozoite antibody titers were associated with low anti-circumsporozoite antibody titers after vaccination Within the 5–17 month age group, younger children had higher anti-circumsporozoite antibody titers after vaccination	Maternal antibodies or fetal exposure to malaria parasites inhibit immunogenicity	[44]
RTS,S/AS01	Immuno-informatic tools were used to compare T helper	CSP component of the RTS,S vaccine exhibited a low degree of T-cell epitope relatedness to circulating variants: The prevalence of epitopes restricted by specific HLA-	Reduction in CD4+ T-cell (reduced T-cell help, low T-cell epitope content, reduced presentation of T-cell epitopes by prevalent HLA-DRB1, high human-cross reactivity of T-cell epitopes and	[45]

	epitopes in RTS,S vaccine antigens *vs* 57 CSP variants isolated from infected individuals in Malawi	DRB1 alleles was inversely associated with prevalence of the HLA-DRB1 allele in the Malawi study population T-cell epitope content shared between the vaccine and the Malawian CSP variants was only 34%.	polymorphism of CSP in circulating strains) contributed to tolerance/immune camouflage and have overshadowed the protective efficacy of RTS,S	
ME-TRAP	Kilifi District, 1-6 years-old: one group vaccinated *vs* naturally exposed group	Parasitemia immediately before vaccination suppressed the acquisition of T cell responses (by 15-25%, as measured by IFN-γ production). Parasitemia immediately after vaccination did not suppress T cell response. Concurrent parasitemia did not influence T cell response.	Parasitemia influences initial priming but not subsequent recall and/or boosting of T cell responses. In 1-6 years old, age appears to be a less important consideration; likewise, mild and moderate malnutrition does not appear to reduce	[46]

			immunogenicity	
ChAd63 MVA ME-TRAP	Phase 2 (5-17 month-old Burkinabe children)	Anti-TRAP IgG titers were significantly lower in the parasite positive group compared to the parasite negative group. Anti-AMA titers were significantly higher in the positive parasitemia group at vaccination; but negatively correlated with peak T cell response	Using anti-AMA-1 titers as surrogate marker for prior exposure, acute malaria infection could have reduced humoral immunity to vaccination with ChAd63 MVA ME-TRAP, whilst chronic parasite exposure may have an impact on cell mediated immunity	[47]

968 Table 2. Vaccine immunogenicity and high baseline antibody titers in blood-stage vaccine candidate trials.

Vaccine	Study population	Observation and author's notes	Reference
MSP1, MSP2 and RESA in Montanide ISA720	Phase 1 18-50 years-old East Sepik Province, Papua New Guinea	Humoral response was significantly boosted by the vaccine in individuals with the lowest MSP1 titer at baseline. No change noted for MSP2 and RESA. The lack of boosting of humoral responses was attributed to the high concentration of antibodies prior to vaccination.	[48]
Combination B: MSP1, MSP2 and RESA in Montanide ISA720	Phase 1-11b 5-9 years-old	Vaccine immunogenicity was neither impaired by circulating parasites nor increased after pre-treatment with sulphadoxine-pyrimethamine.	[49]
FMP1/AS02A	Phase 1 18-55 years-old Bandiagara, Mali	Response to vaccine varied and was attributed to the variation in background immunity: in 6 participants that developed high antibody titers (≥ 8-fold increase in antibody titer) the baseline GMT for $MSP1_{42}$ was <3,000 *vs*. those with baseline GMT of >20,000, a <2-fold rise was observed.	[50]
FMP1/AS02	Phase 1	Highest rise in antibody responses was seen in those with low pre-existing antibody titers prior	[51]

	18-55 years-old Kombewa Division, Western Kenya	to immunization. However based on anti-MSP1$_{42}$ antibody titers at Day 90, FMP1/AS02S vaccinees with low and high pre-existing titers were boosted after 3 doses.	
MSP3-LSP	Phase 1b 18-40 years-old Balonghin, Burkina Faso	Total IgG, IgG subclasses and IgM to MSP3 and MSP3-LSP were similar in vaccinees and control (vaccinated with tetanus toxoid vaccine). The absence of response to vaccination was attributed to high pre-existing antibody levels.	[52]
MSP3-LSP	Phase 1b 12-24 months old Balonghin, Saponé, Burkina Faso	IgG1 and IgG3 responses to MSP3-LSP were higher post vaccination than at baseline. Immunogenicity in young children with limited exposure to natural *P. falciparum* infection contrast with [52].	[53]
GMZ2/Al (OH)	(serum samples from 3 phase 1 clinical trials obtained on day 0 and 4 weeks after the last vaccination): German, Gabonese adults and children	GMZ2 vaccination elicited increase in geometric mean antibody titers: 2.8-fold in Gabonese adults; 290-fold in Gabonese children and German adults compared to day 0 (D0). At D0, Gabonese adults have 50-fold higher anti-GMZ2 IgG than Gabonese children and 63.5-fold higher anti-GMZ2 IgG than German adults. There was a strong correlation between antibody titers after vaccination and pre-vaccination level.	[54]

GMZ2	Phase 2 12-60 months old Burkina Faso, Gabon, Ghana and Uganda	Higher baseline level of naturally acquired antibodies in 3-4 years-old resulted to 5.7-fold increase in anti-GMZ antibodies *vs*. 14-fold increase in 1-2 years old	[55]

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