Analysis of the role of TpUB05 antigen from Theileria parva in immune responses to malaria in humans compared to its homologue in Plasmodium falciparum; UB05 antigen

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Abstract

Background: Despite the amount of resources deployed and technological advancements in Molecular Biology, vaccinology, immunology, genetics, and biotechnology, there is still no effective vaccines against malaria. Immunity to either malaria or East Coast fever is usually seen as species- and/or strain-specific. But there is growing body of evidence suggesting the possibility of the existence of cross strain, cross species and cross genus immune responses in apicomplexans. The principle of gene conservations indicates that homologues play similar role in closely related organisms. UB05 antigen (XP_001347656.2) from P. falciparum is part of chimeric UB05-09 antigen; a potential vaccine candidate has been demonstrated to be a marker of protective immunity in malaria. The homologue of UB05 in T. parva is TpUB05 (XP_763711.1) which was also tested and shown to be a potential marker of protective immunity in ECF as well. In a bid to identify potent markers of protective immunity to aid malaria vaccine development, TpUB05 was tested in malaria caused by P. falciparum. Results: It was observed that TpUB05 provoked stronger immune responses in malaria compared to UB05 antigen as tested using ELISA, ex-vivo ELISpot assay and in vitro growth inhibition assay. Conclusion: This study suggests for the first time that TpUB05 from T. parva is a better marker of protective immunity in malaria compared to its homologue UB05 from P. falciparum.

Background

The malaria parasite has a complex life cycle, consisting of three developmental stages in Anopheles mosquitoes (male and female gametocytes), which serves as the transmission vectors, in the liver (sporozoites) and in the blood cells (merozoites, trophozoites and schizonts) of vertebrates (Siciliano and Alano 2015). Therefore, malaria antigens that are identified as vaccine candidates can be broadly grouped into transmission blocking, pre-erythrocytic stage and blood stage antigens. Antigens in the blood stages of malaria parasites represent targets of parasite growth and replication which provoke immune responses that either exacerbate or prevent the growth and development of the parasite in the vertebrate host, hence affecting disease outcome (Gowda and Wu 2018). Clinical manifestations of malaria are attributed to the blood-stages of the parasites that reside within red blood cells (RBCs); thus, vaccines developed against the erythrocytic forms of the parasite are
may contribute considerably to the efficient control of the disease. There are now a number of blood-stage antigens are being characterized for inclusion as vaccine components in clinical development (Miura K 2016). Amongst the most studied and advanced blood-stage vaccine antigens are; Circumsporozoite protein (CSP), serine repeat antigen 5 (SERA-5), merozoite surface protein 3 (MSP-3) and apical membrane antigen 1 (AMA1) (Miura K 2016). These vaccine candidates have not been efficacious in African children (Ogutu et al., 2009, Spring et al., 2009). However, a multistage vaccine made up of CSP and AMA1 reduced the incidence of clinical malaria episodes in vaccinated children by 50% compared to the control group (Cech et al., 2011). The extensive genetic polymorphisms observed in some malaria vaccine candidates are considered to be a serious hindrance for blood-stage vaccine development (Takala and Plowe 2009). RTS,S (Mosquirix™), a leading vaccine candidate that targets the initial infection of the liver, has demonstrated only partial efficacy that wanes rapidly (Olotu et al., 2013). Although a number of erythrocyte stage antigens are under development, it is possible and there is still the need to search for better markers of protective immunity and hence subunit vaccine candidates to improve the outcome of vaccination. Second generation vaccine candidates that could constitute a multivalent rather than a univalent vaccine candidate are required that target the red blood cells stages and other stages of the parasite (Titanji et al., 2017). There is therefore the need to scavenge other vaccine candidates for the development of a very successful malaria vaccine that can confer full and long-lasting protection. Identification of vaccine candidates begins with the identification of markers of protective immunity. One such marker of protective immunity is UB05 antigen from *P. falciparum* that was identified through a differential immunoepidemiology study (Titanji et al., 2009).

One approach of identifying better markers of protective immunity would be to seek homologues of markers of protective immunity in closely related organisms. The idea of the conservation of gene products has been proven to suggest that orthologous gene products appear to perform similar functions in closely related species (Gabaldón and Koonin 2013). Apicomplexans which includes the species *P.*, *Toxoplasma, Babesia* and *Theileria*, amongst others, are known to be the most successful intracellular pathogens known to humans. They share a lot of similarities in their life cycles and
interactions with the immune system of their hosts; including cross-reactivity between antigens from different strains, species or genera. A few studies have studied the cross-reactivity of antigens during the immune response in pre-erythrocytic and erythrocytic stages of different P. species. For example, a circumsporozoite protein (CSP)-based vaccine (VMP001) from P. vivax containing conserved CSP regions generated antibodies that could also recognize the CSP on the surface of P. berghei and P. falciparum. Despite the low level of cross-reactive antibody titers, the same study also revealed that the anti-serum reduced the heterologous P. berghei infection (Yadava et al., 2012). CeITOS is a cell-traversal protein expressed in ookinetes and sporozoites which is another example of a highly conserved molecule across P. species. Antibodies provoked in mice by recombinant PfCeITOS induced cross-species protection against P. berghei challenge by cross-reactivity to the heterologous P. berghei sporozoites (Bergmann-Leitner et al., 2010). Using differential immunoscreening, UB05 antigen was identified as a marker of protective immunity in malaria (Titanji et al., 2009). Knowing that homologues may play similar roles in closely related organisms, the information gotten by studying a marker of protective immunity could be transferred to another antigen or disease. Conceivably, the homologue of UB05 in T. parva; TpUB05 was also shown to be a marker of protective immunity in ECF that affects cattle (Dinga et al., 2015).

In previous studies in our group, the P. falciparum antigens; UB05, UB09 and chimeric UB05-09 and their respective polyclonal antiserum were analysed using ELISA, ELISpot assay and Growth Inhibition Assay with samples from a malaria endemic region (Dinga et al., 2016, 2017, 2018). During these same studies, we included TpUB05 and its polyclonal antiserum in separate wells on the same plates and exposed them to the same experimental conditions as UB05 antigen. Now we present the data obtained and compare the performance of UB05 antigen from P. falciparum vis-à-vis that of TpUB05 from T. parva in human malaria.

Results

**Molecular cloning and characterisation of TpUB05**

Using gene specific primers, TpUB05 was amplified from T. parva schizont RNA using reverse transcription to produce a 291 bp DNA fragment (Dinga et al., 2015) while UB05 was amplified from a
*P. falciparum* cDNA library to produce a 243 bp DNA fragment (Dinga *et al.*, 2016). Both fragments were cloned into a pET32a+ expression vector and overexpressed *E. coli* cells yielding recombinant fusion proteins migrating at 28 KDa and 26 KDa, respectively in SDS-PAGE performed in 15% polyacrylamide. The recombinant fusion proteins contained a 6xHis Tag, a S Tag domain and a 109 amino acid thioredoxin fusion protein partner. However, it was easier to overexpress and purify TpUB05 as compared to UB05 antigen (Dinga *et al.*, 2015 and 2016).

**TpUB05 antigen from *T. parva* possesses human B cell epitopes which are associated with protective immunity against malaria**

Plasma from the study subjects were tested *ex-vivo* with TpUB05 using ELISA and it was shown that SIS possessed more anti-TpUB05 antibody than FSS. Adults (SIS + FSS) produced more antigen-specific antibody to TpUB05 (*p* = 0.0001) than children (SC) (Fig. 1A). When the antibody response to TpUB05 was compared to that of UB05 in the malaria study, it was observed that the former produced a significantly higher antibody response (*p* = 0.006) compared to the latter (Fig. 1B).

**Human antibody levels to TpUB05 negatively correlates with fever and malaria parasitaemia**

When the antibody levels to TpUB05 were plotted as a function of parasitaemia, it was observed that there was a negative correlation between parasitaemia and antibody levels. This implied that the higher the specific antibody level the lower the parasite density in the blood. In other experiments, the antibody levels were shown to be statistically higher in subjects with no fever and then in subjects with fever (Fig. 2A, 2B). Taken together these results suggested that high anti-TpUB05 antibody levels was associated with a state of protective immunity to malaria.

**TpUB05 is a better reagent to distinguish between SIS and FSS compared to UB05**

There is currently no simple assay to distinguish between the semi-immune (SIS) and the frequently
sick status (FSS) in malaria. We therefore compared the diagnostic potential of TpUB05 and its homologue UB05 in distinguishing between these two clinical groups. The results are presented in Table 3 showing that rTpUB05 is a relatively better as a better reagent than UB05 for diagnosing acquisition of some immunity against malaria. While the specificity of the assay with UB05 was 85% that of TpUB05 was 92.5%. and within the cut off range for a good diagnostic test The positive predictive value for UB05 was 57% as compared to 90% by TpUB05. Similar trend was observed for the negative predictive value which was for UB05 was 39.1% and this was increased to 52.1% when using TpUB05 (Table 1). However, the sensitivity of the TpUB05 assay was still too low for TpUB05 to be considered for routine usage.

Table 1: Comparison of the sensitivity, specificity and positive predictive values of using UB05 and TpUB05 antigens as reagents to differentiate between semi-immune subjects (SIS) and frequently sick subjects (FSS) in a malaria-endemic region.

| Test antigen | Sensitivity (%) | Specificity (%) | Positive Predictive Value (%) | Negative Predictive Value (%) |
|--------------|----------------|-----------------|------------------------------|------------------------------|
| UB05         | 13             | 85              | 57                           | 39                           |
| TpUB05       | 44.30          | 92.50           | 90                           | 52                           |

**Human PBMCs recognize T-cell epitopes in TpUB05 in a manner that correlates with immune protection against malaria**

With the presence of human B-cell epitopes in TpUB05, it was worthwhile to test for the possibility of the presence of human T-cell epitopes in TpUB05. Since TpUB05 is the T. parva homologue of UB05 found in P. falciparum, and it has been shown that UB05 stimulates T-cell proliferation in persons exposed to malaria, we decided to test the ability of TpUB05 to recall T-cell function in humans. To do this, PBMCs from 63 subjects were isolated and stimulated with r-TpUB05 to produce IFN-γ and measured using human ELISpot assay. Twenty-seven subjects recognized TpUB05 of which SIS make up 85.2% (Fig. 3A). When the recognition of TpUB05 was compared to that of UB05 (Fig. 3B), there was no significant difference (Fig. 3C).

We also found that there was a significantly higher stimulatory index (SI) of IFN-γ production between
subjects with no fever \((p = 0.002)\) compared to those who had fever (Fig. 4A). The same trend was observed when the absence or presence of parasitaemia was taken into consideration (Fig. 4B). These results suggested that TpUB05 stimulated human PBMCs to produce IFN-\(\gamma\) in a manner that correlated with protection against malaria.

**Online prediction indicates the presence of human epitopes on TpUB05**

Online prediction of human T cell and antibody epitopes revealed the presence of epitopes that could bind to human MHC on TpUB05. The MHC I and II binding peptides with a percentile rank below 1.0 were considered as those with very high affinity for the MHC molecules (Table 2). Using algorithms at www.iedb.org also predicted the presence of human antibody epitopes in TpUB05 (Table 2).

**Rabbit polyclonal antibody raised against recombinant TpUB05 performs better in inhibiting *P. falciparum* parasite growth *in vitro* than antibodies to recombinant UB05**

The ability of the TpUB05-specific antiserum to inhibit parasite growth *in vitro* was tested using the growth inhibition assay (GIA) as described in Materials and Methods and the data compared with that obtained with UB05-specific antiserum. Rabbit anti-TpUB05 antiserum was able to inhibit parasite growth in vitro in a way that was significantly higher than the performance of anti-UB05 antiserum \((p = 0.0001)\) (Figure 5). This inhibition was observed for all the parasite strains tested (Figure 5).

In positive control experiments we tested in parallel two monoclonal antibodies available from Bioresources, namely, anti AMA1 and anti EBA11. Amongst the parasite strains tested, only the *P. falciparum* HB3 strain was not inhibited by the positive control antibodies (anti-AMA1 and anti-EBA175) indicating that these two anti-sera are less cross-reactive that those direct against TpUB05 and UB05. The results presented in figure 5 showed that Anti-TpUB05 antiserum did not significantly inhibited parasite growth more than anti-AMA1 antibody. However, it performed far better than anti-EBA175 antibody in inhibiting in vitro parasite growth \((p = 0.0001)\).

**Discussion**

Malaria vaccine development has been mostly focused on single antigens. But the complex nature of
the malaria parasite life cycle and the mechanisms it uses to evade the human immune responses implies the ideal malaria vaccine should target several antigens expressed in different stages of the parasite’s development (Titanji et al., 2017) as well as homologous antigens from related species in a bid to circumvent the setbacks observed with strain-specific or species-specific protective immune responses. The first step towards this would be the identification and characterization of homologous antigens from phylogenetically related organisms as better or more potent markers of protective immunity against malaria. Here we show for the first time that TpUB05 antigen from T. parva is a better diagnostic reagent for semi immune status in malaria as compared to UB05 antigen from P. falciparum. Antibodies to TpUB05 were more effective than those directed against its homologue UB05 in inhibiting P. falciparum growth in culture.

The principle of the conservation of gene function shows that most orthologous gene products play similar role in closely related organisms (Gabaldón and Koonin 2013). We had earlier shown that the T. parva homologue of UB05; TpUB05, is a marker of protective immunity in ECF. We then decided to characterize TpUB05 in malaria caused by P. falciparum. This observation of effective cross immunisation with homologous antigens has been shown before (Gabaldón and Koonin 2013). In other words, it might be more useful to search for antigens that induce protection against ECF and test in malaria caused by a distant relative like P. falciparum. To verify this hypothesis, we carried out simultaneous testing of immune responses in semi-immune and malaria susceptible subjects and showed TpUB05 is a more potent marker of immune protection in malaria compared to UB05. Although B cells have been seen as contributing little to resistance and protective immunity and resistance to infections with Apicomplexa, many studies have shown that hosts infected with these parasites are capable of producing parasite-specific immunoglobulins which are protective, after recovering from an infection (Hogh 1996). The present study shows that there was a statistically significant higher amount of antibodies in human plasma from SIS that recognised TpUB05 as compared to plasma from FSS, hence an indication of correlation with protection from malaria caused by P. falciparum. When this was compared to the ELISA results obtained with UB05 antigen (Dinga et al., 2017) under the same experimental conditions, a similar trend was observed. However, TpUB05
appeared to be a better marker of protective immunity to malaria than UB05 using ELISA, as TpUB05 was able to detect a higher amount of antigen-specific antibody in human plasma in a manner that was statistically significant.

Studies to elucidate the mechanism(s) of the protective immune response to apicomplexan parasites implicate the role of the production of gamma interferon (IFN-\(\gamma\)) amongst other cytokines and chemokines (Doolan and Martinez-Alier. 2006), which control parasite infectivity and interfere with parasite development. To find out whether TpUB05 from T. parva can induce immune response similar to those occurring during malaria caused by P. falciparum, we tested the ability of TpUB05 to recall T cell function in PBMCs from subjects in a malaria endemic region. Biostatistics analyses revealed that there are T cell epitopes in TpUB05 that could bind and be recognized by human T cells leading to the production of IFN-gamma. This production of IFN-gamma occurred in a manner that correlated to immune protection against malaria as it was preferentially recognized by semi-immunes as compared to frequently sick subjects. When these data were compared to that obtained with UB05 antigen from P. falciparum (Dinga et al., 2016), which were obtained under the same experimental conditions, the same trend of correlation with protection against malaria was observed. There was no significant difference between these two antigens in stimulating the production of IFN-gamma from human PBMCs suggesting that they could stimulate similar reactions in vivo; this however remains to be shown.

The hallmark of an effective malaria subunit vaccine would be its ability to stimulate the cellular as well as the antibody components of the immune system that are protective. This implies that an antigen's ability to preferentially detect antigen-specific antibodies and recall T cell function in people who have acquired limited protective immunity to malaria is an indication that the protein is involved in the immune protection against malaria parasites. TpUB05 was able to recall SIS’s T-cells’ ability to produce IFN-gamma and detect antigen-specific antibodies in these semi-immunes, hence a marker of protective immunity in malaria.

Contamination of antigen preparations with lipopolysaccharide (LPS) have been shown to confound T-cell response assay results to malaria antigens. However, it is unlike there was a contribution of
possible contaminating bacterial LPS to the observed responses in the present study as the responses were specific to each of the groups. The cytokines whose production appears to be influenced by LPS include IL-1beta and IL-6 but not IFN-gamma which was studied herein (Jansk et al., 2003).

Some schools of thought have it that an association is not necessarily causation and as such should not be interpreted in isolation. But when the data obtained in this study is associated with fever and parasitaemia it implies causation. Normal body temperature with little or no parasites in the blood as well as higher IFN-gamma production and antibody amounts have been shown to correlate with immune protection against clinical malaria (Titanji et al., 2009, Doolan et al., 2009, Greenhouse et al., 2011, Dinga et al., 2016). The data obtained in this study strongly suggest that TpUB05 is a better marker of protective immunity in clinical malaria compared to UB05.

Having shown that TpUB05 possesses T cell and B cell epitopes that are bind and recognize human MHC molecules and antibodies, respectively, it was necessary to find out if polyclonal antibodies raised against TpUB05 from T. parva has any effect on malaria parasite development by employing the in vitro growth inhibition assay. Growth inhibition assay which involves impaired merozoite invasion and subsequent development of parasites in erythrocytes is currently being considered as one of most relevant assays to screen potential blood-stage vaccine candidates prior to moving to the stage of clinical development. Purified polyclonal total IgG raised in rabbits against TpUB05 from T. parva was able to significantly inhibit malaria parasite growth in vitro. This inhibition was statistically higher than that observed with anti-UB05 polyclonal antibody as well as anti-EBA175 monoclonal antibody but not anti-AMA1 monoclonal antibody. Hence TpUB05 should be considered a potent and better marker of protective immunity against malaria as compared to UB05 from P. falciparum.

Bioinformatics comparison of TpUB05 and UB05 shows that they exhibit a degree of sequence homology: 43.3% identity and 67% similarity was observed. The in silico analysis predicted presence of human T cell and antibody epitopes on TpUB05 and the cross-reactivity observed was expected in view of the significant homology between the two antigens. However, based on the ELISA and GIA data, TpUB05 may contain more potent B-cell epitopes compared to UB05 that are yet to be identified.
Put together the results from this study implies TpUB05 is a more potent marker of protection against malaria and confirms the notion that homologues could play similar or better roles in related organisms. This is in line with previous studies where *Mycobacterium bovis* (BCG vaccine) was used to vaccinate humans against *M. tuberculosis* (Fine *et al.*, 1999) and humans exposed to *Onchocerca ochengi* were protected against *O. volvulus* infection (Wahl *et al.*, 1998). The potential role of TpUB05 in inducing a more effective immune protection against malaria infection and disease warrants more investigation.

While species-specific vaccine development against either malaria or ECF is expected to effectively reduce transmission, targeting cross-reactive epitopes in potent vaccine candidates may lead to protective immunity that cuts across multiple apicomplexan parasites. The importance of such immune cross-reactivity may even insinuate enhancement of protective immune responses amongst distantly related apicomplexans like *P. falciparum* and *T. parva*. The results obtained in the current study permits the evaluation of such a potential cross-species malaria vaccine candidate at an earlier stage, with the potential to save time and resources for development decisions.

**Methods**

**Table 1**: Study design to show how the study was planned, executed and reported. (see the Supplementary Files)

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**Study site**

The study was carried out in Buea, which is endemic for malaria. Buea is a multi-ethnic town found along the flanks of Mount Cameroon in the South West region of the Republic of Cameroon. The prevalence of malaria in Buea varies between a meso-endemic (dry season) and a hyperendemic (rainy season) and) zone with perennial malaria transmission (Nkuo Akenji *et al.*, 2005).

**Study population**

A general invitation was given to the people staying in the Buea municipality and those who visited
the Buea Regional Hospital for medical attention in order to recruit subjects for this study (Dinga et al., 2016). After explaining the objectives of the project to voluntary individuals, informed consent was obtained from each participant above 18 years of age or parents/guardians of sick children below the age of 5. The medical history of each subject was obtained and recorded by a collaborating Medical Nurse who also assisted in completing a questionnaire form. Blood was then drawn from the subjects by a Medical Laboratory Technician for the diagnosis of malaria, peripheral blood mononuclear cells isolation, and serology as described below.

The following criteria were used to screen and recruit subjects into the study; (i) subjects who were aged 18 years of age or older and who had been living in the study site for at least 3 years; (ii) subjects with no history of a malaria episode in the last 12 months, no fever and parasitaemia at sample collection, no use of mosquito bed net, hence had been exposed to mosquito bites and was on no prophylaxis were designated semi-immune subjects (SIS); (iii) subjects who had had at least one malaria episode in the last 12 months and had fever and parasitaemia at sample collection referred to as frequently sick subjects (FSS); and (iv) children aged 5 or below who had fever and parasitaemia at the time of sample collection were referred to as sick children (SC). This cohort of individuals was highly selected and described previously (Titanji et al., 2009, Dinga et al., 2014). The analyses for fever, parasitaemia and blood sample collection for ELISpot assay and ELISA took place between March–June 2014 (Dinga et al., 2016).

**Haemoglobin measurement**

In order to assess the level of anaemia which be due to malaria, Haemoglobin level of the volunteers was measured using a haemoglobinometer (STAT- Site MHgb metre, Stanbio Laboratory Texas, USA). This was done as earlier reported (Dinga et al., 2014, and 2016).

**Assessment of nutritional status**

It has been shown that being in good health reduces susceptibility to non-communicable and infectious diseases including malaria (Suhrcke et al., 2011). Assessment of the nutritional status of
the study population was done as reported in Dinga et al., 2014 and 2016), as it is assumed that a probable indication of good health and being healthy is having a normal body mass index (BMI) (Sach et al., 2007). It was therefore imperative to measure the BMI of the study subjects as part of the biodata collection and to assess their nutritional status. Only subjects in the normal or pre-obese range were admitted into the study (Dinga et al., 2014 and 2016).

The BMI was calculated by the following formula:

\[ \text{BMI (kg/m}^2\text{)} = \frac{\text{Weight (kg)}}{(\text{Height})^2}\text{(m)} \]

**Parasite strains, antigens and polyclonal antibody**

As mentioned in Dinga et al., 2018, all laboratory strains were generously donated by the MR4/BEI Resources, NIAID, NIH, Manassas, VA. The following *P. falciparum* laboratory strains were used: FCR-1/FVO (MRA-909, contributed by W. Trager), 3D7 (MRA-102, contributed by Daniel J. Carucci), and HB3 (MRA-155, contributed by Thomas E. Wellems) for the in vitro assay. Two field isolates; GH01 and SC01 were also used for the study and were obtained from the Buea District Hospital and Solidarity Clinic, Buea, Cameroon, respectively.

Overexpression of recombinant TpUB05 and its polyclonal antibody production in rabbits was described in Dinga et al., 2015. UB05 and polyclonal antibodies raised in rabbits against UB05 antigen (Dinga et al., 2016), were obtained from the cited studies.

**Preparation of Peripheral Blood Mononuclear Cells from human donors**

The procedure to isolate peripheral blood mononuclear cells (PBMCs) was performed as described by Dinga et al., 2016. Briefly, people who responded to the call were pre-screened for recruitment into the study by assessing their malaria status. Their fingers were pricked to microscope slides and stained with Giemsa. The slides were read under an oil immersion microscope (Unico Microscope; series: G380) by two Microscopists at 1000× and 100 fields counted.

To confirm the status of SIS (absence of parasitaemia and the questionnaire responses which shows that they had not suffered from malaria for at least 12 months), they were monitored for a period of
anywhere between 2 weeks to 4 months prior to blood collection. Microscopic slide examination, medical records and the questionnaire was used to make sure FSS were sick of malaria (presence of parasitaemia and fever) and have had suffered at least one malaria attack in the last 12 months, as of the time of sample collection. It was ensured that participants in both cases had also stayed at the malaria endemic study site for at least three (03) years. The exclusion criteria were; anaemic individuals, obese persons, very elderly and those unable to consent by themselves.

Preparation of PBMCs for use in ELISpot assay was done using percoll gradient method as earlier described (Hamburger et al., 1985, Dinga et al., 2016). Briefly, 8 mL of venous blood was collected from subjects in EDTA containing tubes. An equal volume of sterile PBS was then added to the blood samples and layered on percoll discontinuous gradient solution (5 mL each of 60%, 50% and 40% percoll solution layered in that order). Using a Heraeus Sepatech Minfuge RF, the samples were centrifuged at 1170 x g for 30 min. The white buffy coat at the 40%/50% and the 50%/60% interface were carefully collected and put into a 50 mL falcon tube. These interfaces contain monocytes and lymphocytes, respectively. Washing of the collected buffy coat to remove excess percoll was done twice with 40 mL of sterile PBS supplemented with 5% foetal calf serum at 4000 rpm for 10 min. 2 Cells were then re-suspended in 2 mL of complete RPMI-1640 culture medium (CCM) was used to resuspend the cells and cell viability and quantification was done using Trypan Blue staining and the Improved Neubauer counting chamber (Shanehsazzadeh et al., 2013).

**Enzyme linked immunosorbent spot (ELISpot) assay**

This was done as described by Dinga et al., 2016. Briefly, ELISpot assay was used to determine the proportion of IFN-gamma-secreting PBMCs from subjects *ex-vivo* upon stimulation with recombinant antigens TpUB05 and UB05. The PBMCs were used within 2 h of collection in EDTA-containing tubes, that is, transported to the laboratory, (10 min away), isolated and analysed. Cell and antigen preparations were carried out in a biological safety cabinet prior to use to ensure sterility of the cells and protein samples. 5 µg/mL of antigen was mixed 300 000 PBMCs and tested in triplicates. MABTECH AB kits was used to carry out the ELISpot assay as previously described (Dinga et al.,
The positive control used to recall T cell memory is the anti-CD3-2 monoclonal antibody. The stimulation index (SI) was calculated as follows: \( SI = \frac{\text{mean number of spots in triplicate test (with antigen) wells}}{\text{mean number of spots in triplicate negative control (without antigen) wells}} \).

An SI value of more than two was considered positive (Pinder et al., 2004, Jiang et al., 2007).

**Plasma sample collection**

As indicated in Dinga et al., 2017, plasma was collected from the blood samples from which PBMCs were isolated and stored until further analysis. At the time of performing the ELISA experiments, additional subjects were recruited into the study to obtain more plasma samples and increase sample size for analysis. Plasma samples were also collected in EDTA tubes from children below the age of 5 whose parents gave their consent and tested positive for malaria (fever and parasitaemia).

For the additional adult subjects, 5 mL of venous blood and 1 mL from children were collected in Vacutainer tubes containing EDTA and kept at +4°C for 1 hour. Samples were then centrifuged and the plasma aspirated and stored at −20°C until use.

**Determination of the levels of antibodies recognising r-TpUB05 and r-UB05 by ELISA**

As mentioned, in Dinga et al., 2017, antibody (total IgG) measurement was carried out by ELISA as earlier described (Katende et al., 1998) with modifications. 100 µL of 0.625 µg/mL r-TpUB05 or r-UB05 in PBS was used to coat microtitre plates by incubation at +4°C overnight. Some wells were coated with soluble fraction of crude *E. coli* extract or r-UB05 antigen as positive control for antigen while the Tag only (Fusion partner) antigen was used as a negative control antigen. The plates were washed thrice with 200 µL of Wash buffer (PBS-Tween-20 (0,05%) after overnight incubation, followed by blocking with 150 µL per well of 0.2% casein in PBS with 0.05% Tween-20. After the second washing, plasma was added at 1:150 dilutions in Wash buffer containing 1% skimmed milk. They were then incubated at room temperature for 3 hours. To obtain and record background signals (negative control for plasma), human plasma from malaria-naïve individuals which was kindly given to us by Mrs. Philomena Gwanmesia of the Biotechnology Center, University of Yaounde I, Cameroon was
included in the assay. After the 3 hours’ incubation at room temperature, the plates were washed with Wash buffer three times and 100 μL of anti-rabbit IgG-HRP conjugate (Sigma) diluted at 1:10 000 in Wash buffer containing 1% skimmed milk and incubated for 1 hour at room temperature. After which the plates were washed with Wash buffer and 100 μL of substrate added. Optical density (OD) of the wells were obtained at 405 nm using a microplate reader (LabsystemsMultiskan MCC 340, Helsinki, Finland). A subject was considered positive if its OD value was equal to or greater than the mean control OD +2SD after subtracting background. The experiment was run in duplicates.

**Growth Inhibition Assay**

The ability of purified rabbit IgGs raised against r-TpUB05 and r-UB05 to inhibit the replication of *P. falciparum in vitro*, was tested by measuring parasite lactate dehydrogenase (pLDH) in late trophozoite/early schizont stage cultures as described in Methods_In_Malaria_Research 6th Edition (Moll et al., 2013, Dinga et al., 2018). Briefly, enrichment of late-stage infected erythrocytes was done by performing three 5% sorbitol synchronizations and 60% Percoll gradient centrifugations. The assay was done using late trophozoite or schizont stages. This was ensured by starting and stopped (≈96 hours) the experiment when most of the parasites were at those stages. The start parasitaemia of the culture was at 0.1%-0.3% parasitaemia and 1% haematocrit in a CO₂ incubator for 2 cycles. Polyclonal antibodies raised against r-TpUB05 or r-UB05 was added at the start of the cultured at an optimized dilution of 1:10 (final concentration of anti-TpUB05 total IgG = 0.48 mg/mL and anti-UB05 total IgG = 0.63 mg/mL). The monoclonal antibodies, anti-EBA-175 RII (final concentrations; R217 at 0.133 mg/mL and R218 at 0.153 mg/mL) and anti-AMA1 (final concentration of 0.1 mg/mL) and were used as positive controls in the inhibition assays, while negative control wells contained pre-immune sera (final concentration of 0.7 mg/mL) or no antiserum. Control wells were included in the assay that contained either only parasitized RBCs (pRBC only) or normal RBCs. Percentage growth inhibition was calculated by first of all subtracting the average OD from the normal RBCs wells from all the other OD values obtained before proceeding to the calculation proper.
**Immune epitope prediction using online resources**

*In silico* prediction of immune epitopes from TpUB05 and UB05 was done using algorithms in IEDB Analysis Resources (www.immuneepitope.org). This was already described in Dinga *et al.*, 2015, 2016, and 2017). The MHC I binding predictions were made on 7/30/2019 using the IEDB recommended method which combines predictions from ANN (Andreatta and Nielsen, 2016, Lundegaard *et al.*, 2008a, Lundegaard *et al.*, 2006, Lundegaard *et al.*, 2008b, Nielsen *et al.*, 2003, Buus *et al.*, 2003), SMM (Peters and Sette 2005 ) and Comblib (Sydney *et al.*, 2008). While the MHC II binding predictions were made on 7/30/2019 using the IEDB analysis resource Consensus tool (Wang *et al.*, 2008, 2010). The Emini surface accessibility scale (Emini *et al.*, 1985), Kolaskar and Tongaonkar antigenicity scale (Kolaskar and Tongaonkar 1990), Bepipred-1.0 Linear Epitope Prediction (Larsen *et al.*, 2006), and BepiPred-2.0: Sequential B-Cell Epitope Predictor (Jespersen *et al.*, 2017) were used to predict the presence of human antibody epitopes on TpUB05.

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**Statistical Analysis**

Chi-square, Analysis of Variance and The Mann–Whitney tests were employed to compare differences between the mean SI values between study groups. ANOVA was used to determine the significance in the OD values of the various antigens against the immune status of the study subjects and to assess the effect of the various IgGs on parasite growth. Spearman’s rho was used to determine correlation between data sets. Kruskal–Wallis test was used to assess difference between body temperature, parasitaemia and OD values. All these tests were performed using SPSS software (Version 17.0, Chicago, IL, USA). A value of $p < 0.05$ was considered significant.

**Declaration**

**Ethics approval and consent to participate**

Ethical clearance for this study (Ref: 2013/144/UB/FHS/IRB) was obtained from the Institutional Review Board of the Faculty of Health Sciences, University of Buea. All participating adult subjects read, approved and signed the consent form. A parent or guardian of any child participating in the
study provided informed consent on the child’s behalf. The informed consent given was written.

Ethical clearance for blood collection and polyclonal antibody production in rabbits was approved by the ILRI Institutional Animal Care and Use Committee (ILRI-IACUC) (ref no. 2013.05). ILRI-IACUC provides clearances based on The Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines on the care and use of animals in research.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

JND conceived and designed the experiments. JND, SDG, SNP, FNGC and DLN performed the experiments. JND, SDG, SNP, FNGC, DLN, AD, RP and VPKT analysed the data. JND, DLN, AD, RP and VPKT drafted the manuscript or revised it critically for important intellectual content. JND agrees to be accountable for all aspects of the work. All authors read and approved the final manuscript.

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References
Andreatta M. and Nielsen M. Gapped sequence alignment using artificial neural networks: application to the MHC class I system. Bioinformatics 2016; 32:511-7.

Bergmann-Leitner ES, Mease RM, De La Vega P, Savranskaya T, Polhemus M, Ockenhouse C, Angov E. Immunization with pre-erythrocytic antigen CelTOS from *Plasmodium. falciparum* elicits cross-species protection against heterologous challenge with *P. berghei*. PloS one. 2010; 5(8): e12294.

Buus S, Lauemøller SL, Worning P, Kesmir C, Frimurer T, Corbet S, Fomsgaard A, Hilden J, Holm A, Brunak S. Sensitive quantitative predictions of peptide-MHC binding by a ‘Query by Committee’ artificial neural network approach. *Tissue Antigens* 2003; 62:378-384.

Cech PG, Aebi T, Abdallah MS, Mpina M, Machunda EB, Westerfeld N, Stoffel SA, Zurbriggen R, Pluschke G, Tanner M, Daubenberger C, Genton B, Abdulla S. Virosome-formulated *Plasmodium falciparum* AMA-1 & CSP derived peptides as malaria vaccine: randomized phase 1b trial in semi-immune adults & children. *PLoS ONE*. 2011; 6: e22273.

Dinga JN, Dobgima GS, Titanji VPK. Enhanced acquired antibodies to a chimeric *Plasmodium falciparum* antigen; UB05-09 is associated with protective immunity against malaria. *Parasite Immunol*. 2017; 39:e12445.

Dinga JN, Gamua SD, Ghogomu SM, Titanji VPK. Preclinical efficacy and immunogenicity assessment to show that a chimeric *Plasmodium falciparum* UB05-09 antigen could be a malaria vaccine candidate. *Parasite Immunol*. 2018; e12514.

Dinga JN, Mbandi SK, Cho-Ngwa F, Nde PF, Moliki J, Efeti RM, Nyasa BR, Anong DN, Jojic N, Heckerman D, Wang R, Titanji VPK. Differential T-cell responses of semi-immune and susceptible malaria subjects to in silico predicted and synthetic peptides of *Plasmodium falciparum*. Act Trop 2014; 135: 104-121.

Dinga JN, Njimoh DL, Kiawa B, Djikeng A, Nyasa RB, Nkuo-Akenji T, Pelle R, Titanji VPK. Differential T-cell responses to a chimeric *Plasmodium falciparum* antigen; UB05-09, correlates with acquired immunity to malaria. *Parasite Immunol*. 2016; 38:303-316.

Dinga JN, Wamalwa M, Njimoh DL, Njahira MNN, Djikeng A, Skilton R, Titanji VPK, Pelle R. TpUB05, a
homologue of the immunodominant *Plasmodium falciparum* protein UB05, is a marker of protective immune responses in cattle experimentally vaccinated against east coast fever. *PLoS ONE*. 2015; 10: e0128040.

Doolan DL and Martínez-Alier N. Immune response to pre-erythrocytic stages of malaria parasites. *Current Molecular Medicine*, 2006; 6:169-185.

Doolan DL, Dobaño C, Baird JK. Acquired immunity to malaria. *Clin Microbiol Rev* 2009; 22:13-36.

Emini EA, Hughes JV, Perlow DS, Boger J. Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *J Virol*. 1985; 55:836-9.

Fine PEM, Cameiro IAM, Milstein JB, Clements CJ, World Health Organization. Issues relating to the use of BCG in immunization programs: a discussion document. World Health Organization.

https://apps.who.int/iris/handle/10665/66120. Accessed 15 Aug 2019.

Gabaldón T and Koonin VE. Functional and evolutionary implications of gene orthology. *Nat Rev Genet*. 2013; 14(5): 360 – 366.

Gowda DC and Wu X. 2018. Parasite Recognition and Signaling Mechanisms, innate immune responses to malaria. *Front. Immunol*, 2018; 9:3006. doi: 10.3389/fimmu.2018.03006.

Greenhouse B, author1 Benjamin Ho, corresponding author3 Alan Hubbard, Njama-Meya D, Narum DL, Lanar DE, Dutta S, Rosenthal PJ, Dorsey G, John CC. Antibodies to *Plasmodium falciparum* antigens predict a higher risk of malaria but protection from symptoms once parasitemic. *J Infect Dis*. 2011; 204:19-26.

Siciliano G, Alano P. Enlightening the malaria parasite life cycle: bioluminescent Plasmodium in fundamental and applied research. *Front Microbiol*. 2015;6:391. doi:10.3389/fmicb.2015.00391.

Hogh B. “Clinical and parasitological studies on immunity to *Plasmodium falciparum* malaria in children,” *Scand J Infect Dis Suppl*. 1996;102:1-53.

Jansky L, Reymanova P, Kopecky J. Dynamics of cytokine production in human peripheral blood mononuclear cells stimulated by LPS or infected by Borrelia. *Physiol Res*. 2003;52:593–598.

Jens Erik Pontoppidan Larsen, Ole Lund and Morten Nielsen. Improved method for predicting linear B-cell epitopes. *Immunome Res*. 2006; 2: 2.
Jespersen MC, Peters B, Nielsen M, Marcatili P. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res* 2017.

Jiang G, Charoenvit Y, Moreno A, Baracers MF, Banania G, Richie N, Abot S, Ganeshan H, Fallarme V, Patterson NB, Geall A, Weiss WR, Strobert E, Caro-Aquilar I, Lanar DE, Saul A, Martin LB, Gowda K, Morrissette CR, Kaslow DC, Carucci DJ, Galinski MR, Doolan DL. Induction of multi-antigen multi-stage immune responses against *Plasmodium falciparum* in rhesus monkeys, in the absence of antigen interference, with heterologous DNA prime/poxvirus boost immunization. *Malar J* 2007; 6: 135.

Katende J, Morzaria S, Toye P, Skilton R, Nene V, Nkonge C, Musoke A. An enzyme-linked immunosorbent assay for detection of *Theileria parva* antibodies in cattle using a recombinant polymorphic immunodominant molecule. *Parasitol Res*. 1998; 84:408-416.

Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Lett.* 1990; 276:172-4.

Lundegaard C, Lamberth K, Harndahl M, Buus S, Lund O, and Nielsen M. NetMHC-3.0: Accurate web accessible predictions of Human, Mouse, and Monkey MHC class I affinities for peptides of length 8-11. *NAR* 2008; 36:509-512.

Miura K. Progress and prospects for blood-stage malaria vaccines. *Expert Rev Vaccines*. 2016; 15:765-781.

Lundegaard C, Nielsen M, Lund O. The validity of predicted T-cell epitopes. *Trends Biotechnol* 2006; 24:537-538.

Lundegaard C, Lund O, and Nielsen M. Accurate approximation method for prediction of class I MHC affinities for peptides of length 8, 10 and 11 using prediction tools trained on 9mers. *Bioinformatics* 2008; 24:1397-1398.

Moll K, Kaneko A, Scherf A, Wahlgren M. *Methods in Malaria Research*, 6th edn. Glasgow: EVIMalaR; 2013.

Nielsen M, Lundegaard C, Worning P, Lauemøller SL, Lamberth K, Buus S, Brunak S, Lund O. Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. *Protein Sci* 2003; 12:1007-1017.
Nkou Akenji TK, Ntonifor NN, Ching JK, Kimbi HK, Ndamukong NK, Anong DN, Boyo MG, Titanji VPK. Evaluating a malaria intervention strategy using knowledge, practices and coverage surveys in rural Bolifamba, southwest Cameroon. *Trans R Soc Trop Med Hyg*. 2005; 99: 325-332.

Ogutu BR, Apollo OJ, McKinney D, Okoth W, Siangla J, Dubovsky F, Tucker K, Waitumbi JN, Diggs C, Wittes J, Malkin E, Leach A, Soisson LA, Milman JB, Otieno, L, Holland CA, Polhemus M, Remich SA, Ockenhouse CF, Cohen J, Ballou WR, Martin SK, Angov E, Stewart VA, Lyon JA, Heppner DG, Withers MR. Blood stage malaria vaccine eliciting high antigenspecific antibody concentrations confers no protection to young children in Western Kenya. *PLoS One* 2009; 4: e4708.

Olotu A, Fegan G, Wambua J, Nyangweso G, Awuondo KO, Leach A, Lievens M, Leboulleux D, Njuguna P, Peshu N, Marsh K, Bejon P. Four-year efficacy of RTS,S/AS01E and its interaction with malaria exposure. *N Engl J Med* 2013; 368:1111-1120.

Peters B, Sette A. Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method. *BMC Bioinformatics* 2005; 6:132

Pinder M, Reece WHH, Plebanski M, Akinwunmi P, Flanagan KL, Lee EAM, Doherty T, Milligan P, Jaye A, Tornieporth N, Ballous R, Mcadam KPMJ, Cohen J, Hill AVS. Cellular immunity induced by the recombinant *Plasmodium falciparum* malaria vaccine, RTS, S/AS02, in semi-immune adults in The Gambia. *Clin Exp Immunol* 2004; 135: 286–293.

Sach TH, Barton GR, Doherty M, Muir KR, Jenkinson C & Avery AJ. The relationship between body mass index and healthrelated quality of life: comparing the EQ-5D, EuroQol VAS and SF-6D. *Int J Obes (Lond)* 2007; 31: 189–196.

Shanehsazzadeh S, Oghabian MA, Allen BJ, Amanlou M, Masoudi A & Daha FJ. Evaluating the effect of ultrasmall superparamagnetic iron oxide nanoparticles for a long term magnetic cell labeling. *J Med Phys* 2013; 38: 34–40.

Sidney J, Assarsson E, Moore C, Ngo S, Pinilla C, Sette A, Peters B. Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries. *Immunome Res* 2008; 4:2.

Spring MD, Cummings JF, Ockenhouse CF, Dutta S, Reidler R, Angov E, Bergmann-Leitner E, Stewart
VA, Bittner S, Juompan L, Kortepeter MG, Nielsen R, Krzych U, Tierney E, Ware LA, Dowler M, Hermsen CC, Sauerwein RW, de Vlas SJ, Ofori-Anyinam O, Lanar DE, Williams JL, Kester KE, Tucker K, Shi M, Malkin E, Long C, Diggs CL, Soisson L, Dubois MC, Ballou WR, Cohen J, Heppner DG, Jr. Phase 1/2a study of the malaria vaccine candidate apical membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A. *PLoS ONE* 2009; 4, e5254.

Suhrcke M, Stuckler D, Suk JE, Desai M, Senek M, McKee M, Tsolova S, Basu S, Abubakar I, Hunter P, Rechel B, Semenza JC. The impact of economic crises on communicable disease transmission and control: a systematic review of the evidence. *PLoS One* 2011; 6: e20724. doi:10.1371/journal.pone.0020724

Takala SL, Plowe CV. Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming “vaccine resistant malaria”. *Parasite Immunol.* 2009; 31:560-573.

Titanji VP, Amambua-Ngwa A, Anong DN, Mbandi SK, Tanging E, Tening I, Yengo R. Isolation and expression of UB05, a *Plasmodium falciparum* antigen recognised by antibodies from semi-immune adults in a high transmission endemic area of the cameroonian rainforest. *Clin Chem Lab Med.* 2009;47:1147-58. doi:10.1515/CCLM.2009.255.

Titanji VPK, Dinga JN, Nyasa RB. A rational approach for predicting the minimum composition of anti-parasite sub-unit vaccines: A multiple target vaccine hypothesis. *Journal of the Cameroon Academy of Sciences.* 2017; 14:3-10.

Wahl G, Enyong P, Ngosso A, Schibel JM, Moyou R, Tubbessing H, et al. *Onchocerca ochengi*: epidemiological evidence of cross-protection against *Onchocerca volvulus* in man. *Parasitology* 1998; 116: 349 – 362.

Wang P, Sidney J, Dow C, Mothé B, Sette A, Peters B. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. *PLoS Comput Biol.* 2008; 4:e1000048.

Yadava A, Nurmukhambetova S, Pichugin AV, Lumsden JM. Cross-Species Immunity Following Immunization With a Circumsporozoite Protein-Based Vaccine for Malaria. *Journal of Infectious Diseases.* 2012; 205:1456–63.

Tables
Due to technical limitations the tables are available as a download in the Supplementary Files.

Figures

Figure 1

Human ELISpot assay using TpUB05 to stimulate human PBMCs for IFN-gamma production.
A: r-TpUB05 from T. parva was used to stimulate human T-cells from 63 subjects. B: Human ELISpot assay using r-UB05 from P. falciparum. C: Combining and comparing SI values obtained using r-TpUB05 and r-UB05.
Relationship between T-cell responses (IFN-γ production) to recombinant TpUB05, fever and parasitaemia. A: subjects with no fever (SIS) appear to produce more IFN-gamma in response to stimulation by TpUB05 compared to those with fever (FSS) (p = 0.002) B: production of IFN-gamma correlated with protective immune response against malaria.
Antibody response of TpUB05 in humans. A: Comparison of antigen-specific antibody to TpUB05 in plasma collected from human subjects (SIS, FSS, SC). B: Average OD405 value of TpUB05 by the different immune status groups; SIS (semi-immune subjects), FSS (frequently sick subjects), SC (sick children).
Relation between fever, parasitaemia and antibody response to TpUB05. A: Comparing the absence or presence of fever with the anti-TpUB05 antibody level in human plasma. B: The relationship between anti-TpUB05 antibody levels and parasite load indicates there was a negative correlation.
Figure 5

Comparing anti-TpUB05 and anti-UB05 polyclonal antiserum in Growth Inhibition Assay.

Rabbit antisera against rTpUB05 and rUB05 were used in vitro to test for their ability to inhibit parasite growth. This was done using P. falciparum laboratory strains; 3D7, FCR-1/FV0 and HB3 and 2 field isolates; GH01 and SC01. They were tested at 1:10 dilution.

Standard deviation is indicated with error bars. The experiment was done twice in triplicates.

Supplementary Files

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